ROLE OF SUBSTANCE P IN SEPSIS

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SUMMARY

The pro-inflammatory neuropeptide substance P (SP) is known to play an important role in the pathophysiology of various inflammatory diseases. *Preprotachykinin-A* gene knock-out (*PPTA*$^{-/-}$) mice (lacking SP) are shown to be protected against polymicrobial sepsis. The aim of this study was to evaluate the role of SP in polymicrobial sepsis and associated lung injury and understand the molecular mechanisms involved in the pathogenesis of this serious inflammatory disorder.

Pharmacological blocking of the neurokinin receptors with SR140333, a highly potent and selective antagonist of the neurokinin-1 receptors (NK-1R), and GR159897, a neurokinin-2 receptor (NK-2R) antagonist, were studied 8 h after cecal ligation and puncture surgery that induced polymicrobial sepsis in mice. Lung tissue was collected and analyzed for myeloperoxidase activity, histology, chemokines, cytokines and adhesion molecules. Transcription factors nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) were analyzed to elucidate the mechanism involved. A beneficial effect of treatment with SR140333, but not GR159897, in lung injury in sepsis was observed. NK-1R blocking lowered leukocyte infiltration and lung levels of chemokines, cytokines and adhesion molecules. The mechanistic studies revealed that the inhibition of SP action was mediated through NK-1R and the downstream signaling cascade involving protein kinase C alpha (PKCα) and NF-κB and AP-1 transcription factors modulated the pro-inflammatory mediators in polymicrobial sepsis. The combined data provided further support for the role of SP in polymicrobial sepsis.

In addition to the use of neurokinin receptor blockers, *PPTA* gene knock-out mice were

subjected to polymicrobial sepsis in order to understand the immunological basis of protection enjoyed by these mice lacking SP. Affymetrix high-density oligonucleotide arrays were used for lung gene expression profiling. Genes that were either consistently increased or decreased were shortlisted and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to validate the results for selected differentially expressed genes. Multiplexed bead-based suspension arrays were employed for the time-course measurement of a set of plasma cytokines. Elevated levels of pro- and anti-inflammatory gene and protein expression were observed in the early stages of sepsis in *PPTA* gene knock-out mice. This may help in resolving the infection without excessive immunosuppression in *PPTA* gene knock-out mice. Additional antimicrobial mediators that were observed in the study might have further supported in restoring and maintaining the delicate balance of inflammatory forces.

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ABBREVIATIONS

AP-1	activator protein-1
CLP	cecal ligation and puncture
ELISA	enzyme-linked immunosorbent assay
ERKs	extracellular signal regulated kinases
HMGB-1	high mobility group box-1
IKK	inhibitor kappa B kinase
IL-	interleukin
IL-1ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
JNKs	Jun-N terminal kinases
LPS	lipopolysaccharide
MAPKs	mitogen activated protein kinases
MCP	monocyte chemoattractant protein
MIF	macrophage migration inhibitory factor
MIP	macrophage inflammatory protein
MPO	myeloperoxidase
NF-κB	nuclear factor kappa B
NK	neurokinin
NO	nitric oxide
PKCα	protein kinase C alpha
PPTA$^{-/-}$	*preprotachykinin-A* knockout
RAGE	receptor for advanced glycation end products
RANTES	regulated upon activation normal T cell expressed and secreted
RT-PCR	reverse transcriptase-polymerase chain reaction
s.c	subcutaneous
SEM	standard error of mean
SP	substance P
TLRs	toll-like receptors
TNF	tumor necrosis factor

CHAPTER 1. INTRODUCTION

1.1 General overview

Patients with severe burns, hemorrhage, traumatic injury or abdominal surgery are highly vulnerable to pathogens and opportunistic infections. In addition, critically ill, elderly, pediatric and post-operational patients in the intensive care unit are also susceptible to infections. A minor wound infection in these patients can easily end up in sepsis (Kobayashi *et al.,* 2006). In the United States alone, approximately 750000 people develop sepsis annually and one third of them die of the resulting multiple organ failure (Angus *et al.,* 2001). The incidence of sepsis has been estimated to increase by 1.5% every year with a current annual cost of $16.7 billion for sepsis treatment in the USA (Angus *et al.,* 2001; Martin *et al.,* 2003). Sepsis has a high death rate (30-47%) especially in immunocompromised conditions such as patients with malignancies, organ transplants or AIDS (Kobayashi *et al.,* 2006; Riedemann *et al.*, 2003). Even in this modern era of medical science, sepsis associated complications are the most common cause of fatalities in the intensive care units. Predominantly supportive treatment, with no effective therapies so far, helps little in reducing the mortality rate of sepsis (Ness *et al.,* 2004).

Sepsis is the intense systemic inflammatory response syndrome caused usually by bacterial infection (Ashare *et al.,* 2005). When the immune defenses of the body fail to eliminate pathogens, infection spreads through blood circulation. The resultant production of pro-inflammatory cytokines and chemokines leads to recruitment of neutrophils, tissue damage and multiple organ failure (Ness *et al.,* 2004). However, it is the subsequent excessive production of anti-inflammatory mediators that induces

immunosuppression and fatalities in sepsis (Ashare *et al.,* 2005; van der Poll and Deventer 1999). Balance between pro-inflammatory and anti-inflammatory mediators plays an important role in the pathophysiology of sepsis.

Sepsis is generally caused by mixed infection (Sriskandan and Altmann 2008) and multiple mediators have been reported to be involved in the development of sepsis (Okazaki and Matsukawa 2009; Marshall *et al.,* 2003). Substance P (SP), a *preprotachykinin-A* (*PPTA*) gene product, is an immunoregulatory neuropeptide implicated in various inflammatory diseases. Recent literature reports illustrate evidence of a role for SP in acute pancreatitis, endotoxemia, and polymicrobial sepsis (Ramnath *et al.,* 2006; Ng *et* al., 2008; Puneet *et al.,* 2006; Zhang *et al.,* 2007). However understanding the molecular mechanisms and therapeutic implications of the role of SP in sepsis and associated lung injury remains a challenge.

1.2 Literature review

1.2.1 Polymicrobial sepsis

Sepsis is a state of systemic inflammatory response syndrome with a known focus of infection resulting from bacteria, virus, fungus or parasites (Matsuda and Hattori, 2006). Severe sepsis refers to sepsis with at least one organ or organ system dysfunction and septic shock is severe sepsis associated with hypotension (Remick 2007). A staging system has been developed for sepsis based on 4 characteristics represented by the acronym PIRO: P- for the predisposition (pre-existing co-morbid conditions); I- for the insult/infection (clinical knowledge about the pathogen); R- for the response to the infectious challenge (including the development of systemic

inflammatory response syndrome); and O- for organ dysfunction (including coagulation cascade) (Levy *et al.,* 2003).

Sepsis is a common, expensive and often fatal clinical illness caused by an imbalance in the inflammatory response of the body mainly against sustained and overwhelming bacterial infection. The initial pro-inflammatory response to eliminate pathogens is followed by an excessive production of anti-inflammatory mediators that also contributes to the pathophysiology of sepsis (Ashare *et al.,* 2005; Reddy *et al.,* 2001; van der Poll and Deventer 1999). Sepsis is manifested by a varying degree of low blood pressure, coagulopathy, reduced oxygen supply to the tissues and often multiple organ failure. Hemodynamic features of sepsis include cardiovascular instability, reduced ejection fraction and decreased systemic vascular resistance (Wilson *et al.*, 1998).

1.2.1.1 Pathophysiology of polymicrobial sepsis

Pathophysiology of polymicrobial sepsis is generally agreed to be complex and so far no single mediator/system/pathway/pathogen has been reported to drive this multifactorial disease on its own (Remick 2007). It comprises a continuum of clinical and pathophysiological severity induced by a score of pathogens (Sriskandan and Altmann 2008). Although recently gram-positive infections have been documented to be more frequent (Martin *et al.,* 2003), both gram-positive and gram-negative sepsis are common (Sriskandan and Altmann 2008). *Staphylococcus aureus* and *Escherichia coli* are the most common causes of sepsis and *Pseudomonas aeruginosa* is reported to be the most lethal (Vincent *et al.,* 2000). But many cases of sepsis are due to mixed infection and polymicrobial in origin.

Several facets of the interaction between host and pathogen must be considered before unraveling the immune events leading to sepsis. The survival of the host depends on its recognition of invading organism and a quick response. The innate immune response is the primary line of defence against infectious assault. The innate defenses of the host immune system such as neutrophils, macrophages and natural killer cells recognize biochemical patterns displayed by pathogens and trigger active responses either directly or by releasing cytokines or by activating T and B cells. Innate immune system utilizes pattern-recognition receptors (PRRs) to recognize these highly conserved pathogen-associated molecular patterns (PAMPs) such as bacterial cell-wall components (lipopolysaccharide, LPS) and peptidoglycans. Toll-like receptors (TLRs) are a family of pattern-recognition receptors present on mammalian macrophages, dendritic cells and other cells triggering innate immune responses. So far 10 TLRs have been identified with TLR-4 being the first in 1997. TLR-4s recognize endotoxin (LPS) while TLR-2s detect peptidoglycan and lipoteichoic acid (Beutler 2002). Once activated, TLRs trigger a cascade of cellular signals and engage a sequence of cytoplasmic interaction that result in activation of transcription factor NF-κB and transcription of target genes (Wiersinga and van der Poll 2007). The resulting gene products act on T and B cells to initiate adaptive immunity. However under pathological conditions, this activation may lead to septic shock as heavy load of pathogens can induce massive quantities of inflammatory cytokines and activation of innate immunity throughout the host. Innate immune sensing is beneficial to the host when the bacterial inoculum is limited, but with massive systemic bacterial contamination the innate immune sensing could be damaging (Beutler 2002).

1.2.1.2 Dysregulated coagulation

Although the coagulation system is not conventionally a part of the immune response to pathogens, both are linked closely in sepsis (Sriskandan and Altmann 2008). Septic patients reportedly have coagulation abnormalities, endothelial cell injury and abnormal blood flow (Remick 2007). The clotting system is activated by the invading pathogens in the blood stream along with a down-regulation of anticoagulant system and fibrinolysis (Wiersinga and van der Poll 2007). Thrombus and clot formation is promoted which in turn leads to more inflammation (Schouten *et al.,* 2008). Although limited intravascular coagulation benefits survival by walling off the damaged and infected tissue, disseminated intravascular coagulation leads to microvascular clogging and impaired organ perfusion (Sriskandan and Altmann 2008; Opal 2004). Thus it is important to limit excessive coagulopathic damage and simultaneously maintain localized clot formation (Esmon 2000). Recombinant human activated protein C (APC) has been introduced as a therapeutic intervention for its anti-coagulant and anti-inflammatory properties (Bernard *et al.,* 2001). It is also reported to inhibit neutrophil chemotaxis and NF-κB activation (Amaral *et al.,* 2004). However it is not useful in all septic patients and the beneficial effects are still not completely convincing (Eichacker *et al.,* 2006).

1.2.1.3 Endothelial cell dysfunction

The blocking of peripheral blood vessels and the resulting ischaemia leads to endothelial cell damage (Sriskandan and Altmann 2008). It is worsened by the direct bacterial invasion and loss of barrier function. Platelets bind to the damaged surface resulting in platelet and fibrin accumulation and adhesion molecules attract neutrophils leading to endothelial disruption and plasma extravasations (Remick 2007). However

endothelial cells have not been shown to undergo apoptosis during sepsis in *in vivo* animal models (Hotchkiss *et al.,* 2001).

1.2.1.4 Inflammatory mediators in sepsis

The inflammatory response is an integral part of sepsis. An ideal situation is when the inflammatory response eliminates the infectious assault without harming the host. Inflammatory mediators such as chemokines, cytokines, adhesion molecules, nitric oxide, hydrogen sulfide, SP have been reported to play varied roles in the progression of sepsis. A balance between anti- and pro-inflammatory mediators is crucial in deciding the outcome of sepsis.

1.2.1.4.1 Chemokines

Chemokines are a family of small (8-14 kDa), inducible cytokines secreted by a variety of cells. They play a crucial role in trafficking and recruiting antibacterial leukocytes to the primary sites of innate immune response. Chemokines are classified into 4 subfamilies depending on the relative position of cysteine residues. CC and CXC chemokines are the most widely studied peptides in sepsis. Chemokines produced by bacterial infections play an important role on the improvement or impairment of host antibacterial resistances. While chemokines help to boost the host defenses against invading pathogens, overwhelming levels of the same can lead to the pathogenesis of sepsis. Chemokines bind to a family of G protein-coupled transmembrane receptors on the surface of leukocytes (Ramnath *et al.,* 2006). CCR1, CCR2, CCR4, CXCR1 and CXCR2 are the important chemokine receptors in sepsis.

Chemokine production is increased in animals and humans exposed to pathogens. CXCL8 (interleukin-8, IL-8) systemic levels are reported to be elevated in humans during bacterial sepsis (Aalto *et al.,* 2004). Similarly, plasma levels of CXCL9

(monokine-induced by interferon-γ, MIG) (Shin *et al.,* 2003), CCL2 (monocyte chemoattractant protein-1, MCP-1) (Bossink *et al.,* 1995), CCL3 (macrophage inflammatory protein-1α, MIP-1α) (O'Grady *et al.,* 1999), CCL4 (MIP-1β) (O'Grady *et al.,* 1999; Proulx *et al., 2*002) and CCL8 (MCP-2) (Bossink *et al.,* 1995) were demonstrated to increase during sepsis. Our group has also reported elevated levels of chemokines in polymicrobial sepsis (Puneet et al., 2006; Zhang et al., 2007). In this perspective, various chemokine receptor antagonists have been tried and tested in animal models of sepsis and found to be promising.

1.2.1.4.2 Cytokines

Cytokines are soluble, low-molecular-weight glycoproteins that are synthesized and released in response to tissue damage. Inflammatory mediators released in sepsis are aimed to enhance leukocyte trafficking to the site of infection. Neutrophil mediates clearance of bacteria, but if the neutrophil transmigration is unchecked, significant organ damage may occur by the release of pro-inflammatory granules and enzymes (Sriskandan and Altmann 2008). Activation of TLRs by microorganisms triggers a signaling cascade culminating in the activation of NF-κB and transcription of various pro-inflammatory cytokines and chemokines. Tumor necrosis factor alpha (TNF-α), IL-1 and IL-6 coordinate the initiation of acute phase response in sepsis (Sriskandan and Altmann 2008). The acute phase response that is triggered by the pathogen recognition is important for survival in sepsis. TNF-α and IL-1 exert profound effects on the endothelium, vasculature and coagulation cascade. TNF-α acting through type I and II TNF receptors results in further NF-κB mediated transcription of pro-inflammatory mediators.

The host simultaneously produces anti-inflammatory mediators such as IL-10, sTNFRs to counter-balance the pro-inflammatory response. Mechanisms of the anti-inflammatory response have not been clearly understood so far (Reddy *et al.,* 2001).

1.2.1.4.3 Novel cytokines

Recently, two soluble cytokines and a transmembrane receptor (discussed below) have also been identified as a critical mediator of sepsis.

1.2.1.4.3.1 High Mobility Group Box-1 (HMGB-1)

HMGB-1, a nonhistone, nuclear DNA-binding protein involved in gene transcription, is reported as a late mediator of sepsis (Cinel and Opal 2009). Elevated levels of HMGB-1 are detected in septic patients and are correlated with the degree of organ dysfunction (Sundén-Cullberg *et al.,* 2005; Gibot *et al.,* 2007). When released in large quantities into the extracellular environment in sepsis, it can have harmful pro-inflammatory effects (Sriskandan and Altmann 2008; Ulloa and Tracey 2005). Apoptotic tissue damage possibly induces the release of HMGB-1, which then binds to bacterial substances and also initiates a second wave of TLR signaling (Zimmerman *et al.,* 2004; Yu *et al.,* 2006; Qin *et al.,* 2006). Macrophages and neutrophils are also capable of actively secreting HMGB-1 to trigger inflammation (Cinel and Opal 2009). LPS and various cytokines release HMGB-1 from activated macrophages and binding of HMGB-1 to receptor for advanced glycation end products (RAGE), TLR-2, and -4, is reported to activate NF-κB and extracellular regulated kinase 1/2 (Hori *et al.,* 1995; Park *et al.,* 2004). HMGB-1, being a late emerging cytokine of sepsis, provides a wide therapeutic window and is an attractive target for treatment.

1.2.1.4.3.2 Macrophage Migration Inhibitory Factor (MIF)

MIF, originally found in T cells, is a stress response mediator and pro-inflammatory cytokine (Calandra *et al.,* 1995). It has been detected in clinical sepsis and may have a role in the pathogenesis of sepsis (Cinel and Opal 2009). During endotoxaemia, MIF was shown to be produced by the anterior pituitary gland (Calandra and Roger 2003) although the source of trigger of MIF in humans is not clear (Sprong *et al.,* 2007). Injection of MIF reportedly increased mortality and inhibition of MIF decreased TNF-α and IL-1β and improved survival in endotoxin-induced shock and sepsis models (Calandra and Roger 2003; Calandra *et al.,* 2000; Bozza *et al.,* 1999). In normal conditions, MIF is found to sensitize macrophages to LPS by promoting the expression of TLR-4 (Roger *et al.,* 2001) and regulate activation-induced apoptosis (Mitchell *et al.,* 2002). However, elevated MIF in sepsis delays the clearance of activated monocytes/macrophages by apoptosis and consequently increases cytokine production and pro-inflammatory response (Cinel and Opal 2009).

*1.2.1.4.3.3 R*eceptor for advanced glycation end products (RAGE)

HMGB-1 is known to activate RAGE, an immunoglobulin superfamily receptor and a part of the innate immune system (Harris and Raucci 2006). RAGE has been shown to decrease inflammation, neutrophil extravasation and migration (Chavakis *et al.,* 2003). Administration of anti-RAGE antibody much later in polymicrobial sepsis in mice has shown to promote survival and thus provided a therapeutic rationale (Lutterloh *et al.,* 2007).

1.2.1.4.4 Nitric Oxide (NO)

NO, a ubiquitous biological molecule produced by various cells, is believed to play a key role in the pathogenesis of sepsis, especially the cardiovascular alterations (Panas

et al., 1998). At high concentrations, NO forms peroxynitrite which is reported to be pro-inflammatory and cytotoxic and may cause tissue damage (Chandra *et al.,* 2006). NO contributes to septic shock by means of vasodilatation, myocardial dysfunction, oxidative damage, increased intestinal permeability and subsequent translocation of bacteria, and inhibition of mitochondrial respiration (Vincent *et al.,* 2000). Genetic deletion of inducible NO synthase (iNOS), the enzyme that synthesizes NO from L-arginine, has been reported to protect mice from endotoxin-induced mortality (Wei *et al.,* 1995). Selective iNOS inhibitors, Aminoguanidine (Wu *et al.,* 1996) and L-canavanine (Liaudet *et al.,* 1997) have also been shown to benefit in experimental sepsis. L-NAME (N omega-nitro-L-arginine methyl ester), an NO antagonist, is reported to improve arterial pressure and oxygenation in septic patients (Avontuur *et al.,* 1995). However, few other studies demonstrated that nitric oxide donors may improve splanchnic microcirculation in sepsis (Assadi *et al.,* 2008; Siegemund *et al.,* 2007; Baumgart *et al.,* 2009).With continuing debate over the pros and cons of NO inhibition, more targeted and selective inhibition of NOS isoforms should help better in the management of sepsis (Chandra *et al.,* 2006).

1.2.1.4.5 Carbon monoxide (CO)

Carbon monoxide is a toxic gas that binds strongly to the iron centers of heme-containing proteins such as hemoglobin, catalase, myoglobin, and cytochrome-c-oxidase (Alonso *et al.,* 2003; Foresti *et al.,* 2008). Carbon monoxide promotes vasodilatation and the effects are particularly pronounced in the hepatosplanchnic system (Baumgart *et al.,* 2009). It also shows anti-inflammatory properties (Baumgart *et al.,* 2009). Severe sepsis patients have reportedly higher carboxyhemoglobin levels than nonseptic intensive care unit controls (Zegdi *et al.,* 2002). Further,

carboxyhemoglobin levels were higher in patients who survived (Zegdi *et al.,* 2002). However, inhalation of carbon monoxide for 1 h by volunteers before endotoxin injection failed to produce any anti-inflammatory effects (Mayr *et al.,* 2005).

1.2.1.4.6 Hydrogen Sulphide (H_2S)

Endogenous hydrogen sulphide is synthesized in mammals from L-cysteine by cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (Szabó 2007). Vasodilator hydrogen sulphide has been shown to regulate hemodynamic parameters in animal models of septic shock (Hui *et al.,* 2003). Pro-inflammatory role of hydrogen sulphide has been reported in polymicrobial sepsis (Zhang *et al.,* 2006) which is mediated through SP and also other mediators (Zhang *et al.,* 2007).

1.2.2 Substance P (SP)

Neuropeptide SP belongs to the tachykinin family, along with other members such as neurokinin A (NKA), neurokinin B (NKB), and two elongated forms of neurokinin A: neuropeptide γ and neuropeptide K (Bhatia *et al.,* 2003; Eulberg *et al.,* 2005). Tachykinins share a common C-terminal sequence Phe-Xaa-Gly-Leu-Met-NH2 that is needed for their interaction with specific receptors and is important for producing most of their biological effects (Bhatia 2003; Kimura *et al.,* 1984). In mammals, the gene *PPTA* or *PPT-I* encodes both SP and NKA, and neurokinin B is encoded by the *preprotachykinin-B* gene (*PPTB* or *PPT-II*) (Harrison and Geppetti 2001; Severini *et al.,* 2002; Bhatia 2003). Another *preprotachykinin* gene (*PPTC*) has been described that encodes a novel tachykinin termed hemokinin-I (Zhang et al., 2000). The *PPTA* gene has been detected in both central and peripheral nervous system, in enteric neurons of the gut and in various cells of the immune system (Bhatia 2003). The *PPTB* gene is expressed almost exclusively in the central nervous system (Bhatia 2003).

SP was discovered in 1931 by Von Euler and Gaddum as an active compound from horse intestine and brain extract that caused intestinal smooth muscle contractions (Maggio 1988). The 11 amino acid sequence of SP was discovered in 1970 by Chang and Leeman (Chang and Leeman 1970). SP is localized in the central nervous system as well as released from nerve endings in several peripheral tissues, including the entire length of the gastrointestinal tract, the pancreas as well as the colon. Immunoregulatory peptide SP is produced at various inflammation sites. It is found in resident macrophages, circulating leukocytes and dendritic cells (Ho *et al.,* 1997; Lai *et al.,* 1998; O'Connor *et al.,* 2004) and is known to have a role in neurogenic inflammation (Chavolla-Calderon *et al.,* 2003). SP is reported to increase postcapillary venule permeability, immune cell influx, and glandular secretion in mammalian airways (Rizzo *et al.,* 1999). It also induces the release of pro-inflammatory mediators, lymphocyte proliferation and chemotaxis (Gronberg *et al.,* 2004). Increased SP immunoreactivity has been found in bronchoalveolar lavage samples from patients suffering from lung diseases (Espiritu *et al.,* 1992).

Three distinct receptors, NK-1R, NK-2R and NK-3R, mediate the biological actions of tachykinins. SP, NKA and NKB are generally considered to be the preferred ligands for NK-1R, NK-2R and NK-3R, respectively (Regoli *et al.,* 1989). However, both NKA and NKB have been shown to be potent in stimulating NK-1R (Hastrup and Schwartz 1999) and all the three mammalian tachykinins (SP, NKA and NKB) are reported to be capable of acting as full agonists on each of the three receptors, with different potencies (Bhatia 2003). SP binds with high affinity to NK-1R, and with low affinity to NK-2 and 3 receptors (Koon and Pothoulakis 2006). The NK receptors

belong to the superfamily of rhodopsin-like, G-protein-coupled receptors (GPCRs) with seven transmembrane spanning segments.

Activation of NK receptors results in the exchange of GDP bound to Gα subunit of the G protein for GTP and dissociation into Gα and Gβγ subunits (Johnston and Siderovski, 2007; Oldham *et al.,* 2007; Rozengurt 2007). GTP-Gα complex activates the β isoforms of phospholipase C (PLC) which catalyses the hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP2) in the plasma membrane resulting in inositol 1,4,5 trisphosphate (IP3) and 1,2,diacylglycerol (DAG) (Exton 1996; Rozengurt 1998). Inositol 1,4,5 trisphosphate binds to its intracellular receptor, a ligand gated calcium channel, found in the endoplasmic reticulum to release calcium from the internal stores (Mikoshiba 1997). 1,2,diacylglycerol directly activates enzyme PKC (Nishizuka 1995). Stimulation of peripheral tachykinin receptors leads to smooth muscle contraction, neuronal stimulation, endothelium-dependent vasodilation, plasma protein extravasation, chemotaxis and activation of immune and inflammatory cells, and stimulation of secretion (Maggio 1988; Patacchini and Maggi 2001).

An elevated expression of SP receptor binding sites has been observed in the submucosa of patients suffering from inflammatory bowel disease (Mantyh *et al.,* 1988) and increased NK-1R in lymphoid aggregates, small blood vessels, and enteric neurons was reported in patients with Crohn's disease (Mantyh *et al.,* 1994; Mantyh *et al.,* 1995). Elevated systemic SP levels have been reported in postoperative septic patients (Beer *et al.,* 2002). SP, acting through NK-1R, is reported to play an important role in the pathogenesis of acute pancreatitis (Bhatia *et al.,* 2003; Patto *et al.,* 1992). Genetic deletion of NK-1R as well as pharmacological blockade of NK-1R has been shown to protect mice against acute pancreatitis and associated lung injury (Lau *et al.,*

2005; Bhatia *et al.,* 1998). Genetic deletion of *PPTA* has been reported to ameliorate acute pancreatitis and associated lung injury (Bhatia *et al.,* 2003). Further, *PPTA* knockout mice are protected against polymicrobial sepsis (Puneet *et al.,* 2006) and LPS-induced endotoxemia (Ng *et al.,* 2008). Deletion of *PPTA* gene in those mice significantly attenuated inflammation and damage in the lungs (Puneet *et al.,* 2006; Ng *et al.,* 2008).

1.2.3 Nuclear Factor-κB (NF-κB) transcription factor

1.2.3.1 The NF-κB family

NF-κB is a general term that corresponds to various dimeric complexes of Rel protein family members (Hayden and Gosh 2004; Ghosh and Karin 2002). Various home- and heterodimeric combinations are formed by c-Rel, RelA (p65), RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100), but the p50/p65 heterodimer is the most commonly detected form (Calzado *et al.,* 2007). The DNA binding, dimerization, and nuclear translocation of NF-κB are mediated by N-terminal Rel-homology domain (RHD), shared by all the members (Calzado *et al.,* 2007). NF-κB is regulated through its localization in the cell. In normal resting cells, NF-κB is bound to inhibitory IκB proteins such as IκBα, IκBβ and IκBε in the cytoplasm (Ghosh and Karin 2002). IκB proteins interact with NF-κB through multiple ankyrin repeats and inhibit its DNA binding (Calzado *et al.,* 2007). IκBα blocks the nuclear translocation of only p65 and IκBβ masks p65 and p50 (Calzado *et al.,* 2007). IκBα also provides a negative feedback mechanism for the termination of NF-κB response (Arenzana-Seisdedos *et al.,* 1995).

1.2.3.2 Activation of NF-κB

NF-κB is a ubiquitous transcription factor that is activated by a wide range of signals such as LPS, cytokines, growth factors, viral infection and DNA damage, mediated through three major pathways: canonical and non-canonical pathways (Pomerantz and Baltimore 2002) and a cascade triggered by DNA damage (Janssens and Tschopp 2006). A proteasome-dependent step to generate DNA-binding dimers is common to all the pathways (Ben-Neriah and Schmitz 2004). Canonical pathway involves a cascade of adaptor proteins and protein kinases, stimulating inhibitor kappa B kinase (IKK) complex comprising enzymatically active subunits IKK-α and β (IκB kinases) and their regulatory subunit IKKγ/NEMO (NF-κB essential modifier) (Akira and Takeda 2004; Karin and Ben-Neriah 2000). The IKKγ/NF-κB essential modifier subunit binds to Lys 63-linked polyubiquitination allowing subsequent activation of IKK (Wu *et al.,* 2006; Ea *et al.,* 2006). Activated IKKβ phosphorylates IκB, enabling subsequent lysine-48-linked polyubiquitination of IκBα and proteolytic degradation (Calzado *et al.,* 2007). The unmasked nuclear localization sequence of p65 protein allows translocation of NF-κB into the nucleus and binding to its consensus decameric sequence in the promoter region of genes involved in the pro-inflammatory response, encoding immunoreceptors, cell adhesion molecules, cytokines and chemokines (Baeuerle and Baichwal 1997).

The noncanonical activation pathway involves IKKα protein and is independent of IKKβ (Calzado *et al.,* 2007). It induces a delayed and sustained activation of primarily RelB-containing NF-κB dimmers, unlike the rapid activation observed in canonical pathway (Calzado *et al.,* 2007). DNA damage-triggered NF-κB activation is not very clear yet as ultraviolet-induced NF-κB signaling is independent of IKK activation, but

most other DNA-damaging substances involve an IKK-dependent IκBα phosphorylation (Li and Karin 1998).

1.2.3.3 NF-κB and diseases

NF-κB is suggested to have an important role in innate and acquired immunity (Lenardo and Baltimore 1989). Dysregulated NF-κB activity is reported in various diseases including chronic inflammation and cancer. NF-κB activation is a key mediator of the inflammatory response in pancreatitis (Chen *et al.,* 2002). In rheumatoid arthritis, NF-κB activation is reported to precede the onset of disease, and inhibition of NF-κB decreased the production of inflammatory cytokines and ameliorated the disease severity (Firestein *et al.,* 2004; Bacher and Schmitz 2004). Various studies have evaluated the role of NF-κB in sepsis. Elevation of inflammatory mediators during septic shock has been shown to depend on NF-κB activation (Calzado *et al.,* 2007). Bacteria and their components activate NF-κB via TLRs through the classical IKK pathway (Hayden and Gosh 2004). LPS is reported to activate NF-κB via TLR-4 (Murthy *et al.,* 2004) and peptidoglycan, a major component of gram-positive bacteria, utilizing TLR-2. Although NF-κB is an important target in treating diseases, cell-type specific inhibition of NF-κB pathway is a better strategy for drug development as NF-κB is also important for its anti-apoptotic activities and host-defense immune responses.

1.2.4 Activator protein – 1 (AP-1) transcription factor

The transcription factor AP-1 consists of a mixture of heterodimers composed of members of the Jun, Fos and activating transcription factor protein families. AP-1 family includes ATF1-4, c-Fos, c-Jun, c-Myc and C/EBP (Shaywitz and Greenberg 1999; Wisdom 1999). Phosphorylation of AP-1 family members by kinases leads to

transactivation activity. Growth factors, neurotransmitters, polypeptide hormones, inflammatory cytokines, bacterial and viral infections as well as a variety of physical and chemical stresses induce AP-1 via mitogen-activated protein kinase to translate external stimuli into changes of gene expression (Chang and Karin 2001). Transcription factor complex consisting of AP-1 and nuclear factor of activated T cells (NFAT) regulate cytokine genes (Zenz *et al.,* 2008). Nuclear factor of activated T cell-dependent gene regulation has been demonstrated for IL-2, IL-3, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-13, interferon-γ (IFN-γ), TNF-α, CD40L, FasL, CD5, Igκ, CD25 and the chemokines IL-8 and MIP1α (Zenz *et al.,* 2008).

1.2.5 Mitogen activated protein kinases (MAPKs)

MAPKs are a family of serine/threonine kinases that transduce signals from the cell surface to the nucleus (Chang and Karin 2001; Dong *et al.,* 2002; Hazzalin and Mahadevan 2002). Phosphorylation of MAPKs is necessary to activate them. Extracellular signal regulated kinases (ERKs), Jun-N terminal kinases (JNKs) and p38 MAPKs are the 3 major types of MAPKs. ERKs are believed to be involved in the control of cell division; JNKs regulate transcription; and p38 MAPKs are activated by inflammatory cytokines and environmental stress. MAPK cascade consists of MAPKs, the kinases that activate the MAPKs (MAPK kinases such as MEKs) and MAPKK kinase or MEK kinase (MAPKKK or MEKK) (English *et al.,* 1999). MEKs are dual-specificity kinases that recognise and phosphorylate the MAPKs. MEK kinases (MEKKs) located upstream of MEKs activate them (Schramek 2002). Two isoforms of JNK, JNK1 and JNK2, and two isoforms of ERKs, p44 MAPK (ERK1) and p42 MAPK (ERK2) are known. Activated MAPKs lead to the activation of various

transcription factors such as NF-κB and AP-1, protein kinases, phospholipases, cytoskeleton-associated proteins, and thus result in biological responses.

1.2.6 Animal models of sepsis

Although various animal models of sepsis have been developed, cecal ligation and puncture (CLP) - induced sepsis model in rodents remains the gold standard (Rittirsch *et al.,* 2007; Remick *et al.,* 2000; Deitch 2005; Buras *et al.,* 2005). Administration of endotoxin, LPS, is practiced by many researchers to study lethal sepsis. However, the endotoxemia model is different from polymicrobial sepsis with respect to a number of characteristics (Riedemann *et al.,* 2003). The kinetics and magnitude of peritoneal and systemic cytokine and chemokine levels in LPS model has been demonstrated to differ from CLP model (Remick *et al.,* 2000).

CLP closely mimics symptoms of clinical sepsis such as hypothermia, tachycardia and tachypnea in rodents. Perforation of cecum serves as an endogenous source of bacterial infection, resulting in bacterial peritonitis, followed by systemic mixed enteric bacteria load (Rittirsch *et al.,* 2009). Bacteremia leads to systemic activation of the inflammatory response, septic shock, multiorgan dysfunction and eventually failure. CLP model has the advantage of inducing sepsis with varied severity for investigating both acute and chronic sepsis (Benjamin *et al.,* 2004; Xiao *et al.,* 2006). However, it is important to use the model with high consistency to obtain reliable and reproducible results, as length of the cecum ligated, size of the needle used and the number of punctures determine the outcome of resulting sepsis (Singleton and Wischmeyer 2003; Baker *et al.,* 1983).

Possible approaches of studying the effects of SP in animal models of sepsis include, depletion of SP with capsaicin, pharmacological blocking of SP receptors and

silencing of gene encoding SP (Bhatia *et al.,* 2003). Capsaicin pre-treatment is reported to inhibit microvascular leakage induced by toxic gases in rats and guinea pigs (Solway and Leff 1991). Capsaicin, the active component of chilli pepper, selectively binds to transient receptor potential vanilloid (TRPV)-1 receptors on sensory nerves, depleting presynaptic stored SP from nerve endings. Thus ablation of sensory nerves by capsaicin helps to study neurogenic inflammation. Specific blocking of NK receptors provides another good approach to study SP in various disease models (Bhatia *et al.,* 2003). Further, my access to *PPTA*$^{-/-}$ mice is a great advantage as silencing of gene encoding SP provides a valuable animal model to study the role of SP in diseases. Indeed, *PPTA*$^{-/-}$ mice have been reported to be successfully used earlier in disease models such as acute pancreatitis (Bhatia *et al.,* 2003), endotoxemia (Ng *et al.,* 2008), sepsis (Puneet *et al.,* 2006), and nociception (Martin *et al.,* 2004).

1.3 Objectives

The aim of this study is to:

1. investigate the role of SP in the pathogenesis of sepsis and associated lung injury in mice
2. evaluate the effect of pharmacological blocking of the SP receptors on the severity of sepsis
3. analyze the genetic signature of *PPTA* knock-out animal model of sepsis
4. explore the underlying molecular mechanism of SP-NK receptor activation.

Two different strategies were employed:

1. Inhibition of the NK receptor by treating the mice with receptor blockers.

2. *PPTA* gene knock-out mice were used to study polymicrobial sepsis.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

SP ELISA kit was purchased from Peninsula Laboratories, Inc., Bachem, San Carlos, CA, USA. SR140333 was provided by Sanofi Synthelabo, France. TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), RNeasy® mini kit (QIAGEN, USA) and GelRed™ (Biotium, Hayward, CA, USA), were used for the RNA isolation and quantification. GeneChip® Mouse Genome 430 2.0 array, GeneChip® Hybridization Wash and Stain kit, One-cycle Target labeling and Control Reagents (containing IVT labeling kit, One cycle cDNA Synthesis kit, Sample Cleanup module, Poly-A RNA Control kit, Hybridization Controls) were purchased from Affymetrix, Inc. Santa Clara, CA, USA. NanoDrop® ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) was used for RNA quantification. PCR was performed in MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) was used for cDNA synthesis. Primers were synthesized by 1st BASE Pte. Ltd., Singapore. GeneChip® Fluidics Station 450, GeneChip® Hybridization Oven 640, and GeneChip® Scanner 3000 at the DSO were used for data collection. Procarta™ Cytokine Assay kit (Panomics, Inc.) was used for plasma cytokine profiling.

2.2 Animal Ethics

All animal experiments performed were in accordance with the guidelines of the DSO Animal Care and Use Committee (DSOACUC), DMERI, Singapore, which follows

the established International Guiding Principles for Animal Research. Mice were maintained at a controlled temperature (21-24° C) and lighting (12 h light/dark cycle) and fed with standard laboratory chow and drinking water, provided *ad libitum*. Animals were randomly assigned to control or experimental groups using six or more mice for each group. Before the experiment, two days of acclimatization was allowed for all mice.

2.3 Induction of polymicrobial sepsis

Mice were anesthetized lightly with mouse anesthesia cocktail (0.75 ml ketamine (100 mg/ml) and 1 ml medetomindine (1 mg/ml) dissolved in 8.25 ml distilled water) (7.5 ml/kg body weight) (Animal Holding Unit, NUS, Singapore). Polymicrobial sepsis was induced by CLP as described elsewhere (Ayala *et al.,* 1996; Zhou *et al.,* 2001; Baker *et al.,* 1983). Following strict aseptic conditions, the anterior abdomen was shaved and a midline incision was made in the lower part of the abdomen. The peritoneum was opened and the cecum was ligated 3-5 mm below the ileocecal valve with 4/0 silk suture without obstructing the bowel (**Fig. 2.1**). The cecum was punctured twice with a 22-gauge needle distal to the point of ligation and squeezed gently to extrude the cecal contents. The cecum was placed back in the abdomen and the muscle and skin incision were sutured separately with sterile Permilene 5/0 thread. All the mice were given saline (1 ml, s.c) after the surgery and kept on heat pads for recovery. The same surgical procedure except the cecal ligation and puncture was performed on sham-operated animals. The animals were sacrificed 8 h after surgery by an i.p. injection of a lethal dose of pentobarbitone. Blood was collected by cardiac puncture, heparinized, centrifuged, plasma removed and stored at -80° C. Samples of

Fig. 2.1

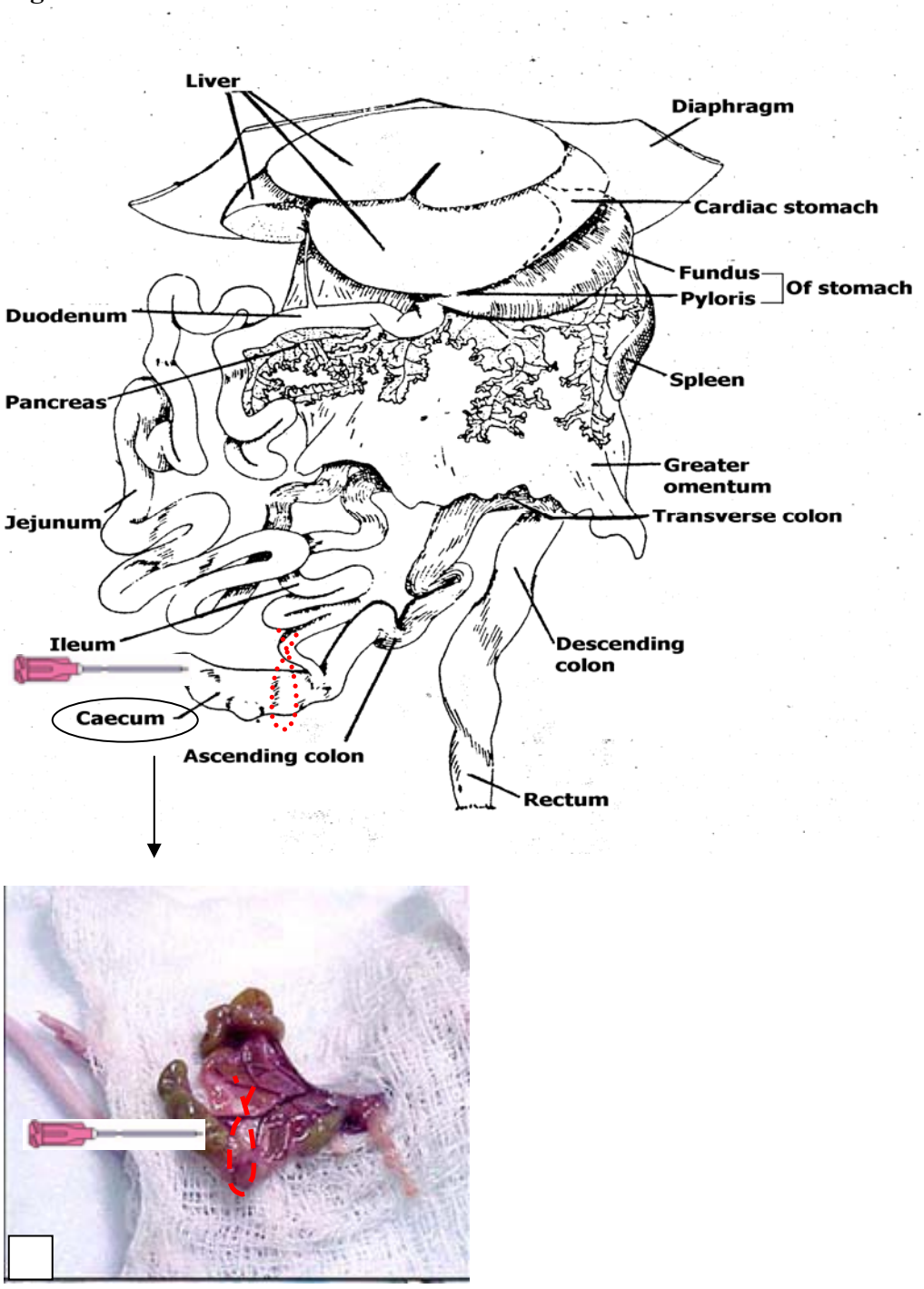


Fig. 2.1 Cecal Ligation and Puncture. The cecum was ligated 3-5 mm below the ileocecal valve with 4/0 silk suture without obstructing the bowel. The cecum was punctured twice with a 22-gauge needle distal to the point of ligation and squeezed gently to extrude the cecal contents.

lung were snap frozen in liquid nitrogen and stored at -80° C for subsequent measurements.

2.4 Myeloperoxidase estimation

Myeloperoxidase (MPO) activity as a measure of neutrophil sequestration in lung was quantified as described previously (Bhatia *et al.,* 2000; Bhatia *et al.,* 1998). Lung tissue samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 × g, 10 min, 4° C) and the resulting pellet resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, USA). The suspension was subject to four cycles of freezing and thawing and further disrupted by sonication (40 sec). The sample was then centrifuged (10,000 × g, 5 min, 4° C) and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mM tetramethylbenzidine (Sigma, St. Louis, MO, USA), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. This mixture was incubated at 37° C for 110 sec, the reaction terminated with 2 M H_2SO_4 and the absorbance measured at 450 nm. The absorbance was then corrected for the DNA content of the tissue sample (Labarca and Paigen 1980). Results were expressed as fold increase over control.

2.5 ELISA analysis

Plasma and lung tissue homogenates were assayed to evaluate the level of chemokines, cytokines and adhesion molecules by a sandwich ELISA according to the manufacturer's instructions. Lung sample was homogenized in 1 ml phosphate buffer 20 mM, pH 7.4, centrifuged and the resultant supernatant was used for the assay.

DuoSet ELISA kits with matched antibody pairs against mouse chemokine/cytokine/adhesion molecule were obtained from R&D systems, Inc., Minneapolis, MN, USA. Briefly, anti-chemokine/cytokine/adhesion molecule primary antibody was coated onto 96-well ELISA plates and incubated overnight at room temperature. Samples and standards were added to the wells and incubated for 2 h, the wells were washed, and a biotinylated goat anti-mouse chemokine/cytokine/adhesion molecule antibody was added for 2 h. Plates were washed again, and streptavidin conjugated to horseradish peroxidase was added for 20 min. After a further wash, tetramethylbenzidine was added for color development and the reaction was terminated with 2 N H_2SO_4. Absorbance was measured at 450 nm. Sample concentration was estimated from the standard curve. DNA assay was performed fluorometrically by using Hoechst dye 33256 (Labarca and Paigen 1980). The sample concentration was then corrected for the DNA content of the tissue (Labarca and Paigen 1980).

2.6 Histopathology

Paraffin-embedded lung samples were sectioned at 5-µm thickness, stained with hematoxylin / eosin (H&E) and qualitatively evaluated by light microscopy and documented by photographs. Eight randomly chosen microscopic fields (x125) were examined for each tissue sample and the extent of cell injury/necrosis, represented by the destruction of histo-architecture of the cells, vacuolization and swelling of cells, all of which have been associated with an inflammatory reaction, was evaluated.

2.7 Substance P estimation

The level of SP in lung tissue was measured using competitive Enzyme Immunoassay kit (Bachem, Peninsula Laboratories, USA) as per the manufacturer's protocol. Briefly, the lung tissue (about 100 mg) was homogenized in 1 ml ice-cold SP assay buffer for 20 s. The homogenate was centrifuged (13,000 rpm, 20 min, 4°C) and the supernatant was separated. SP in the supernatant was adsorbed on C18 separation column containing 200 mg C18 (Bachem, Peninsula Laboratories, USA) as described (Castagliuolo *et al.,* 1997). The adsorbed peptide was then eluted with 1.5 ml of 75% v/v acetonitrile and freeze-dried overnight. The lyophilized sample was reconstituted in SP assay buffer and the SP content was then determined using the SP ELISA kit (Bachem, Peninsula Laboratories, USA) according to the manufacturer's instructions. Non-biotinylated SP in the sample competes for the limited amount of immobilized antibody and the color intensity produced by the substrate depends on the quantity of biotinylated SP bound to the immobilized antibody. Absorbance was measured at 450 nm and SP level was read from a standard curve. It was expressed as picograms per milliliter for plasma and picograms per microgram of DNA for lung. DNA assay was performed fluorometrically by using Hoechst dye 33256 (Labarca and Paigen 1980).

2.8 Nitric oxide measurement

Nitrite is determined as an indicator of nitric oxide production in the tissue as NO is rapidly converted to nitrite and nitrate. Formation of nitrite was determined spectrophotometrically by Griess assay as described (Marzinzig *et al.,* 1997). The assay provides for enzymatic reduction of nitrate to nitrite by nitrate reductase, followed by spectrophotometric analysis of total nitrite using Griess reagent.

Absorbance was measured at 540 nm and the total nitrite concentration was then corrected for the DNA content of the tissue.

2.9 Preparation of nuclear extract

Active Motif nuclear extraction kit (SciMed, Carlsbad, CA, USA) was used to prepare nuclear extracts from lung following the instructions from the manufacturer. Briefly, lung tissue (50 mg) was homogenized in hypotonic buffer containing detergent, incubated for 15 min on ice, and then centrifuged at 850 g, 4°C for 10 min. The pellets were resuspended in hypotonic buffer, treated with detergent and centrifuged at 14,000 g, 4°C for 30 s. The nuclei in the pellets were lysed with complete lysis buffer and the nuclear proteins solubilized in the buffer containing protease inhibitors. The nuclear fraction was separated by centrifuging at 14,000 g, 4°C for 10 min and collecting the supernatant. Protein concentration in the nuclear extract was determined by using protein assay kit (Bio-Rad Laboratories, CA, USA). Sample (5 µl) was added to 250 µl of Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA), incubated for 5 min and read at 595 nm. Protein concentration was calculated from a standard curve.

2.10 NF-κB DNA-binding activity

To measure NF-κB binding to DNA and activation, ELISA-based TransAM NF-κB p65 transcription factor assay kit (Active Motif, SciMed, Carlsbad, CA, USA) was used. Nuclear proteins (5 µg) from the nuclear extract were added to each well coated with an unlabeled oligonucleotide containing the consensus binding site for NF-κB (5'-GGGACTTTCC-3') (Parry and Mackman 1994) and incubated for 1 h at room temperature to allow the active form of NF-κB to bind. A primary antibody directed

against activated NF-κB p65 subunit was added to detect the NF-κB complex bound to the oligonucleotide. Addition of a secondary antibody conjugated to horseradish peroxidase provided a sensitive colorimetric estimation by spectrophotometry. Absorbance was measured at 450 nm using microplate reader (Tecan Systems Inc., San Jose, CA, USA). Results were expressed as fold increase over the control group.

2.11 AP-1 DNA-binding activity

TransAM AP-1 c-Jun transcription factor assay kits (Active Motif, SciMed, Carlsbad, CA, USA) were used to detect and quantify AP-1 activation. AP-1 dimers in the nuclear extract (5 μg of nuclear protein) were added to the 96-well microplate with immobilized oligonucleotide that had a 12-O-tetradecanoyl-phorbol-13-acetate (TPA) - responsive element (TRE) (5'-TGA(C/G)TCA-3') to specifically bind to the oligonucleotide. Primary antibody was used to recognize accessible epitopes on c-Jun proteins upon DNA binding. Secondary antibody conjugated to horseradish peroxidase was added for the colorimetric reaction. Absorbance was read at 450 nm using microplate reader (Tecan Systems Inc., San Jose, CA, USA). Results were expressed as fold increase over the control group.

2.12 Western blot experiment

Lung tissue (50 mg) was homogenised in lysis buffer containing protease inhibitor cocktail (Sigma Chemical Co.) and phosphate inhibitor cocktail (Sigma Chemical Co.). The homogenate was centrifuged at 13,000 g, 4° C for 10 min and the protein concentration in the supernatant was determined using Bradford reagent. 80 μg of the protein in the supernatant was separated on a 12% SDS-polyacrylamide gel

(Invitrogen) and transferred to PVDF membranes (Millipore) by electrophoresis. Non-specific binding was blocked by incubating the membrane at room temperature in 5% non-fat dry milk in phosphate buffered saline Tween 20 (PBST) (0.05% Tween 20 in phosphate buffered saline) for 1 h. The blots were incubated overnight at 4° C with primary antibody (rabbit anti-mouse antibody; Cell Signalling Technology) at 1:1000 dilutions in 2.5% non-fat dry milk in phosphate buffered saline Tween 20. The membranes were then washed four times with phosphate buffered saline Tween 20 and incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilutions in 2.5% non-fat dry milk in phosphate buffered saline Tween 20 for 2 h. Visualization of the blot was done using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL, USA) and exposure to X-ray films (CL-XPosure™, Pierce). Hypoxanthine guanine phosphoribosyl transferase (HPRT) (Santa Cruz Biotechnology; 1:1000 dilution) was used as the housekeeping protein. The band densities were quantified using a UVP® bioimaging system (UVP, Upland, CA, USA). The intensity of bands was analyzed using LabWorks™ Image Analysis software (UVP, CA, USA).

2.13 RNA isolation and quantification

Total RNA was isolated from the lung tissue ($n > 6$ for each group) using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNeasy® mini kit was used to clean up the total RNA after extraction. Briefly, extracted RNA sample was lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer to inactivate RNases leaving intact RNA. Ethanol was added for appropriate binding and the sample was applied to an

RNeasy Mini spin column to bind total RNA to the membrane. Contaminants were washed away and high-quality RNA was eluted in 30-100 µl water. As all RNA molecules longer than 200 nucleotide were purified by this method, mRNA was enriched and most other RNAs (15-20% of total RNA) were selectively excluded. The quantity of extracted RNA was determined by spectrophotometric analysis (NanoDrop®ND1000). RNA samples used were of highest purity with A_{260}/A_{280} ratios close to 2.0 (range: 1.9–2.1). The integrity of RNA was assessed by 1% w/v denaturing agarose gel electrophoresis using GelRed dye to stain 18S and 28S rRNA bands. The RNA sample was stored at −80° C until microarray analysis or RT-PCR.

2.14 Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA (1 µg) was reversely transcribed using iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA, USA) at 25° C for 5 minutes, 42° C for 30 minutes, followed by 85° C for 5 minutes. The cDNA was used as a template for PCR amplification by iQ™ Supermix (Biorad, Hercules, CA, USA). PCR amplification was carried out in MyCycler (Bio-Rad). The reaction mixture was first subjected to 95° C for 3-5 min, followed by an optimal cycle of amplification (denaturation, annealing, and elongation) and a final extension at 72° C for 5-7 min. PCR products were analyzed on 1.5% w/v agarose gel containing 0.1 µl/ml GelRed and visualized by the UVP® bioimaging system (UVP, Upland, CA, USA). The intensity of bands was analyzed using LabWorks™ Image Analysis software (UVP). Densitometry results from PCR products were normalized to the mouse 18S band densities.

2.15 Microarray experiments

GeneChips were prepared individually for each of the mouse lung sample (n = 3 for each group; n = 12 total) according to Affymetrix GeneChip® Expression Analysis Technical Manual. Briefly, double-stranded cDNA was synthesized from total RNA extracted from the mouse lung; an *in vitro* transcription (IVT) and One-cycle Target labeling were done to obtain biotin-labeled cRNA from the cDNA, purified and fragmented before hybridization to the arrays. Biotin labeled RNA fragments ("target") were hybridized to the oligonucleotide probes on the array and stained with streptavidin phycoerythrin conjugate using the Genechip® fluidics station. Fluorescent hybridization signal was detected by the GeneChip® Scanner 3000, enabled for high-resolution scanning. The amount of light emitted at 570 nm was proportional to the bound target at each location on the probe array. GeneChip Operating Software (GCOS) with Affymetrix Microarray Suite 5.0 (MAS5) algorithm was used to define the probe cells and compute intensity for each cell. Each complete probe array was imaged and stored in a separate data file.

2.16 Microarray data analysis

The Expression Console™ software was used to enable probe set summarization and to verify if the hybridization results and initial data were of sufficient quality for secondary analysis. Using GeneSpring™ 7.3 software (Agilent Technologies, CA, USA), differentially expressed genes that showed a fold change of ≥ 2 against sham in at least 1 of 3 mice were selected. Differentially expressed genes were further grouped based on Gene ontology-Biological Process [DAVID Bioinformatics Resources 2008, National Institute of Allergy and Infectious Diseases (NIAID), NIH, Gene function

classification Tool (http://niaid.abcc.ncifcrf.gov/)]. Representation of specific inflammatory and immunoregulatory pathways among the differentially expressed genes was analyzed with Pathway Studio® software (Ariadne Genomics, Rockville, MD) version 5.0. The software uses information available in the current literature to identify common pathways, targets or regulators that are associated with the altered genes to generate biological interaction networks. Microarray expression data was imported into Pathway Studio® to graphically represent all known relationships and potential interactions between the differentially expressed genes. Biological network pathway was proposed according to Pathway Studio® definitions regarding gene expression, interactions and regulations. The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/projects/geo/index.cgi) and are accessible through a GEO Series accession number.

2.17 Statistics

Data were expressed as the mean ± standard error of the mean (SEM). In all figures, vertical bars denote SEM. The significance of changes was evaluated by using ANOVA when comparing three or more groups and Tukey's method as a post hoc test for comparison among different groups. A $p < 0.05$ was taken as significant.

CHAPTER 3. NEUROKININ-1 RECEPTOR ANTAGONIST TREATMENT IN POLYMICROBIAL SEPSIS

3.1 Introduction

SP binds to NK-1 G protein-coupled receptors on the surface of effector cells and acts as a pro-inflammatory mediator in many inflammatory states (Lai *et al.,* 1998; Bhatia *et al.,* 2003). NK-1R activation has been shown to enhance inflammation by decreasing the vascular tone, increasing the endothelial microvascular permeability and transport of inflammatory cells (Chavolla-Calderón *et al.,* 2003). SP and NK-1R have been implicated in the up-regulation of ICAM-1 on vascular endothelial cells and neutrophil infiltration (Nakagawa *et al.,* 1995) and leukocyte adhesion to the endothelial or epithelial cells in the airways (Baluk *et al.,* 1995; DeRose *et al.,* 1994) in inflammation. Furthermore, *PPTA* gene deletion protected against lung injury and mortality in polymicrobial sepsis (Puneet *et al.,* 2006).

Thus in addition to the use of gene knock-out animal models, it was imperative to pharmacologically block the SP receptor to understand the mechanism of action of SP in sepsis. SR140333 (nolpitantium) is a highly potent and selective antagonist of the tachykinin NK-1R in humans and other animals (Emonds-Alt *et al.,* 1993). It has been shown to reduce the severity of inflammation in trinitrobenzene sulfonic acid-induced colitis in rat colon (Di Sebastiano *et al.,* 1999). SR140333 inhibited mustard oil-induced plasma protein extravasations in the dorsal skin of the rat hind paw (Amann *et al.,* 1995). It is also reported to reduce arachidonate release from alveolar macrophages

in guinea-pigs exposed to SP (Boichot *et al.,* 1998). Recently, SR140333 was found to be effective in the modulation of the inflammatory response and airway remodeling in mice (Veron *et al.,* 2004). Moreover, SR140333 is reported to cause antagonism of the SP-induced relaxations of human isolated intralobar pulmonary arterial rings (Pedersen *et al.,* 2000).

Therefore, this part of the study was aimed at evaluating the role of SP and NK-1R in polymicrobial sepsis in mice. As bowel perforation-induced peritonitis patients are reported to have infection resulting from a mixed intestinal flora (Ellaban *et al.,* 2004), a similar model of polymicrobial sepsis that is reliable and clinically relevant was selected. Thus CLP surgery was used to cause polymicrobial sepsis in mice.

3.2 Materials and Methods

3.2.1 Animal ethics

All animal experiments performed were in accordance with the guidelines of the DSO Animal Care and Use Committee (DSOACUC), DMERI, Singapore as mentioned in **Section 2.2.**

3.2.2 Induction of polymicrobial sepsis

Swiss male mice (25-30 g) used for the study were randomly assigned to sham or CLP experimental groups (n > 6 in each group). Polymicrobial sepsis was induced in mice by CLP as described in **Section 2.3**. The **s**ame surgical procedure except the cecal ligation and puncture was performed on sham-operated animals. Vehicle (DMSO diluted in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml, s.c.) was administered to CLP-operated mice either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The animals were sacrificed 8 h after surgery by an i.p. injection of a lethal

dose of pentobarbitone. Blood was collected by cardiac puncture, heparinized, centrifuged, plasma removed and stored at -80° C. Samples of lung were snap frozen in liquid nitrogen and stored at -80° C for subsequent measurement of tissue MPO activity and chemokine, cytokine and adhesion molecule levels. Random cross-sections of lung were fixed in 4 % neutral phosphate-buffered formalin and embedded in paraffin wax for histopathology examination.

3.2.3 Myeloperoxidase estimation

MPO activity as a measure of neutrophil sequestration in lung was quantified as described in **Section 2.4**.

3.2.4 Histopathology

Paraffin-embedded lung sections were stained with hematoxylin / eosin and evaluated by light microscopy as explained in **Section 2.6**.

3.2.5 ELISA analysis of chemokines, cytokines and adhesion molecules

Plasma and tissue homogenates were assayed to evaluate the level of chemokines (MCP-1, MIP-2 and RANTES), cytokines (IL-6, IL-1β and TNF-α) and adhesion molecules (E- and P-selectins, ICAM-1 and VCAM-1) by a sandwich ELISA according to **Section 2.5**. Sample concentration was estimated from the respective standard curve.

3.2.6 Statistical analysis

All values were expressed as mean ± SEM. The significance of changes was evaluated by using ANOVA when comparing three or more groups and Tukey's method as a post hoc test for comparison among different groups. A p value of < 0.05 was considered to indicate a significant difference.

3.3 Results

3.3.1 Effect of SR140333 treatment on neutrophil sequestration in lung in CLP mice

Neutrophil infiltration was quantified by measuring tissue MPO activity. Increased MPO activities represent recruitment of neutrophils and a state of inflammation. Following 8 h after CLP, MPO activity in lung was significantly increased in vehicle treated, in both pre- and post-CLP, animals when compared to the sham mice (**Fig. 3.1**). Treatment with SR140333, 30 min before or 1 h after CLP, significantly reduced the MPO activity in lung (**Fig. 3.1**).

Figure 3.2(a-e) shows representative hematoxylin / eosin stained lung sections from sham and CLP operated mice. Histological evaluation of lung sections showed a significant increase in alveolar thickening, an indicator of edema, as well as inflammatory infiltration in CLP animals treated only with vehicle (**Fig. 3.2b and d**). Lung section from sham group showed little or no edema and inflammatory infiltration (**Fig. 3.2a**). Prophylactic and therapeutic treatment with SR140333 significantly reduced the lung injury, represented by reduced lung edema and neutrophil infiltration (**Fig. 3.2c and e**).

3.3.2 Effect of SR140333 treatment on chemokine levels in lung

Chemokines are produced in response to infection. They act as chemoattractants to various inflammatory cells in sepsis. I determined the levels of CXC chemokine MIP-2 and CC chemokines MCP-1 and RANTES in lung tissue. As expected, lung MIP-2 levels in CLP mice without SR140333 treatment were significantly higher compared to the sham levels (**Fig. 3.3a**). Administration of SR140333, both pre- and post-CLP resulted in a significant decrease in lung MIP-2 levels compared to the corresponding

Fig. 3.1

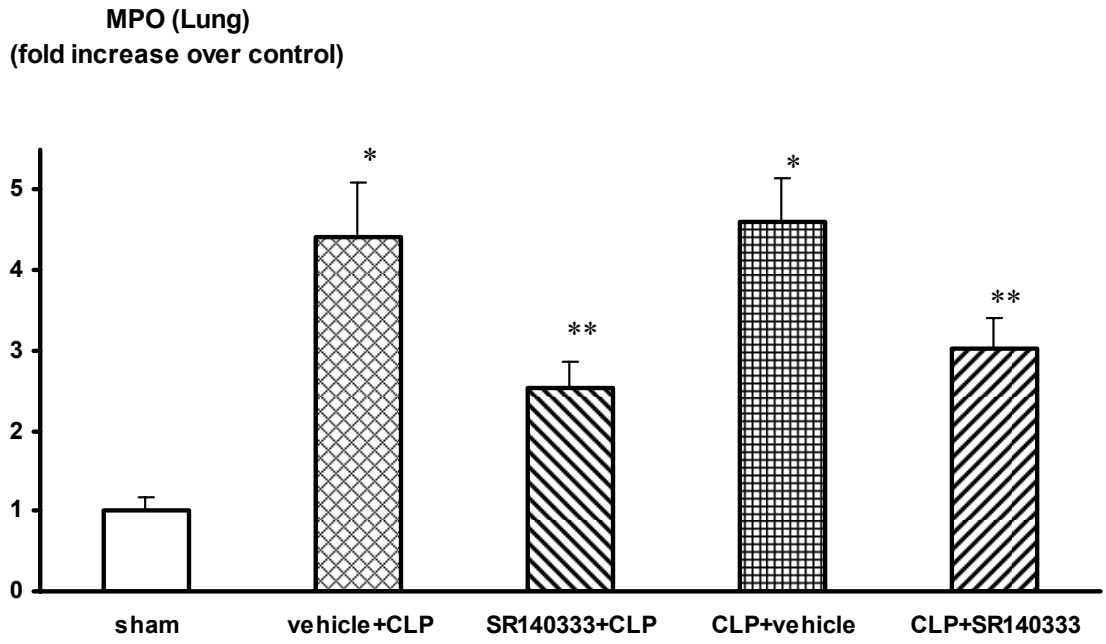


Figure 3.1 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung neutrophil infiltration. Mice (n = 6-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung MPO activity was determined. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

Fig. 3.2 (a-e)

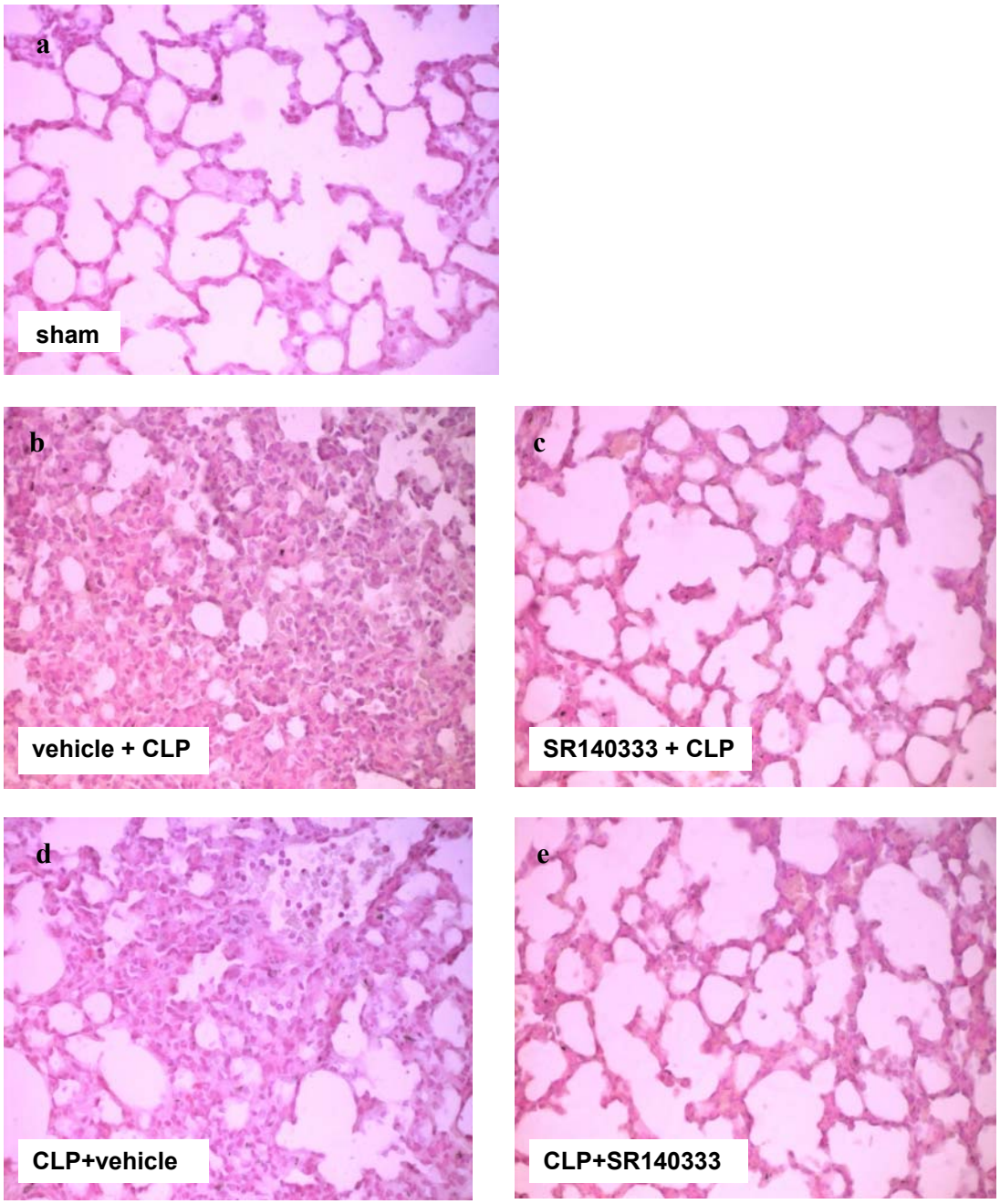


Figure 3.2 Morphological changes in H & E stained mouse lung on induction of sepsis. Panel a, sham: no CLP; Panel b, vehicle (DMSO in PBS, 0.25% v/v) administered 30 min before CLP – Pre-treatment control; Panel c, SR140333 (1 mg/kg) administered 30 min before CLP – SR140333 pre-treatment; Panel d, vehicle (DMSO in PBS, 0.25% v/v) administered 1 h after CLP – Post-treatment control; Panel e, SR140333 (1 mg/kg) administered 1 h after CLP – SR140333 post-treatment.

Fig. 3.3a

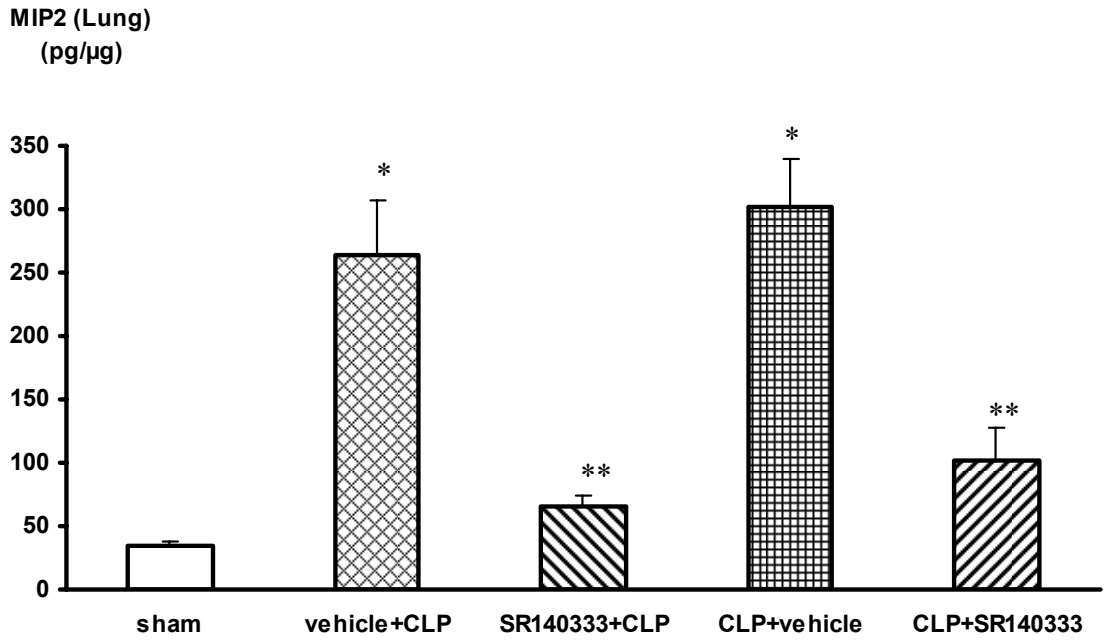


Fig. 3.3b

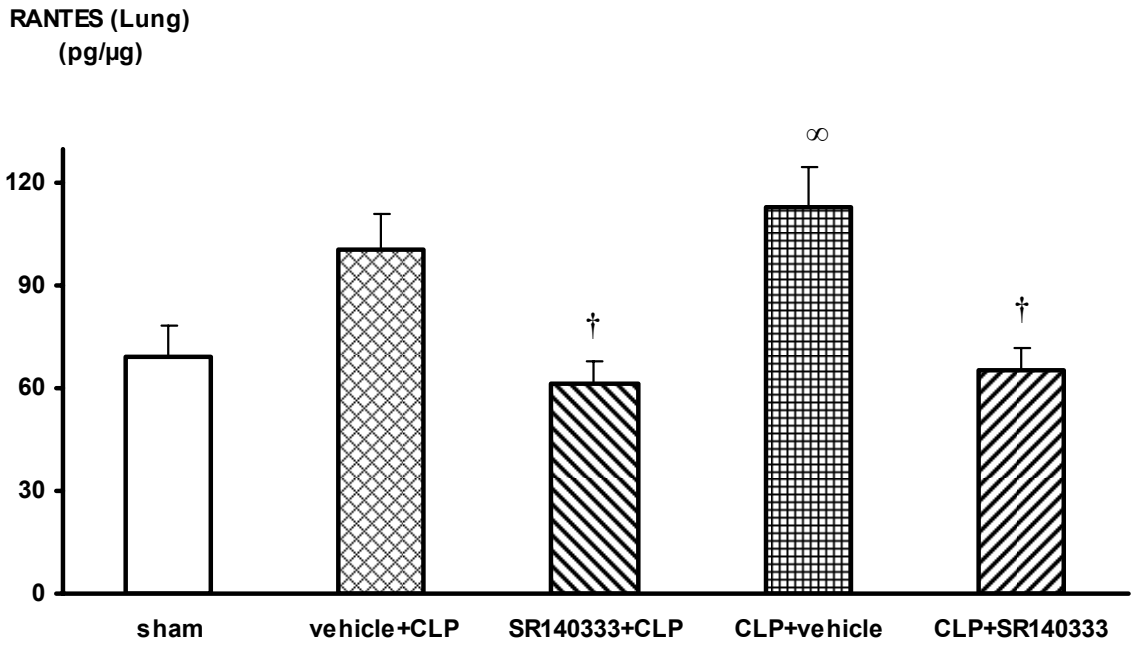


Figure 3.3 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung MIP-2 (Fig. 3.3a) and RANTES (Fig. 3.3b) levels in mice. Mice (n = 6-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-

operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung MIP-2 and RANTES levels were determined. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.001$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; $^{\infty}$ $p < 0.05$ when vehicle-treated CLP animals were compared with sham group animals; † $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

levels in vehicle treated CLP mice (**Fig. 3.3a**). CLP procedure slightly increased the production of lung RANTES compared to that in sham animals, with the increase being significant in animals treated with vehicle 1 h after CLP (**Fig. 3.3b**). However, SR140333 when injected either 30 min or 1 h after CLP resulted in a significant decrease in lung RANTES levels compared to the corresponding levels in the absence of SR140333 (**Fig. 3.3b**).

Similarly MCP-1 production increased significantly in the lungs of CLP mice compared to that of sham animals (**Fig. 3.4a**). This increase in MCP-1 levels was reversed significantly by SR140333 administered either pre- or post-CLP surgery (**Fig. 3.4a**). A similar trend was also found in plasma samples (**Fig. 3.4b**). The high level of plasma MCP-1 found in CLP mice was decreased by SR140333 treatment. The reduction observed was especially significant when SR140333 was injected 30 min before CLP (**Fig. 3.4b**).

3.3.3 Effect of SR140333 treatment on cytokine levels in lung

As a primary line of defense against invading pathogens, cytokines are released in large amount by the host immune system. In the CLP sepsis model, I measured the major cytokines, IL-1β, IL-6, and TNF-α, in lung tissue. As shown in **Fig 3.5a**, CLP animals injected only with the vehicle showed a significant increase in lung IL-1β levels compared to that in sham mice. Administration of SR140333, both 30 min before and 1 h after CLP, resulted in a significant reduction in the lung IL-1β levels. Another important cytokine studied - IL-6, showed a similar pattern of increase in CLP induced sepsis (**Fig. 3.5b**). The lung levels of IL-6 in CLP mice injected only with vehicle, either 30 min before or 1 h after CLP, were significantly higher compared to that in sham operated group. SR140333 when injected either 30 min before or 1 h after

Fig. 3.4a

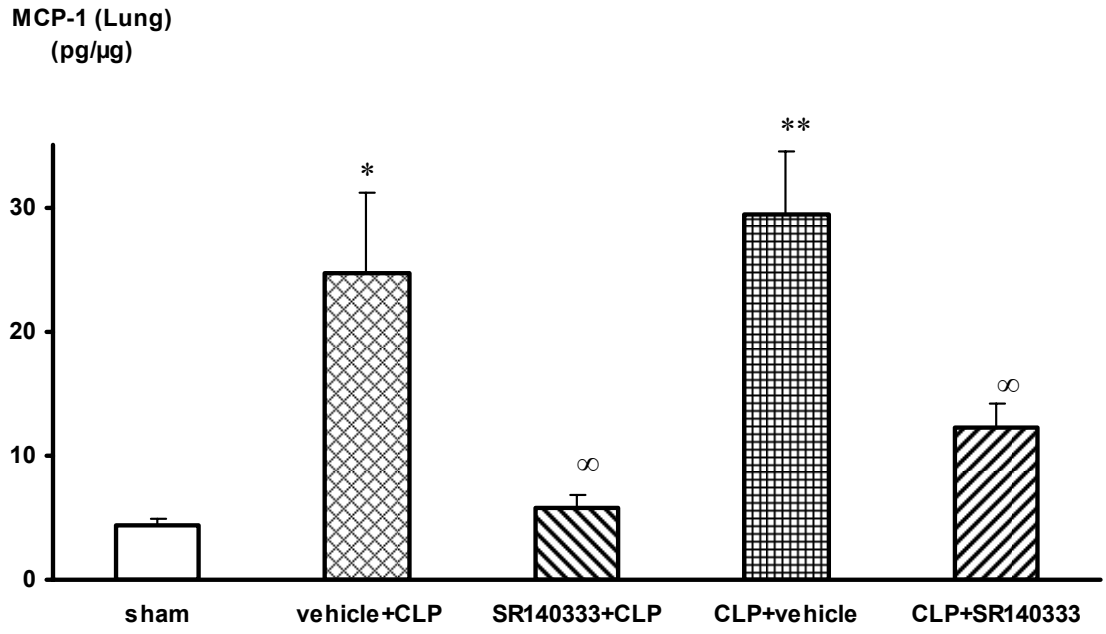


Fig. 3.4b

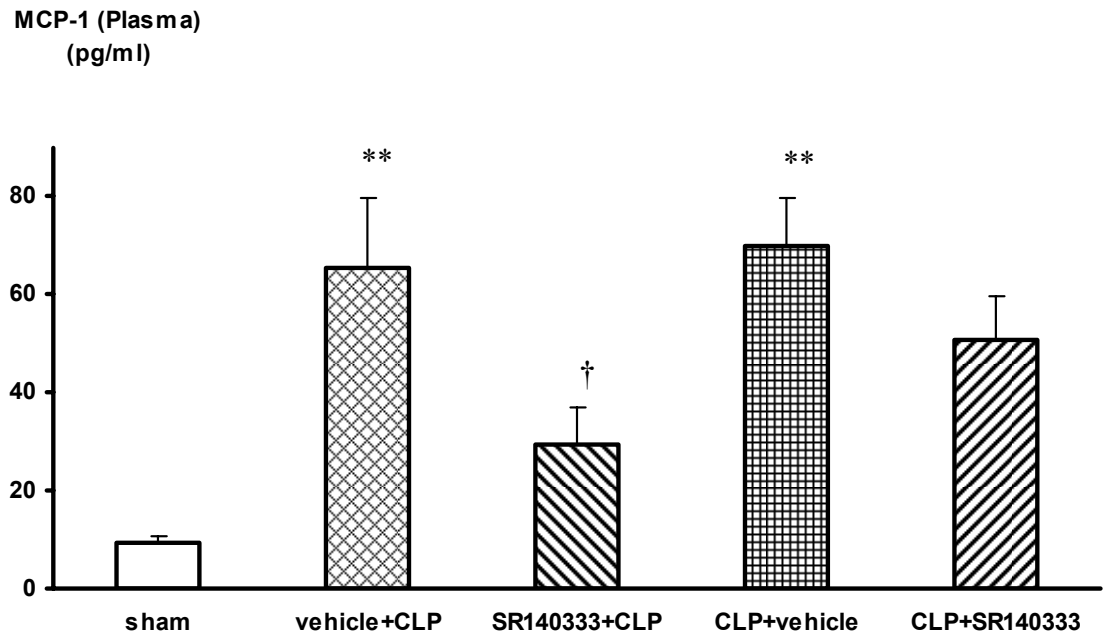


Figure 3.4 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung (Fig. 3.4a) and plasma (Fig. 3.4b) MCP-1 levels in mice. Mice (n = 6-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-

operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and plasma and lung MCP-1 levels were estimated as described in Methods. Results shown are the mean ± SEM. * $p < 0.01$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals; $^{\infty}$ $p < 0.01$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; † $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

Fig. 3.5a

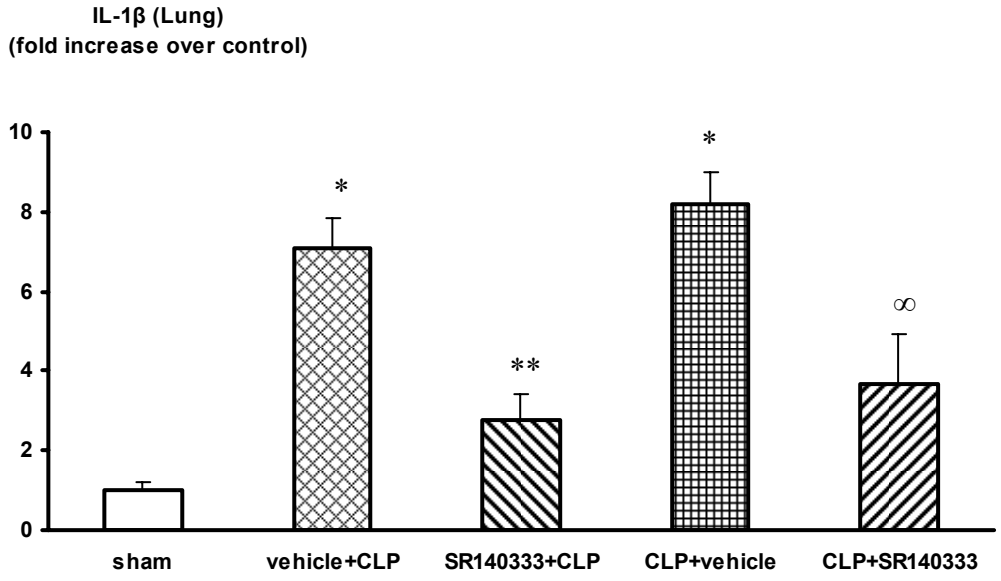


Fig. 3.5b

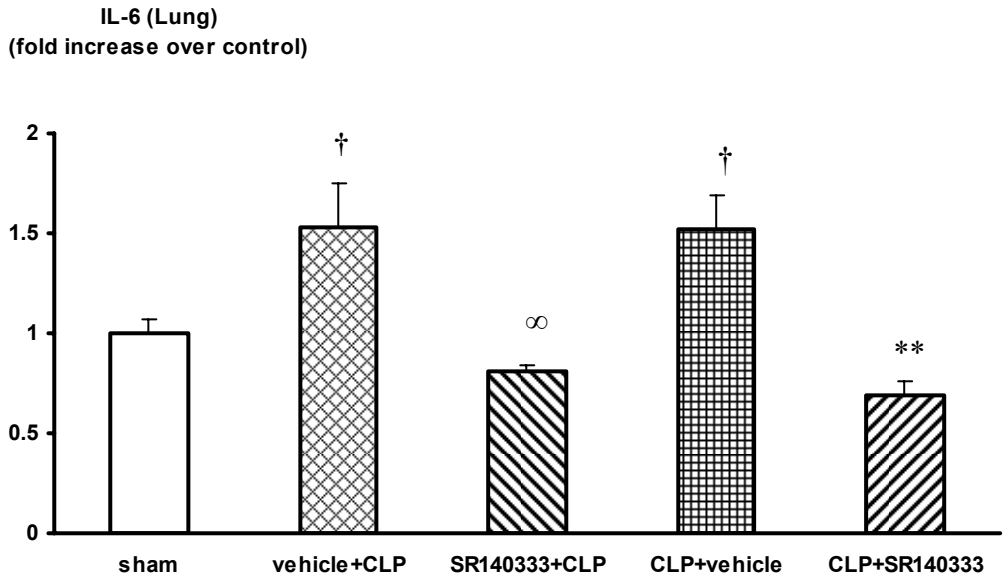


Fig. 3.5c

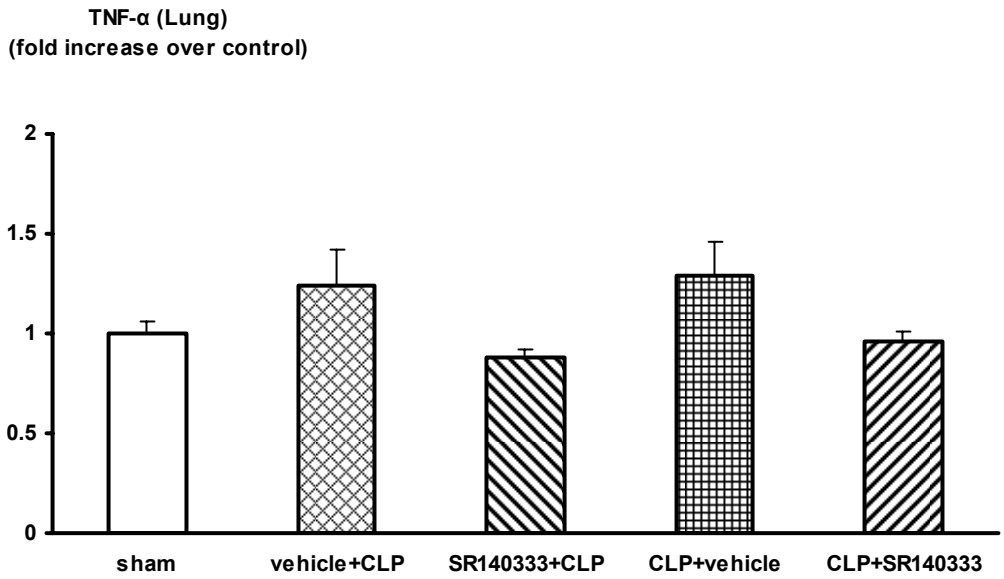


Figure 3.5 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung levels of pro-inflammatory cytokines, IL-1β (Fig. 3.5a), IL-6 (Fig. 3.5b) and TNF-α (Fig. 3.5c), in mice. Mice (n = 6-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung IL-1β, IL-6 and TNF-α level were estimated. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.001$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; $^{\infty}$ $p < 0.01$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; † $p < 0.05$ when vehicle-treated CLP animals were compared with sham group animals.

CLP significantly decreased the lung IL-6 levels compared to the corresponding values in the absence of SR140333 treatment in CLP mice (**Fig. 3.5b**). The lung TNF-α level was not significantly different between sham operated animals and CLP operated mice treated only with the vehicle (**Fig. 3.5c**). Further, treatment with SR140333 did not lower the lung TNF-α level significantly in both the treatment groups 8 h after CLP, as shown in **Fig 3.5c**.

3.3.4 Effect of SR140333 treatment on adhesion molecules in lung

Adhesion molecules play an important role in the leukocyte-endothelial interactions and resulting leukocyte migration into the site of injury or infection. ELISA assay was performed to analyze the lung levels of adhesion molecules such as ICAM-1, VCAM-1, and E- and P-selectin in sepsis. **Figure 3.6a** represents ICAM-1 levels in lung of sham- operated or CLP-operated mice. Animals with CLP surgery had a significantly higher level of ICAM-1 in lung compared to the level in sham surgery group (**Fig. 3.6a**). SR1403333 injected either 30 min before or 1 h after CLP lowered this increase significantly (**Fig. 3.6a**). Lung VCAM-1 level showed no statistically significant increase with CLP operation compared to the levels in sham operated mice (**Fig. 3.6b**). Selectins are a major class of adhesion molecules known to play an important role in early inflammation stages in recruiting the leukocytes to the site of inflammation. Next, I investigated the changes in the production of selectins in lungs. Lung E-selectin level was significantly higher after CLP surgery compared to that in sham-operated mice (**Fig. 3.7a**). This increase was almost completely reversed by SR1403333 when injected either 30 min or 1 h after CLP (**Fig. 3.7b**). A similar trend was observed in case of another selectin, the P-selectin. When P-selectin levels were determined 8 h after CLP procedure, there was a significant increase in both pre- and

Fig. 3.6a

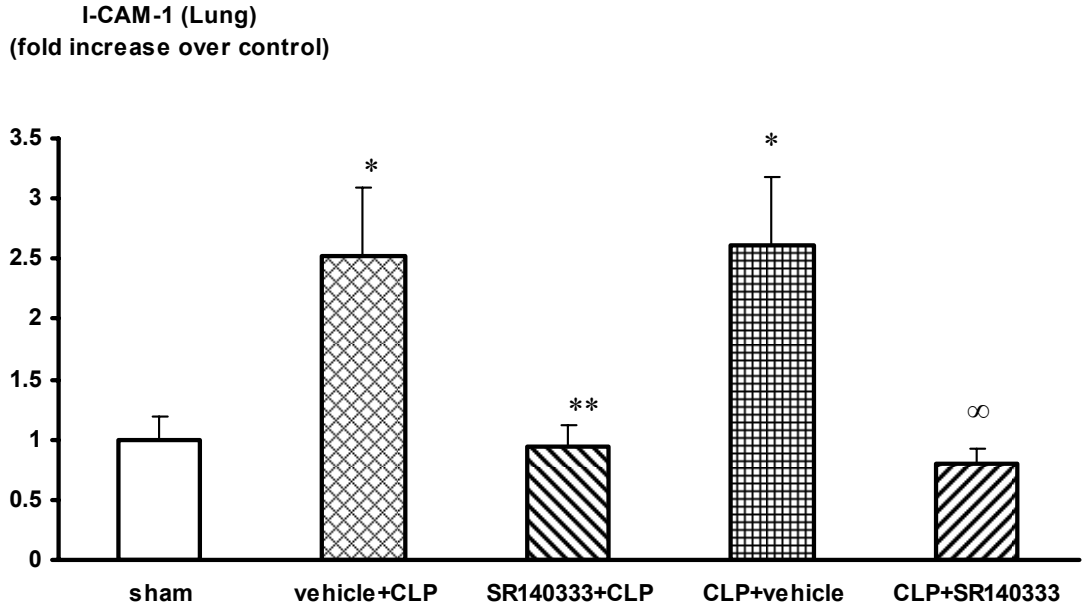


Fig. 3.6b

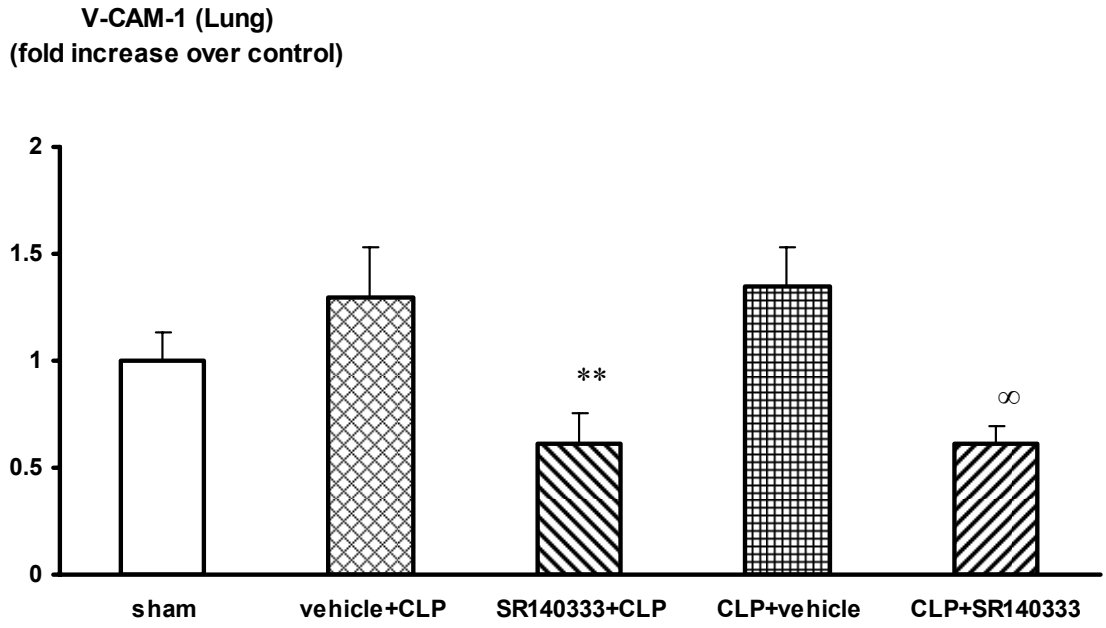


Figure 3.6 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung levels of adhesion molecules, ICAM-1 and VCAM-1, in mice. Mice (n = 6-9 in each group) were divided into CLP-operated and sham-operated groups.

CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung ICAM-1 and VCAM-1 level were estimated. Results shown are the mean ± SEM. * $p < 0.05$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; $^{\infty}$ $p < 0.01$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

Fig. 3.7a

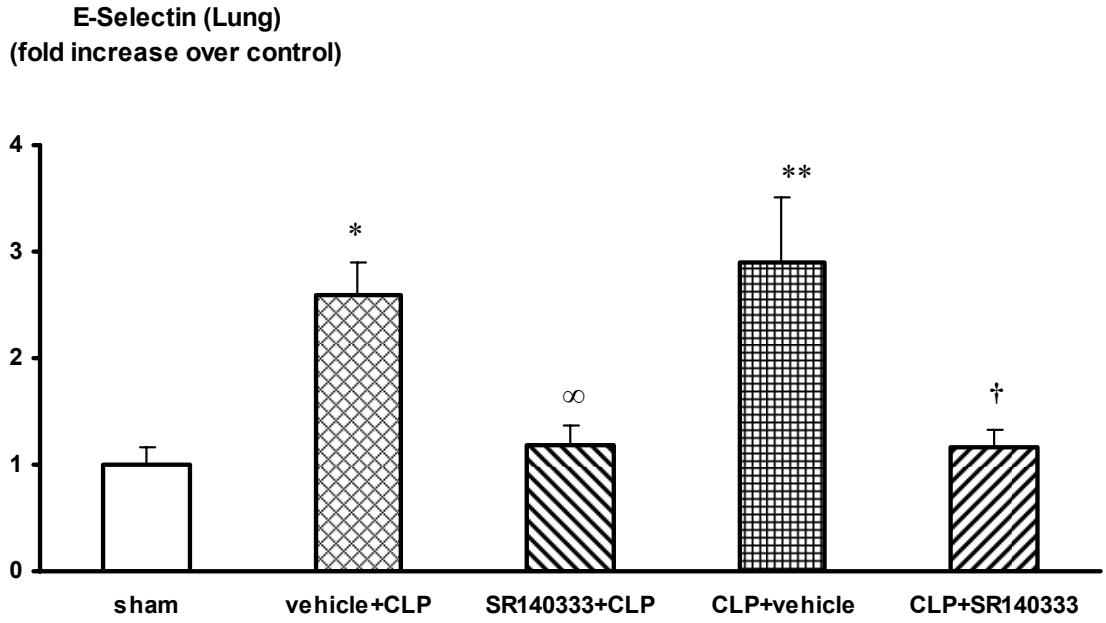


Fig. 3.7b

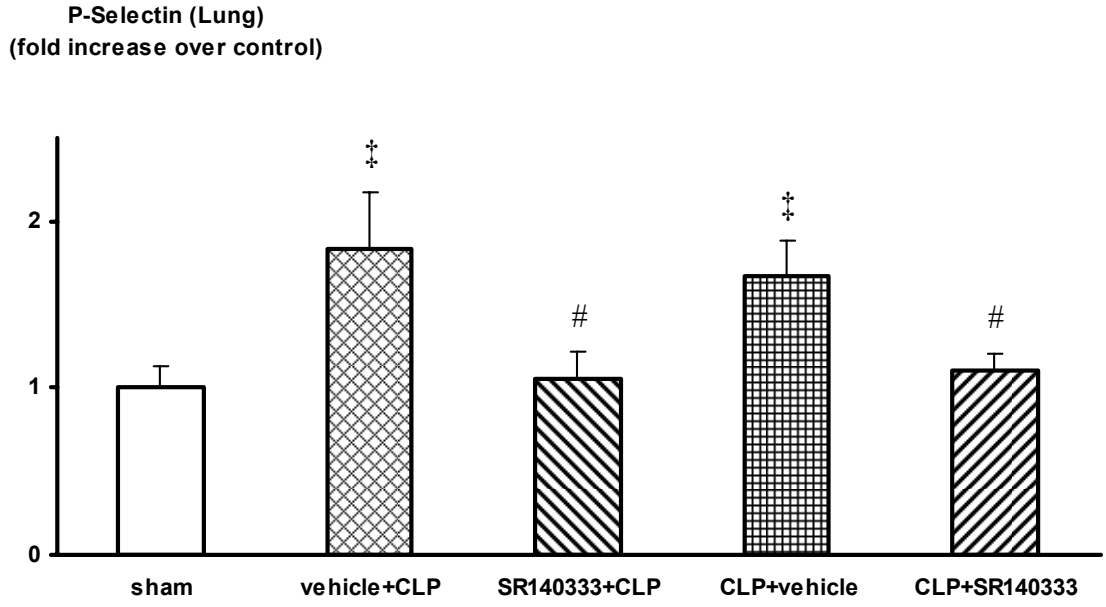


Figure 3.7 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung levels of adhesion molecules, E-selectin (Fig. 3.7a) and P-selectin (Fig. 3.7b), in mice. Mice (n = 6-9 in each group) were divided into CLP-operated and

sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung E-selectin and P-selectin levels were estimated. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.01$ when vehicle-treated CLP animals were compared with sham group animals; $^{\infty}$ $p < 0.01$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; † $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; ‡ $p < 0.05$ when vehicle-treated CLP animals were compared with sham group animals; $^{\#}$ $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

post-vehicle control groups compared to sham operated mice. Administration of SR140333 30 min before or 1 h after CLP resulted in a significant reduction in lung P-selectin levels. These results indicate that treatment with SR140333 reduced the lung inflammation in CLP sepsis, in terms of neutrophil infiltration, levels of chemokines, cytokines and adhesion molecules.

3.4 Discussion

Accumulating evidence over the years has emphasized the pro-inflammatory role of SP in various inflammatory states. SP, acting via NK-1R, has been shown to enhance inflammation. SP is also known to activate mast cells and thus stimulate neutrophil extravasation (Yano *et al.,* 1989; Walsh *et al.,* 1995). The importance of SP and NK-1R in inflammation has been evident from the earlier studies using mice deficient in NK-1R (Bhatia *et al.,* 1998) and *PPTA* gene (Bhatia *et al.,* 2003), which demonstrated a key role of SP in acute pancreatitis and associated lung injury. In addition, treatment with NK-1R antagonist, CP-96345, has been shown to be protective against acute pancreatitis and associated lung injury (Lau *et al.,* 2005; Lau and Bhatia 2006). Recently the role of *PPTA* gene products as key mediators of lung injury in polymicrobial sepsis was reported (Puneet *et al.,* 2006). The study showed that the deletion of *PPTA* gene in mice delayed the pathology of sepsis with protection against pulmonary tissue damage (Puneet *et al.,* 2006). Results from the present study further substantiate the role of SP in sepsis and demonstrate SP as a potential therapeutic target in sepsis.

SR140333 is a highly selective and potent non-peptide antagonist of NK-1R compared to CP96345 and RP67580, two proto-typical non-peptide antagonists of NK-1R

(Emonds-Alt *et al.,* 1993). This competitive blocker has the advantage of being species independent in its potency (Emonds-Alt *et al.,* 1993). Thus we used SR140333 at a dose sufficient enough to inhibit the SP effects of bronchoconstriction and plasma extravasations (Emonds-Alt *et al.,* 1993). CLP was used as the model of polymicrobial sepsis and SR140333 was injected either 30 min before or 1 h after the CLP surgery. The experiment was designed to study the effects of inhibition when SR140333 was given before the surgery as well as after the pathogenic assault has set in. The animals were sacrificed 8 h after CLP to collect blood and tissue as the MPO activity was observed to peak at this time point. Since lung is the main target for damage in sepsis (Cohen 2002), we have focused primarily on pulmonary injury and the levels of inflammatory mediators in lung.

Neutrophil migration to the site of infection is important in the control of infection in sepsis. Neutrophils achieve host defense by releasing proteolytic enzymes and producing reactive oxygen species to degrade invading pathogens. However, excessive production of these factors by overwhelmingly activated neutrophils may lead to host tissue damage during sepsis. MPO activity in lung as a measure of neutrophil infiltration was evaluated 8 h after CLP. MPO activity increased after CLP and was reduced significantly by treatment with SR140333, 30 min before or 1 h after CLP. This was further supported by the histological sections of lung. SR140333 injection clearly reduced the leukocyte infiltration and edema, the signs of lung injury in sepsis.

Recruitment of various inflammatory cells including neutrophils is mediated by chemokines (Salkowski *et al.,* 1998). Chemokines, MCP-1 and MIP-2 are known to orchestrate migration of leukocytes during sepsis and lead to tissue injury. We have shown that MCP-1 and MIP-2 level in lung correlates with neutrophil infiltration in the

lung (Puneet *et al.,* 2006). Consistent with the earlier reports, I found a significant increase in the production of MCP-1 and MIP-2 in lung. SR140333 treatment significantly lowered the lung levels of these two chemokines. RANTES levels also reduced significantly with SR140333 administration.

Pro-inflammatory cytokines such as IL-1β and TNF-α are needed to control infection in sepsis (Ashare *et al.,* 2005). Although these cytokines recruit and activate cells that defend against pathogens during the early phase of infection, if produced in excess, the same cytokines can damage the tissue (Ness *et al.,* 2004). Further, MCP-1 is known to attract neutrophils by activating resident macrophages, which are the source of many inflammatory cytokines and chemokines (Matsukawa *et al.,* 1999). Therefore, next I studied the production of major cytokines IL-6, IL-1β and TNF-α in lung after SR140333 treatment. As the cytokines produced locally in tissue inflammation are more important than that in the serum, I analyzed the levels mainly in lung tissues.

There was a significant increase in the lung levels of both IL-6 and IL-1β 8 h after CLP. Neuropeptides are known to stimulate cytokine production in macrophages, lymphocytes and mast cells (Dickerson *et al.,* 1998). In addition, SP is reported to influence LPS induced production of pro-inflammatory cytokines which was abolished by NK-1R blocking (Dickerson *et al.,* 1998). SR140333 administration either 30 min before or 1 h after CLP in the present study reversed the increase in IL-6 and IL-1β. However, unlike in LPS induced endotoxemia, in CLP model of sepsis TNF-α is not the main mediator of mortality (Eskandari *et al.,* 1992; Villa *et al.,* 1995). Consistently, the present results showed no significant difference in lung TNF-α level after CLP.

Adhesion molecules are important in the activation and adhesion of leukocytes to the endothelium and infiltration into the tissue to fight the infectious organisms (Chandra *et al.,* 2006). Selectins, a major group of adhesion molecules, are involved in the earliest step of acute inflammatory process mediating the rolling of leukocytes (Chandra *et al.,* 2006). High level of pro-inflammatory mediators in sepsis is reported to up-regulate various adhesion molecules (Parent and Eichacker 1999). Absence of ICAM-1 in knock-out mice has been reported to reduce the severity of sepsis by impairing the leukocyte migration and damage of organs (Hildebrand *et al.,* 2005). I found a significant increase in the lung levels of ICAM-1, E- and P-selectin in mice with CLP induced sepsis compared to sham operated group. Treatment with SR140333 lowered the lung levels of ICAM-1, E- and P-selectin significantly. It has been shown that SP induces leukocyte trafficking via the up-regulation of adhesion molecules and treatment with SR140333 reduced the leukocyte rolling, adhesion and emigration (McLean *et al.,* 2000). Thus SR140333 treatment in the present study could have reduced the severity of sepsis by impairing the leukocyte migration via modulating the levels of adhesion molecules. Further there was a significant reduction in VCAM-1 level with SR140333 treatment in CLP mice which is consistent with the reported blocking of SP-induced endothelial VCAM-1 expression in skin cells by a NK-1R antagonist (Quinlan *et al.,* 1999).

As SP levels are known to be increased in sepsis, it can be speculated from the present data that SP acting through NK-1R is one of the major players in sepsis, responsible for the leukocyte responses, inflammatory processes and pulmonary damage. I further hypothesize that chemokines (MCP-1, MIP-2), cytokines (IL-1β, IL-6) and adhesion molecules (ICAM-1, E-selectin, and P-selectin) are modulated downstream by the

action of SP on NK-1R. Thus blocking of NK-1R by SR140333 could ameliorate the inflammatory effects in sepsis.

In summary, data from the present study shows a beneficial role of SR140333 treatment in lung injury in CLP induced mouse sepsis model. SR140333 injected either 30 min before or 1 h after CLP significantly reduced the lung levels of MPO, MIP-2, MCP-1, IL-1β, IL-6, ICAM-1, E-selectin, and P-selectin. As septic lung injury involves various mediators, therapeutic strategies should be targeted at multiple mediators for a successive outcome and NK-1R blocking has a potential therapeutic benefit by lowering the leukocyte infiltration and lung levels of chemokines, cytokines and adhesion molecules. However further clinical studies are needed to establish the benefits of NK-1R blockade in sepsis. Next section provides mechanistic insights into the actions of SP-NK-1R in sepsis.

CHAPTER 4. MECHANISTIC STUDIES

4.1 Introduction

NF-κB transcription factor system is known to control the expression of a number of genes involved in the innate immune response of the body against infection and inflammation. Genes responsible for immunoreceptors, cytokines, chemokines and apoptosis are all modulated by this important family of transcription factor (Viatour *et al.,* 2005). NF-κB activity is reported to be impaired in chronic inflammation (Calzado *et al.,* 2007) and inhibition of NF-κB has been suggested to be beneficial in maintaining the balance between pro- and anti-inflammatory cytokines in inflammatory diseases (Amos *et al.,* 2006). AP-1 is another transcription factor that is induced by inflammatory cytokines and cellular stress. Phosphorylation of AP-1 is necessary for transcriptional activity.

Phosphorylation of NF-κB and AP-1 and thus transcription of pro-inflammatory mediators is facilitated by the activation of various MAPKs. MAPKs in turn are activated by bacterial products, cytokines and chemokines (Brown and Jones 2004; Kyriakis and Avruch 2001). AP-1 c-Jun is reported to be phosphorylated *in vitro* by ERK1 and ERK2 (Pulverer *et al.,* 1993; Pulverer *et al.,* 1991). ERK is also shown to be a regulator of NF-κB activity (Jiang *et al.,* 2004). ERK1/2 reportedly induce NF-κB activation by stimulating downstream MAPK-activated protein kinases (MKs) (Panta *et al.,* 2004; Hayden and Gosh 2004). p38 MAPKs are also activated by inflammatory cytokines and environmental stress.

With NK-1R antagonist treatment showing a beneficial role in lung injury in CLP-induced mouse sepsis model, I next explored the possible mechanism by which SP contributes to sepsis by investigating downstream mediators and transcription factors involved in this effect. Apart from analyzing the activation of NF-κB and AP-1, protein levels of second messengers and MAPKs, mRNA levels of NK-1R and SP concentrations were also evaluated.

4.2 Materials and Methods

4.2.1 Animal ethics

All animal experiments performed were in accordance with the guidelines of the DSO Animal Care and Use Committee (DSOACUC), DMERI, Singapore as mentioned in **Section 2.2.**

4.2.2 Induction of polymicrobial sepsis

Swiss mice (male, 25-30 g) used for the study were randomly assigned to sham or CLP experimental groups (n > 6 in each group). Polymicrobial sepsis was induced in mice by CLP as described in **Section 2.3**. The **s**ame surgical procedure except the cecal ligation and puncture was performed on sham-operated animals. Vehicle (DMSO diluted in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml, s.c.) was administered to CLP-operated mice either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The animals were sacrificed 8 h after surgery by an i.p. injection of a lethal dose of pentobarbitone. Blood was collected by cardiac puncture, heparinized, centrifuged, plasma removed and stored at -80° C. Samples of lung were snap frozen in liquid nitrogen and stored at -80° C for subsequent measurement.

4.2.3 Preparation of nuclear extract

Nuclear extracts were prepared from lung tissue and protein concentrations in the extracts were measured as explained in **Section 2.9**.

4.2.4 NF-κB DNA-binding activity

ELISA-based TransAM NF-κB p65 transcription factor assay kit (Active Motif, SciMed, Carlsbad, CA, USA) was used to measure NF-κB DNA-binding activity from the nuclear extract (reference: **Section 2.10)**.

4.2.5 AP-1 DNA-binding activity

TransAM AP-1 c-Jun transcription factor assay kits (Active Motif, SciMed, Carlsbad, CA, USA) were used to quantify AP-1 activation in the nuclear extract according to **Section 2.11**.

4.2.6 Western blot experiment

Protein levels of IκBα, ERK1/2, JNK, p38, PKCα in lung homogenates were analyzed by Western blot according to **Section 2.12**.

4.2.7 RNA isolation and quantification

Total RNA was isolated from the lung tissue and quantified as described in **Section 2.13**. RNA samples with A_{260}/A_{280} ratios close to 2.0 (range: 1.9–2.1) and integrity were used for RT-PCR.

4.2.8 Semiquantitative RT-PCR

Isolated lung RNA (1 μg) was reversely transcribed and PCR amplified (**Section 2.14)**. The primer sequences and optimal amplification conditions for NK-1R and NK-2R are given in **Table 4.1**.

Table 4.1 Primer sequences and optimal conditions used in PCR analysis

Gene name	Sense primer sequence (5'-3')	Antisense primer sequence (5'-3')	Amplification conditions	No. of amplifi-cation cycles
NK-1R	CTT GCC TTT TGG AAC CGT GTG	CAC TGT CCT CAT TCTCTT GTGGG	95°C 30s; 59°C 30s; 72°C 30s	38
NK-2R	TGC TGT CAT CTG GCT GGT AG	TCT TCC TCG GTT GGT GTC CC	95°C 30s; 61°C 30s; 72°C 30s	42
18S	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG	95°C 30s; 59°C 30s; 72°C 30s	24

4.2.9 Substance P estimation

SP levels were measured in lung and plasma by competitive ELISA method according to **Section 2.7**. The lung SP concentration was then corrected for the DNA content of the tissue.

4.2.10 Nitric oxide measurement

Lung and plasma nitrite levels were determined as an indicator of NO production by spectrophotometric analysis using Griess reagent as described in **Section 2.8**.

4.2.11 Statistical analysis

Statistical analysis was performed as mentioned in **Section 2.17**.

4.3 Results

4.3.1 Effect of SR140333 treatment on lung NF-κB activation after sepsis

As NF-κB is an important transcription factor involved in inflammatory diseases, its activation and nuclear translocation was measured after induction of sepsis and treatment with the NK-1R antagonist. NF-κB activity was significantly increased ($p < 0.001$) in vehicle treated (both prophylactic and therapeutic) mice 8 h after CLP compared to the sham group **(Fig 4.1a)**. Injection of SR140333, both 30 min before and 1 h after CLP, reduced the NF-κB activity significantly ($p < 0.001$) **(Fig 4.1a)**.

Western blot analysis was performed to evaluate the activation and degradation of IκBα. When the inhibitory protein IκBα is phosphorylated and degraded, NF-κB is freed for nuclear translocation. As expected, I observed a significant reduction in IκB levels ($p < 0.01$) in vehicle treated (both prophylactic and therapeutic) mice 8 h after

Fig. 4.1a

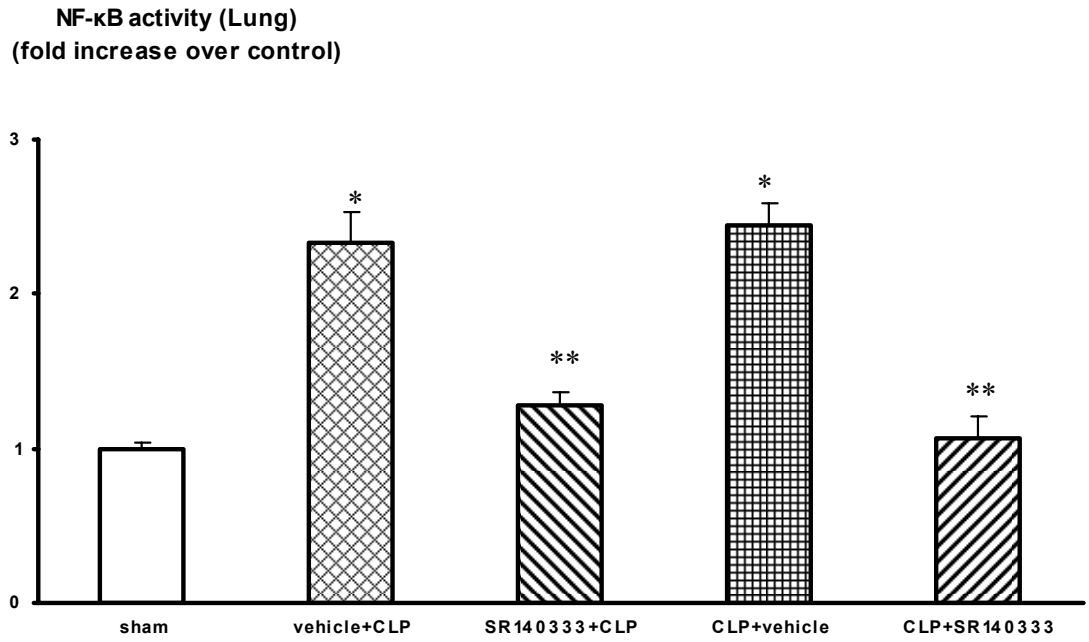


Fig. 4.1b

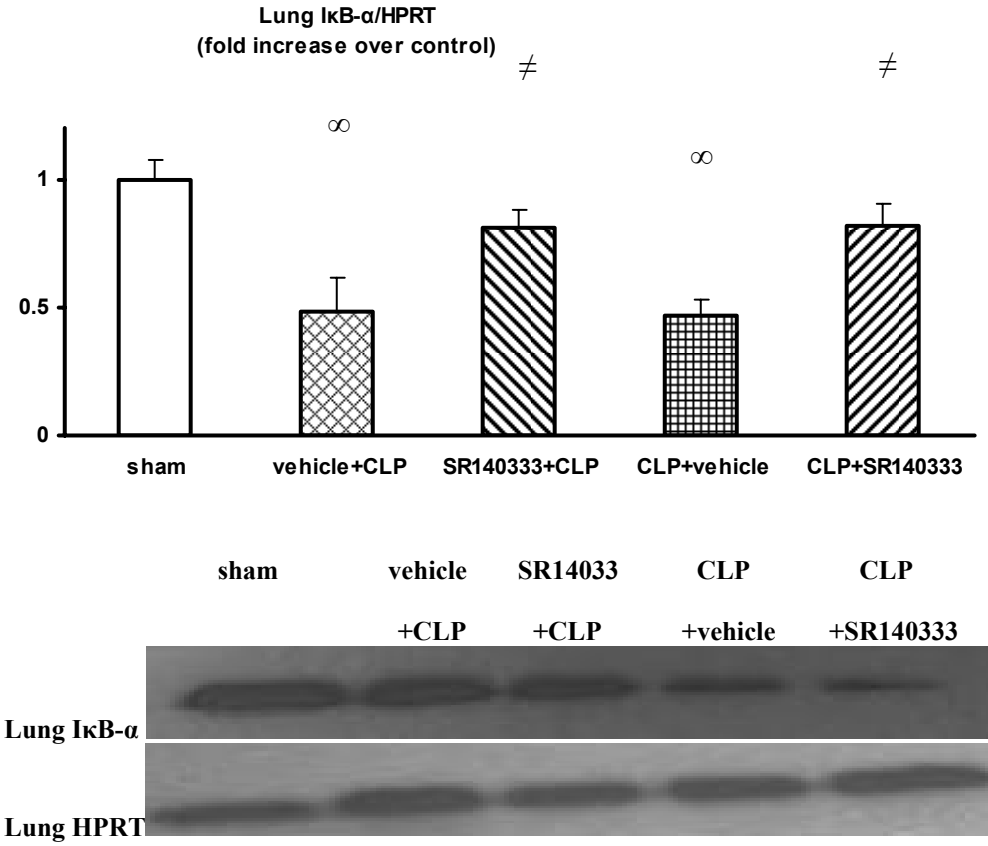


Figure 4.1 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung NF-κB DNA-binding activity and IκB-α level. Mice (n = 6-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung NF-κB DNA-binding activity and IκB-α level were determined. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.001$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; $^{\infty}$ $p < 0.01$ when vehicle-treated CLP animals were compared with sham group animals; $^{\neq}$ $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

CLP compared to the sham group **(Fig 4.1b)**. SR140333 treatment, both 30 min before and 1 h after CLP, restored the IκB levels significantly ($p < 0.05$) **(Fig 4.1a)**.

4.3.2 Effect of SR140333 treatment on lung AP-1 activation after sepsis

Activation of another transcription factor that is involved in sepsis, AP-1 c-Jun, was also measured after induction of sepsis and treatment with the NK-1R antagonist. 8 h after CLP, AP-1 activity was significantly increased ($p < 0.001$) compared to the sham group in vehicle treated mice **(Fig 4.2)**. S.c administration of the NK-1R antagonist, SR140333, both 30 min before and 1 h after CLP, reduced the AP-1 activity significantly ($p < 0.05$).

4.3.3 Effect of SR140333 treatment on MAPKs and PKCα in sepsis

To evaluate the link between NK-1R antagonist treatment and transcription factor inhibition, western blot analysis was performed for various MAPKs: ERK1/2, p38 and JNK. Significant activation of ERK1/2 to the phosphorylated form was detected 8 h after CLP in vehicle treated mice lung homogenates ($p < 0.05$) **(Fig 4.3a)**. SR140333 treatment, both 30 min before and 1 h after CLP, showed a trend to reduce the phospho ERK1/2 levels, although the reduction was not statistically significant **(Fig 4.3a)**.

p38 and JNK MAPKs showed very weak signals and did not show significant differences between the groups (data not shown).

The enzyme PKCα involved in signal transduction of G-protein-coupled receptors was also evaluated in sepsis. Significant phosphorylation and activation of PKCα was observed 8 h after sepsis in mice injected with only vehicle compared to the sham group ($p < 0.05$) **(Fig 4.3b)**. Prophylactic and therapeutic blocking of NK-1R with SR140333 resulted in a significant reduction in lung PKCα phosphorylation in mice

Fig. 4.2

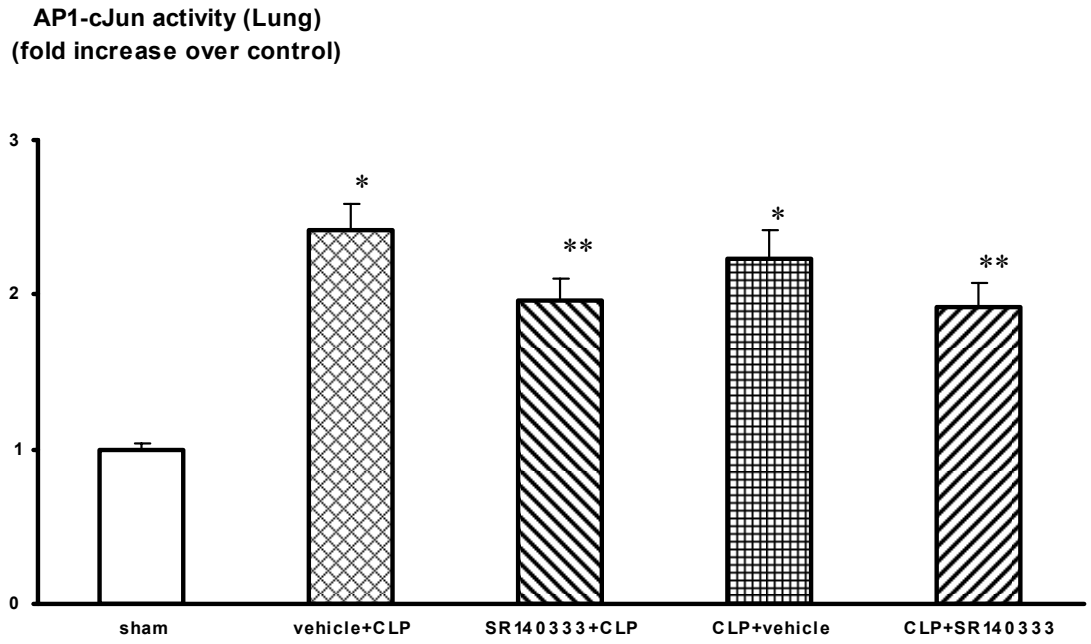


Figure 4.2 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung AP-1 activity. Mice (n = 8-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung AP-1 cJun activity was determined. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

Fig. 4.3a

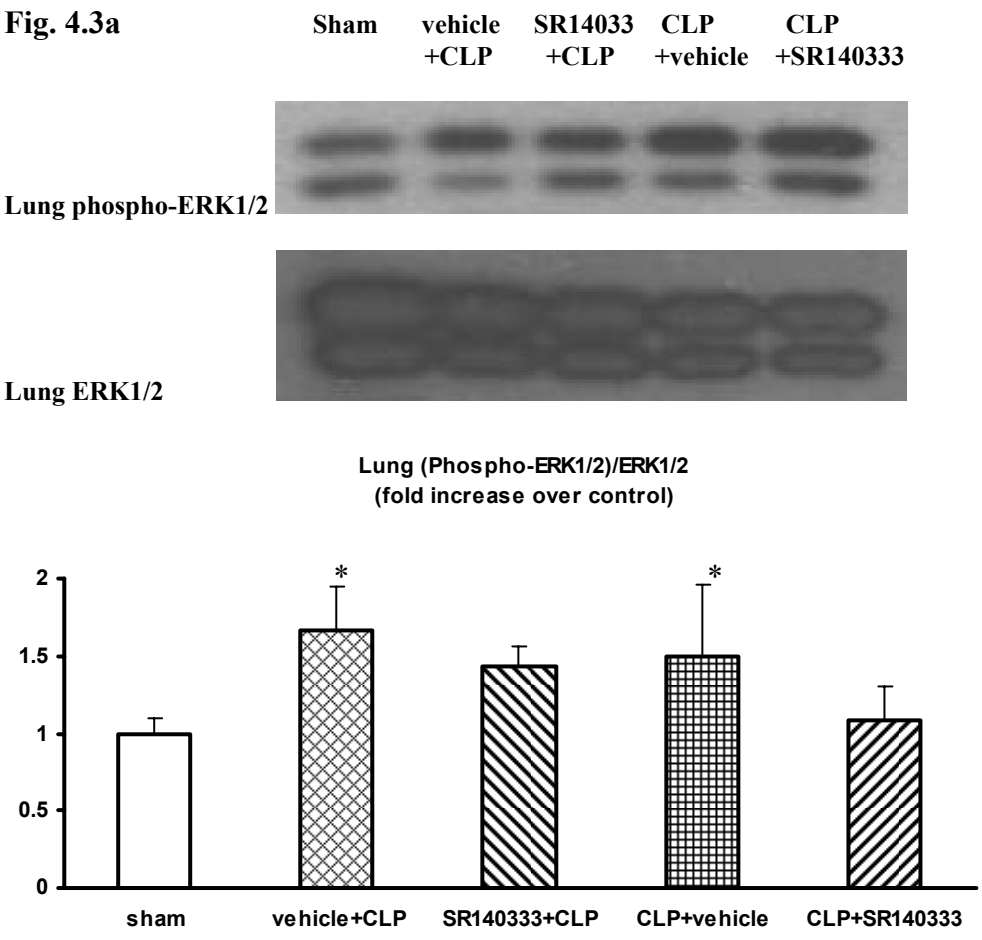
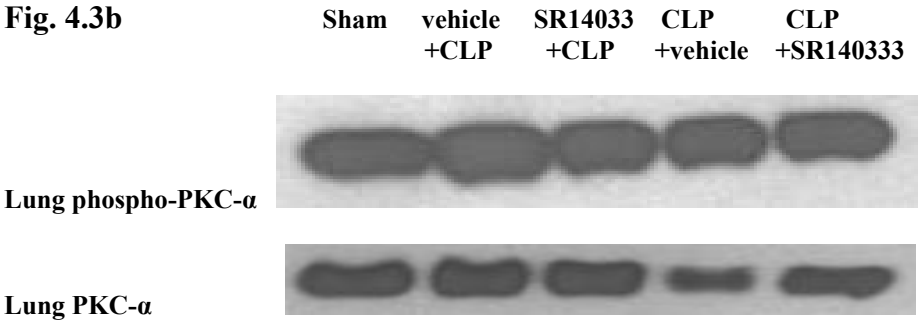


Fig. 4.3b



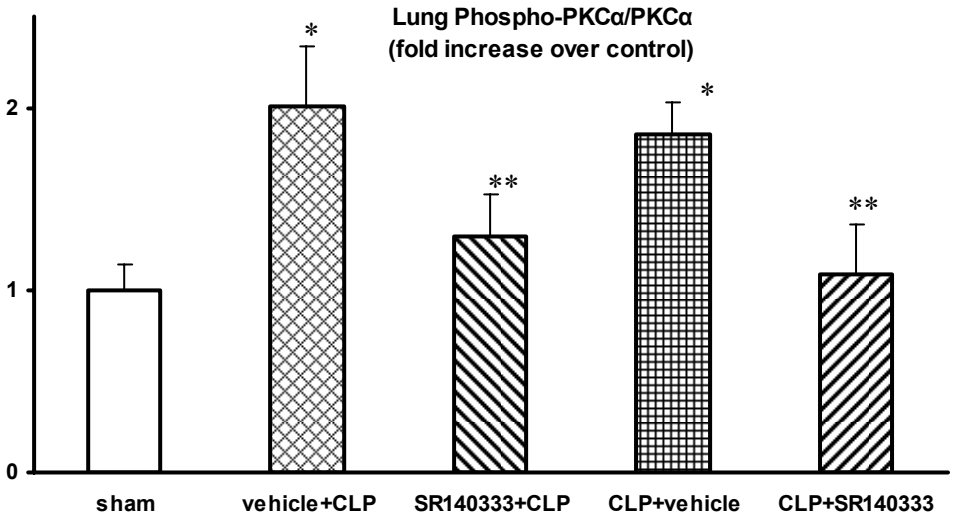


Figure 4.3 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung Phospho ERK1/2 (Fig.4.3a) and Phospho-PKCα (Fig. 4.3b). Mice (n = 6 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung Phospho-ERK1/2 and Phospho-PKCα were determined. Results shown are the mean ± SEM. * $p < 0.05$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

8 h after sepsis induction ($p < 0.05$).

4.3.4 Effect of SR140333 treatment on lung NK receptors after sepsis

mRNA levels of NK receptors, NK-1R and NK-2R, were analyzed by semiquantitative RT-PCR. CLP-induced sepsis resulted in a significant up-regulation of both the NK receptors in vehicle-treated mice compared to sham group ($p < 0.05$) **(Fig 4.4a and b)**. NK-1R blocker had no significant changes on the expression of NK-1R and NK-2R.

4.3.5 Effect of SR140333 treatment on SP levels in sepsis

Next I measured the protein levels of SP in plasma and lung. Both systemic ($p < 0.01$) **(Fig 4.5a)** and lung tissue ($p < 0.01$) **(Fig 4.5b)** SP levels were elevated in mice subjected to CLP surgery as shown previously (Puneet *et al.,* 2006). Treatment with SR140333 did not affect the lung SP levels **(Fig 4.5b)**. However, plasma SP levels were significantly reduced by the NK-1R antagonist, both prophylactically and therapeutically ($p < 0.05$) **(Fig 4.5a)**.

4.3.6 Effect of SR140333 treatment on NO levels in sepsis

Lung and plasma nitrite levels as a measure of NO were measured. As expected in sepsis, NO levels were significantly higher in plasma ($p < 0.05$) and lung ($p < 0.001$) 8 h after CLP procedure **(Fig 4.6a and b)**. SR140333 administered s.c. failed to lower the elevated NO levels in lung **(Fig 4.6b)**. However, the reduction was significant in plasma for both, prophylactic and therapeutic groups ($p < 0.01$) **(Fig 4.6a)**.

4.4 Discussion

In the previous chapter, the data indicated that SP acting through NK-1R was responsible for the leukocyte responses, inflammatory processes and pulmonary damage in polymicrobial sepsis and various chemokines, cytokines and adhesion

Fig. 4.4a

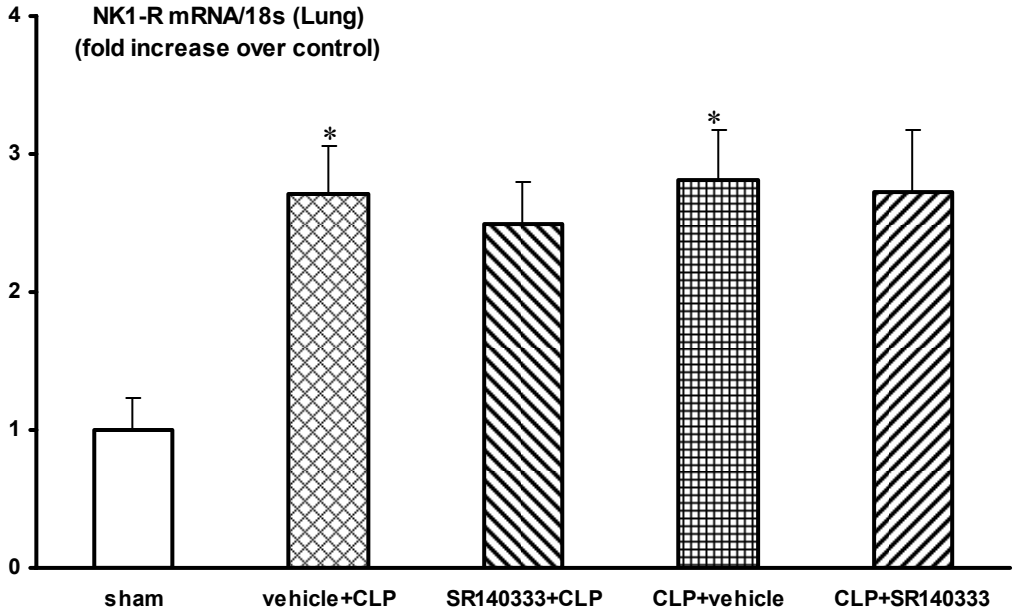


Fig. 4.4b

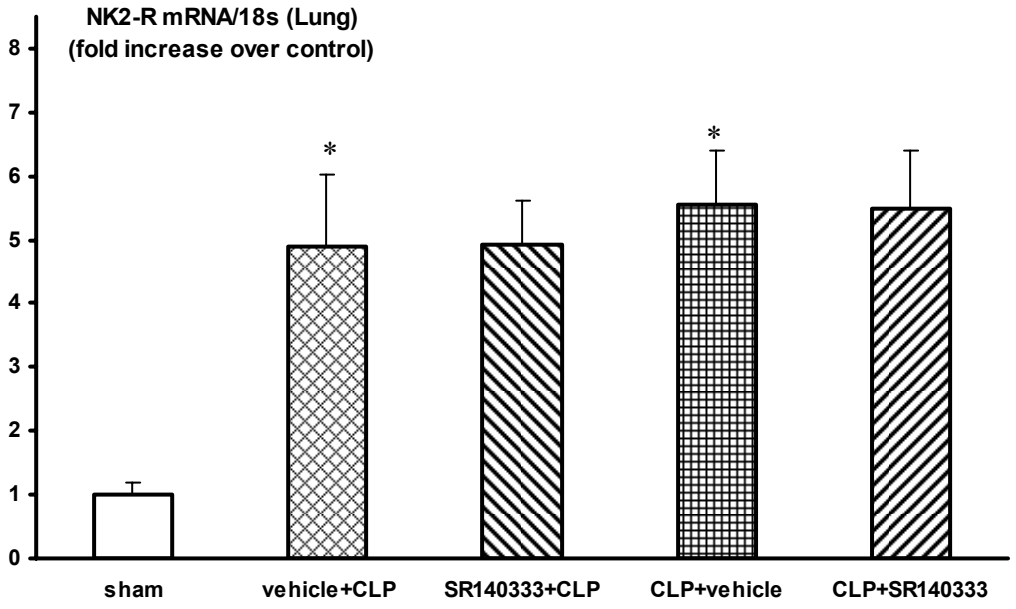


Figure 4.4 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on lung NK-1R (Fig.4.4a) and NK-2R (Fig. 4.4b) mRNA levels. Mice (n = 6-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal

ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung NK-1R and NK-2R mRNA were determined. Results shown are the mean ± SEM. * $p < 0.05$ when vehicle-treated CLP animals were compared with sham group animals.

Fig. 4.5a

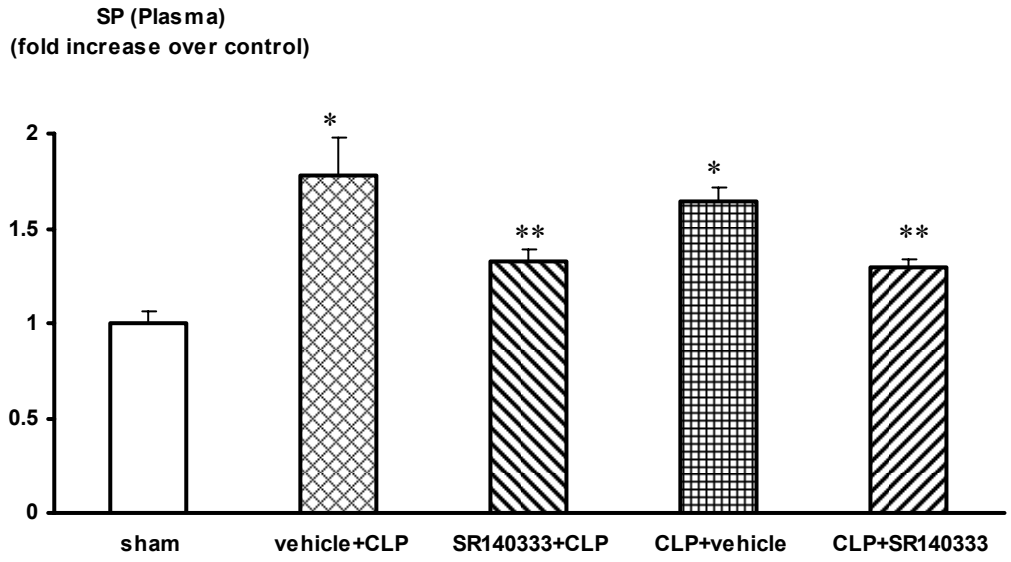


Fig. 4.5b

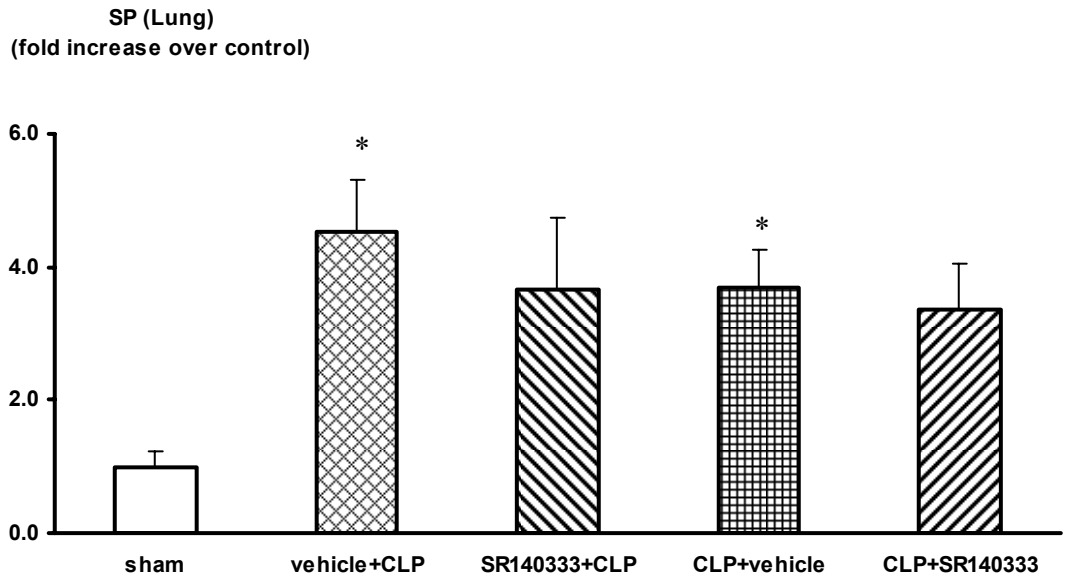


Figure 4.5 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on plasma (Fig 4.5a) and lung (Fig. 4.5b) SP levels. Mice (n = 6-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were

sacrificed and plasma and lung SP levels were determined. Results shown are the mean ± SEM. * $p < 0.01$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.05$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals.

Fig. 4.6a

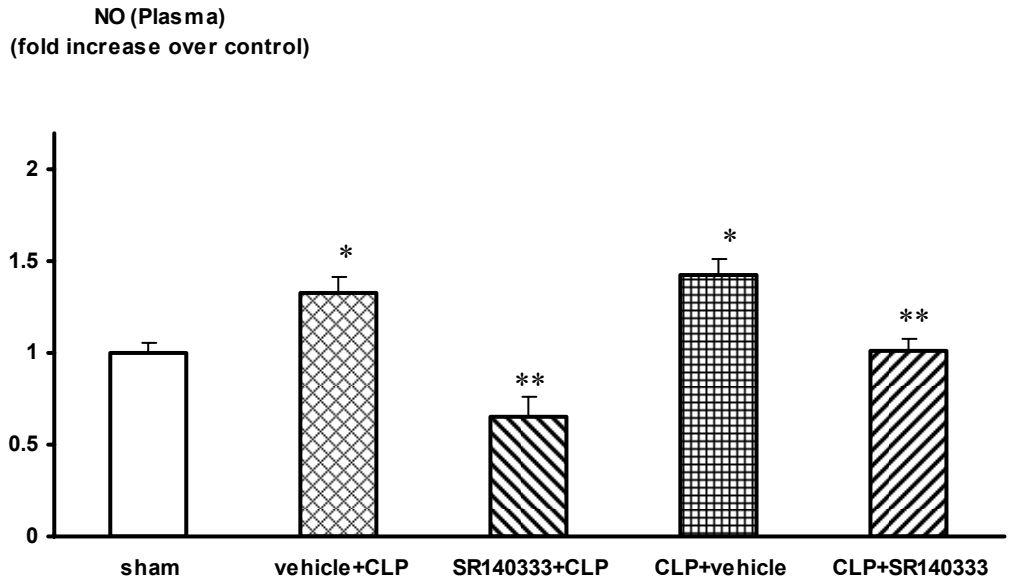


Fig. 4.6b

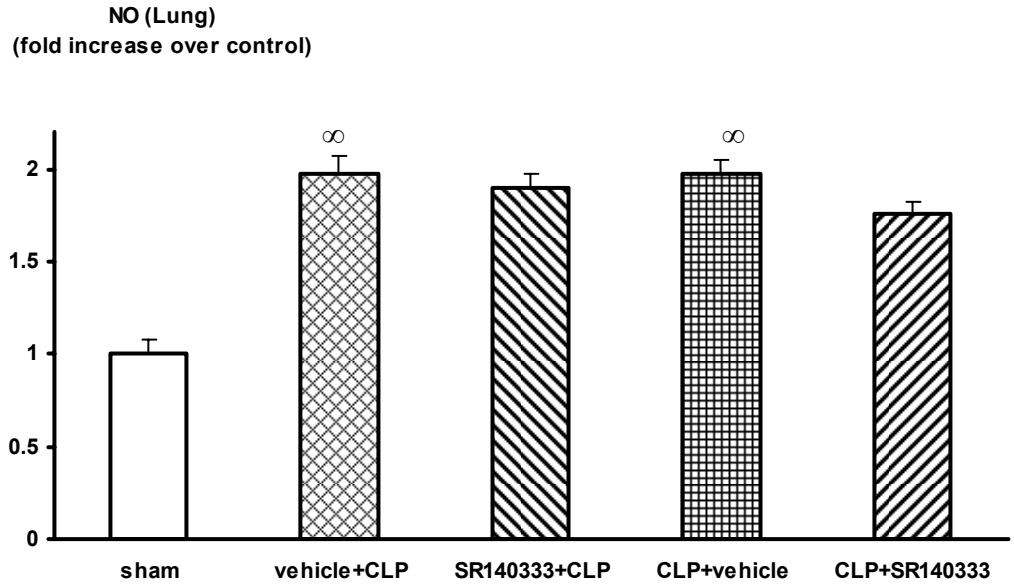


Figure 4.6 Effect of SR140333 administration, either 30 min before or 1 h after CLP, on plasma (Fig 4.6a) and lung (Fig. 4.6b) NO levels. Mice (n = 6-7 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or SR140333 (1 mg/kg; 0.25 mg/ml) s.c. either 30 min before (pre-treatment) or 1 h after (post-treatment) the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and plasma and lung NO levels were determined. Results shown are the

mean ± SEM. * $p < 0.05$ when vehicle-treated CLP animals were compared with sham group animals; ** $p < 0.01$ when SR140333-treated CLP animals were compared with vehicle-treated CLP animals; $^{\infty}$ $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals.

molecules modulated the downstream signaling pathway. Blocking of NK-1R was beneficial to the mice in managing the inflammatory effects in sepsis. So my next goal was to explore the underlying mechanisms for these beneficial effects of SR140333 in sepsis. It was important to see how the downstream intracellular signaling was propagated in order to achieve this effect.

It is well known that SP, after binding to NK-1R, up-regulates pro-inflammatory cytokines (Williams *et al.,* 2007). Also, activation of inflammatory mediators in sepsis depends mainly on the activation of pro-inflammatory transcription factor NF-κB (Calzado *et al.,* 2007). NF-κB is activated by a wide range of signals including bacterial LPS, cytokines, viral infection and lung injury. Furthermore, lung epithelial cells have been reported to highly express cytokine genes in response to stimuli or injury (Chang *et al.,* 1998; Hierholzer *et al.,* 1997). My data on NF-κB shows that SP acting through NK-1R was responsible for NF-κB activation and thus expression of pro-inflammatory mediators in sepsis. Treatment of septic mice with NK-1R antagonist resulted in lowering of IκB degradation and reduction in nuclear translocation of NF-κB. Although NF-κB inhibition has been reported to improve survival in endotoxin models, the situation is not so straight forward in CLP-induced sepsis (Calzado *et al.,* 2007). Impaired survival has been reported when NF-κB was inhibited by the metal chelator PDTC (Joshi *et al.,* 2002). While inhibition of NF-κB decreases the inflammatory mediators, complete loss of anti-apoptotic actions of NF-κB might be detrimental in the host-defense against invading pathogens (Calzado *et al.,* 2007). I found a lowering of NF-κB activation, but the levels were still elevated compared to the basal levels. SR140333 treatment lowered the activity of AP-1 transcription factor as well in sepsis. AP-1 is reported to regulate various cytokine and

chemokine genes (Zenz *et al.,* 2008). Thus SR140333 appears to modulate inflammatory mediators by regulating the activation of transcription factors NF-κB and AP-1.

MAPKs signaling cascade, especially ERK1/2, is known to activate NF-κB transcription (Kyriakis and Avruch 2001; Chen *et al.,* 2004; Wang *et al.,* 2005). *In vitro* treatment of Tacr1-expressing cells with SP is shown to increase phosphorylation of ERK1/2 (Williams *et al.,* 2007). I investigated the potential involvement of ERK in mediating SP-NK-1R induced NF-κB activation in sepsis. ERK phosphorylation was significantly increased in CLP induced sepsis, indicating a possible activation of NF-κB mediated by this MAPK in sepsis. However, NK-1R blocking did not reduce the ERK level to a significant extent. Tachykinins have the ability to activate NF-κB by multiple mechanisms (Williams *et al.,* 2007). In colonic epithelial cells, SP-induced NF-κB activation was dependent on the activity of PKCδ but not calcium or ERK (Koon *et al.,* 2005; Zhao *et al.,* 2002). Thus, possibly other mechanisms might be involved in the SP-NK-1R mediated signaling in sepsis in addition to ERK.

NK-1R, which mediates the actions of SP, is a G-protein-coupled receptor (Mizuta *et al.,* 2008; Williams *et al.,* 2007) and PKC is a downstream signalling molecule activated by G-protein-coupled receptors (Nishizuka 1995). The serine/threonine PKC family has at least 11 mammalian isozymes and are important components of intracellular signal transduction pathways (Lee *et al.,* 2008). PKC is involved in regulating transcription and mediating immune response (Tan and Parker 2003). It is known to aggregate platelets and constrict bronchial smooth muscles (Harper and Poole 2007; Dempsey *et al.,* 2007). A PKC inhibitor is reported to suppress angiotensin-induced NF-κB expression in vascular smooth muscles (Ji *et al.,* 2009).

PKCα acting upstream of PKCθ is reported to activate the IKK complex and NF-κB in T lymphocytes (Trushin *et al.,* 2003). PKC inhibitor has been shown to block SP induced activation of NF-κB *in vitro* (Williams *et al.,* 2007). I observed a significant phosphorylation and activation of PKCα, 8 h after sepsis and blocking of NK-1R with SR140333 resulted in a significant reduction in lung PKCα levels. Thus it is possible to conclude that SP acting through NK-1R promotes inflammation in polymicrobial sepsis via NF-κB and AP-1 activation, mediated also by PKCα.

NK-1R is implicated in mediating pro-inflammatory processes and its expression is reported to be up-regulated in inflammatory conditions (O'Connor *et al.,* 2003; Chu *et al.,* 2000). NK-1R inhibitor is shown to diminish lung inflammation in rats infected with respiratory syncytial virus (King *et al.,* 2001). Increased expression of NK-1 (Adcock *et al.,* 1993) and NK-2 (Bai *et al.,* 1995) receptor mRNA has been reported in asthmatic airways. As expected, expression of NK-1R and NK-2R in the present study was elevated 8 h after CLP-induced sepsis. Treatment with NK-1R antagonist had no significant change in the receptor expression, although it reduced the lung inflammation in sepsis.

Similarly lung and plasma SP levels were elevated in septic mice in the absence of NK-1R blocker. We have earlier shown SP to be a key mediator of sepsis and associated lung damage (Puneet *et al.,* 2006). Tissue (lung) levels of SP were not affected by blocking the actions of SP. However it is intriguing that plasma SP levels were lowered by the NK-1R antagonism. It is possible that blocking of SP actions resulted in its increased clearance from the bloodstream, but the local levels at the site of injury remained elevated; as such the increase in SP levels in CLP was of higher magnitude in tissue compared to plasma. Expression of *PPTA* gene that encodes for SP

and NKA was also observed to be up-regulated 8 h after CLP-induced sepsis and the levels correlated with the corresponding lung and plasma SP concentrations (data not shown).

NO is considered to be a mediator of sepsis and associated tissue damage (Vincent *et al.,* 2000). In normal conditions, vascular endothelial cells produce low levels of NO that regulate blood pressure by mediating smooth-muscle relaxation (Kuhl and Rosen 1998). However, in sepsis, LPS and cytokines induce iNOS to synthesize high levels of NO leading to smooth-muscle relaxation, pressor refractory vasodilation, and shock (Kuhl and Rosen 1998). The present data shows that CLP induced sepsis resulted in a significant increase in lung NO levels and NK-1R blocking had no effect on tissue NO levels. However, plasma NO levels were reduced by SR140333 treatment. The reason for this effect is not clear.

In conclusion, the data reveal that SP acting via NK-1R initiates signaling cascade that is mediated by PKCα and ERK and leads to NF-κB and AP-1 activation and further modulates pro-inflammatory mediators in polymicrobial sepsis and the effect of SP is blocked by NK-1R antagonist SR140333. Next, I studied the role of NK-2R antagonism in sepsis.

CHAPTER 5. NEUROKININ-2 RECEPTOR ANTAGONIST TREATMENT IN POLYMICROBIAL SEPSIS

5.1 Introduction

SP, NKA and NKB are the three major members of tachykinin family, each of which bind to specific neurokinin receptors in a preferential manner and may play a critical role in inflammation. The effects of SP are mediated mainly by NK-1R as it binds NK-1R with high affinity compared to its low affinity to the other tachykinin receptors, NK-2R and NK-3R (Koon and Pothoulakis 2006). NKA and NKB show high binding affinity for NK-2R and NK-3R respectively (Patacchini *et al.,* 2004). NK-2R mRNA, but not NK-3R, has been detected in normal lungs (Lau and Bhatia 2006). A selective NK-2R inhibitor has been reported to inhibit NKA induced bronchoconstriction in asthmatics (Van Schoor *et al.,* 1998).

Although the effects of SP were found to be mediated mainly via NK-1R in sepsis, it was interesting to explore if NK-2R had any role in the actions of SP in sepsis. Both NK-1 and NK-2 receptors are reported to be up-regulated in burn-associated sepsis promoting the formation of oedema and hyperalgesia (Sabato *et al.,* 2003). During the early phase of endotoxemia, endogenous tachykinins have been shown to act through NK-2R affecting lung mechanics and both NK-1 and NK-2 receptors were involved in causing airway microvascular leakage (Tang *et al.,* 2002). GR159897 is a highly potent, selective and long acting non-peptide NK-2R antagonist (Beresford *et al.,* 1995; Advenier 1995). It is proposed to be a useful tool for studying the physiological

and pathophysiological role of NK-2R activation. Thus, I studied the effect of blocking NK-2R with GR159897 in polymicrobial sepsis. Lung MPO activity, chemokine and cytokine levels were measured to evaluate the beneficial effects, if any, of blocking NK-2R in sepsis.

5.2 Materials and Methods

5.2.1 Animal ethics

All animal experiments performed were in accordance with the guidelines of the DSO Animal Care and Use Committee (DSOACUC), DMERI, Singapore as mentioned in **Section 2.2.**

5.2.2 Induction of polymicrobial sepsis

Swiss male mice (25-30 g) used for the study were randomly assigned to sham or CLP experimental groups (n > 6 in each group). Polymicrobial sepsis was induced in mice by CLP as described in **Section 2.3**. The same surgical procedure except the cecal ligation and puncture was performed on sham-operated animals. Vehicle (DMSO diluted in PBS, 0.25% v/v) or GR159897 (0.12 mg/kg; 0.25 mg/ml, s.c.) was administered to CLP-operated mice 1 h after the CLP. The animals were sacrificed 8 h after surgery by an i.p. injection of a lethal dose of pentobarbitone. Samples of lung were snap frozen in liquid nitrogen and stored at -80° C for subsequent measurement of tissue MPO activity and chemokine and cytokine levels.

5.2.3 Myeloperoxidase estimation

MPO activity as a measure of neutrophil sequestration in lung was quantified as described in **Section 2.4**.

5.2.4 ELISA analysis

Lung tissue homogenates were assayed to evaluate the level of chemokines (MCP-1 and MIP-2), and cytokines (IL-6 and IL-1β) by a sandwich ELISA according to **Section 2.5**. Sample concentration was estimated from the respective standard curve.

5.2.5 Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM). The significance of changes was evaluated by using ANOVA when comparing three or more groups and Tukey's method as a post hoc test for comparison among different groups. A p value of < 0.05 was considered to indicate a significant difference.

5.3 Results

5.3.1 Effect of GR159897 treatment on neutrophil sequestration in lung after CLP surgery

Tissue MPO activity as a measure of neutrophil infiltration was quantified as increased MPO activities indicate neutrophil recruitment and a state of inflammation. As expected, 8 h after CLP, MPO activity in lung was significantly increased in vehicle treated animals when compared to the sham mice ($p < 0.001$) (**Fig. 5.1**). However treatment with the NK-2R antagonist, GR159897, 1 h after CLP, did not significantly reduce the MPO activity in lung (**Fig. 5.1**).

5.3.2 Effect of GR159897 treatment on lung chemokine levels in septic mice

Chemokines produced in response to infection attract various inflammatory cells in sepsis. I measured the levels of major CXC chemokine, MIP-2 and CC chemokine,

Fig. 5.1

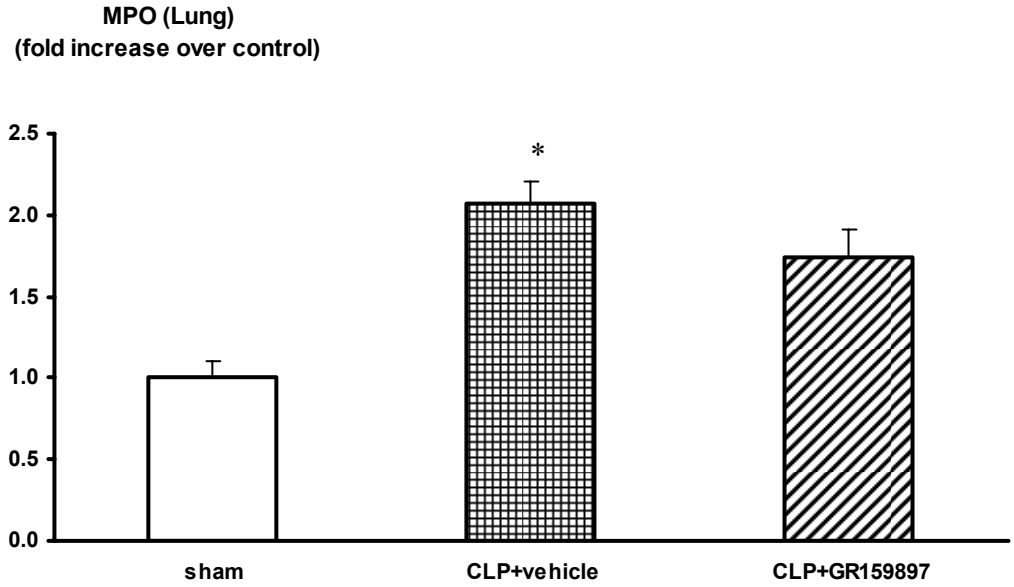


Figure 5.1 Effect of GR159897 administration 1 h after CLP on lung neutrophil infiltration. Mice (n = 6-8 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or GR159897 (0.12 mg/kg; 0.25 mg/ml) s.c. 1 h after the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung MPO activity was determined. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals.

MCP-1 in lung homogenates. CLP-induced sepsis resulted in a significantly higher MIP-2 levels in vehicle treated mice compared to the sham group ($p < 0.001$) (**Fig. 5.2a**). However, GR159897 treatment did not change the elevated lung MIP-2 levels observed 8 h after CLP surgery in vehicle control group (**Fig. 5.2a**). Similarly MCP-1 levels also increased significantly 8 h after CLP surgery without GR159897 administration compared to that of sham animals ($p < 0.001$) (**Fig. 5.2b**). But this increase in MCP-1 levels was not affected significantly by GR159897 administration 1 h after CLP surgery (**Fig. 5.2b**).

5.3.3 Effect of GR159897 treatment on lung cytokine levels in septic mice

Further, I measured the major cytokines, IL-1β and IL-6, in lung tissue. Animals injected only with the vehicle showed a significant increase in lung IL-1β levels 8 h after CLP surgery compared to that in sham mice ($p < 0.001$) (**Fig. 5.3a**). Administration of GR159897 1 h after CLP procedure failed to affect the lung IL-1β levels (**Fig. 5.3a**). IL-6 showed a similar pattern of increase in CLP induced sepsis (**Fig. 5.3b**). The lung IL-6 levels in mice subjected to CLP surgery and injected only with the vehicle 1 h after CLP, were significantly higher compared to that in sham operated group ($p < 0.001$). GR159897 when injected 1 h after CLP surgery did not significantly change the lung IL-6 levels compared to the corresponding levels in the absence of NK-2R antagonist treatment (**Fig. 5.3b**).

5.4 Discussion

NK-2R stimulation has been reported to play a role in bronchoconstriction induced by various agents that induce the release of tachykinins (Advenier *et al.,* 1997; Joos and

Fig. 5.2a

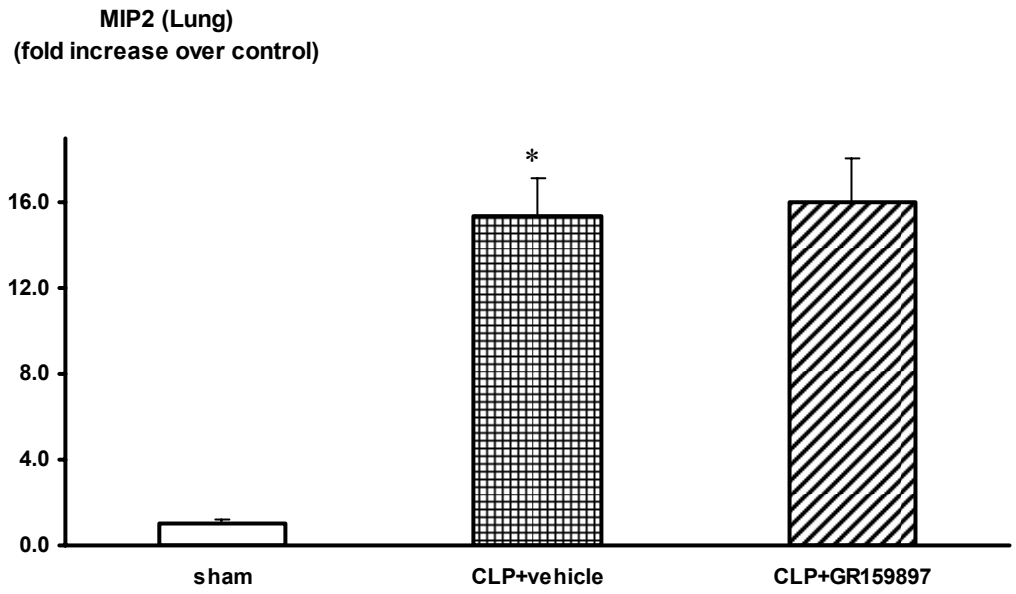


Fig. 5.2b

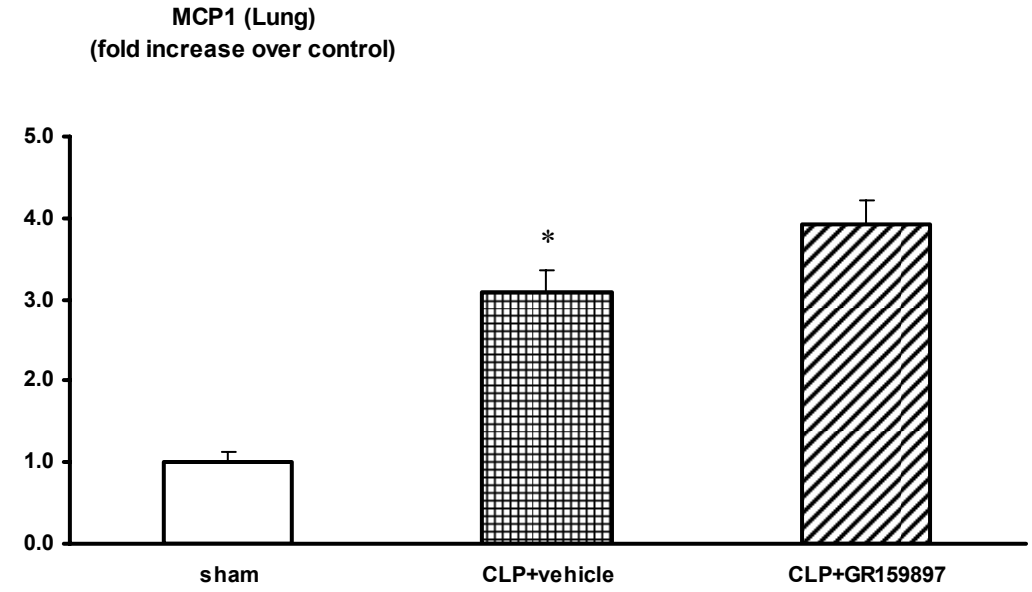


Figure 5.2 Effect of GR159897 administration 1 h after CLP on lung MIP-2 (Fig. 5.2a) and MCP-1 (Fig. 5.2b) levels. Mice (n = 6-8 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or GR159897 (0.12 mg/kg; 0.25 mg/ml) s.c. 1 h after the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung MIP-2 and MCP-1 levels were determined. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals.

Fig. 5.3a

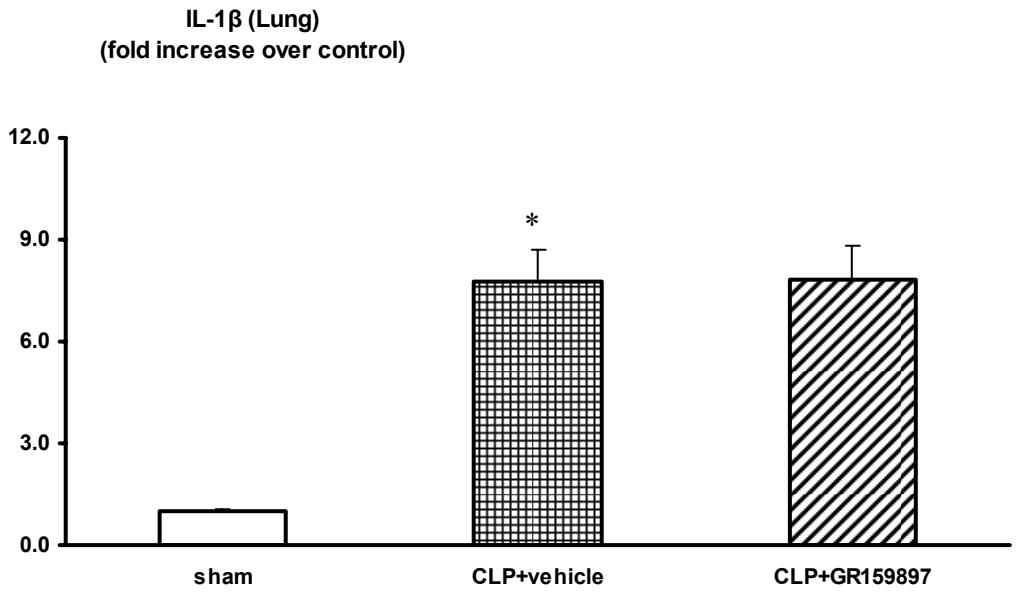


Fig. 5.3b

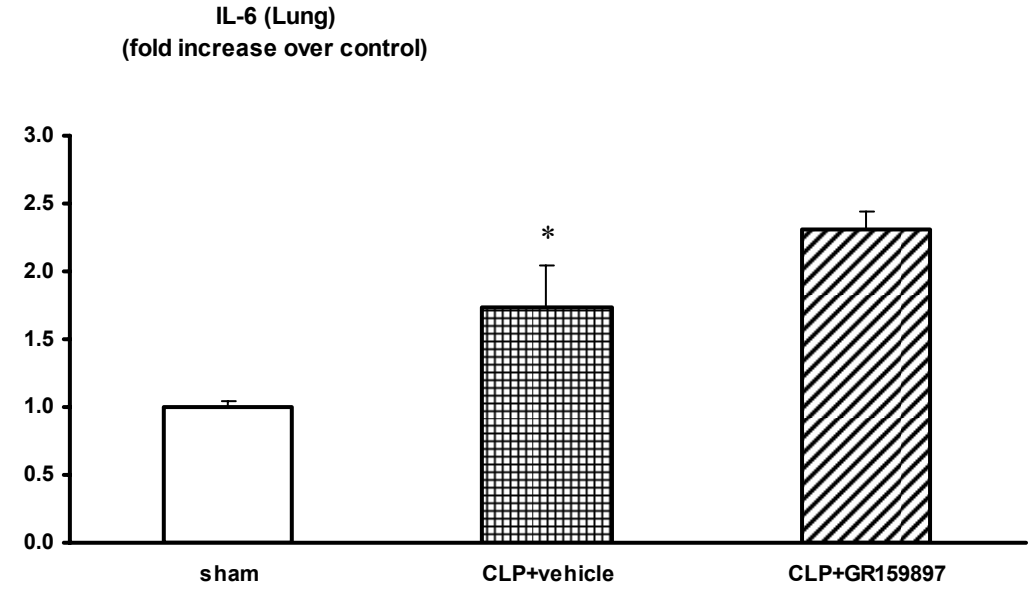


Figure 5.3 Effect of GR159897 administration 1 h after CLP on lung IL-1β (Fig. 5.3a) and IL-6 (Fig. 5.3b) levels. Mice (n = 7-9 in each group) were divided into CLP-operated and sham-operated groups. CLP-operated mice received vehicle (DMSO in PBS, 0.25% v/v) or GR159897 (0.12 mg/kg; 0.25 mg/ml) s.c. 1 h after the CLP. The same surgical procedure as the CLP-operated animals except the cecal ligation and puncture was performed on sham-operated animals. 8 h after the CLP procedure, mice were sacrificed and lung IL-1β and IL-6 levels were determined. Results shown are the mean ± SEM. * $p < 0.001$ when vehicle-treated CLP animals were compared with sham group animals.

Pauwels 2000). Selective NK-2R antagonists are thought to be beneficial in airway disease (Rizzo *et al.,* 1999). Inhibition of NKA mediated or capsaicin-mediated dyspnea by SR 48968, an NK-2R antagonist, has been demonstrated in guinea pigs (Advenier 1995). SR 48968 also is reported to inhibit cough induced by citric acid or capsaicin (Advenier 1995). In addition, SR 48968 is able to abolish the bronchial hyper-reactivity induced by a citric acid challenge or an ovalbumin challenge in guinea pigs (Advenier 1995). Nonpeptide, long-acting NK-2R antagonists are regarded as suitable experimental tools in humans, especially for a determination of the role of tachykinins in asthmatic patients.

GR159897 is a non-peptide NK-2R antagonist that I selected for the study to analyse the role of NK-2R blocking in polymicrobial sepsis-associated lung injury. Since NK-1R antagonism was found to be beneficial in polymicrobial sepsis and associated lung injury, I further evaluated if SP mediated its pro-inflammatory activity in sepsis via NK-2R, in addition to NK-1R. Administration of GR159897 1 h after CLP failed to reduce MPO levels significantly in septic mice. Also, the chemokines such as MCP-1, MIP-2 and cytokines IL-1β and IL-6 were not affected by NK-2R blocking in sepsis. Tachykinins are known to contract smooth muscles mainly by interaction with NK-2R, while the vascular and pro-inflammatory effects are mediated by NK-1R (Joos *et al.,* 2000). In the absence of GR159897, vehicle treated mice showed symptoms of polymicrobial sepsis with elevated MPO activity and lung chemokine and cytokine levels. Thus it seems probable that pro-inflammatory activity of SP in polymicrobial sepsis is mediated mainly by NK-1R. I did not probe the role of NK-3R as it is not found in the lungs (Lau and Bhatia 2006).

Further studies were performed using *PPTA*$^{-/-}$ mice.

CHAPTER 6. *PPTA* GENE DELETION AND POLYMICROBIAL SEPSIS

6.1 Introduction

Gene expression profiling using microarray is a relatively novel approach. Global genome explorations are efficient and feasible tools for understanding the molecular signature of diseases. The aim is to comprehensively analyze various mediators that are differentially expressed in a disease state and visualize the genetic network (Schulze and Downward 2001). This global approach could eventually help understand the complete mechanism involved in the pathogenesis of a disease better. With the help of microarray analysis, researchers can aim to search for diagnostic and therapeutic markers. However, there are few reports of using microarrays to study sepsis *in vivo* (Chung *et al.,* 2006).

It has been shown previously that the pathogenesis of sepsis was delayed in *PPTA*$^{-/-}$ mice and the gene deletion protected against lung injury in sepsis (Puneet *et al.,* 2006). SP is also implicated in various other inflammatory conditions such as acute pancreatitis (Bhatia *et al.,* 2003) and endotoxemia (Ng *et al.,* 2008). To better understand the molecular mechanisms of polymicrobial sepsis and associated lung damage in *PPTA*$^{-/-}$ mice, I employed microarray analysis of pulmonary gene expression. Main focus was on lung injury as respiratory failure is one of the main causes leading to mortality in sepsis. As the lung MPO activity was highest 8 h after CLP (data not shown), lung tissue was collected by sacrificing the mice at this time point. I sought to evaluate the genome-wide tissue-specific differential expression

pattern after the induction of polymicrobial sepsis in *PPTA* knockout mice, with particular focus on chemokines and cytokines. The microarray data was further supported with semi quantitative RT-PCR and ELISA.

6.2 Materials and Methods

6.2.1 Animal Ethics

Reference: **Section 2.2**

6.2.2 Induction of polymicrobial sepsis

PPTA$^{-/-}$ and wild-type Balb/c male mice (25-30 g) were randomly divided into sham or CLP experimental groups (n > 6 in each group). Polymicrobial sepsis was induced by CLP as explained in **Section 2.3**. The same surgical procedure except the cecal ligation and puncture was performed on sham-operated animals. The animals were sacrificed 8 h after surgery by an i.p. injection of pentobarbitone. Samples of lung were snap frozen in liquid nitrogen and stored at -80° C for RNA isolation and ELISA. Blood was collected by cardiac puncture, heparinized, centrifuged, plasma removed and stored at -80° C for subsequent measurement.

6.2.3 RNA isolation and quantification

Total RNA from lung tissue was isolated, purified and quantified as explained in detail in **Section 2.13**, with slight modification for microarray experiments. Briefly, tissue was homogenized in 10 ml Trizol in RNase free tube, incubated at room temperature for 5 min and centrifuged for 5 min at 11,750 g, 4° C. The supernatant was mixed with 2 ml chloroform, incubated at room temperature for 3 min and centrifuged for 15 min at 11,750 g, 4° C. 5 ml of the supernatant was mixed with 5 ml of isopropanol, incubated at room temperature for 10 min and centrifuged for 10 min at 11,750 g,

4° C. After discarding the supernatant, the pellets were washed with 1ml 75% ethanol, centrifuged for 5 min at 7500 g, 4° C and the total RNA pellets were subjected to clean-up as explained in **Section 2.13**. Only RNA samples passing the purity and integrity test were used further for microarray and RT-PCR analysis.

6.2.4 Microarray experiments

GeneChip hybridization and scanning were performed individually for each of the mouse lung sample from the four groups (n = 3 for each group; n = 12 total) according to Affymetrix GeneChip® Expression Analysis Technical Manual as mentioned in **Section 2.15**. All the 12 samples were processed simultaneously to avoid batch variations and errors.

6.2.5 Microarray data analysis

Microarray data was analyzed and biological network pathway was created for both *PPTA*$^{-/-}$ and wild-type septic mice as described in **Section 2.16**.

6.2.6 Semiquantitative Reverse transcriptase-polymerase chain reaction (RT-PCR)

Lung RNA (1 μg) was reverse transcribed and the cDNA was subjected to PCR amplification and analysis as briefed in **Section 2.14** for selected differentially expressed genes. The primer sequences for detection of IL-1β, MCP-1, MIP-2, interleukin-1 receptor antagonist (IL-1ra), MIP-1α, MIP-1β, interferon inducible protein-10 (IP-10), serum amyloid A3 (SAA3), chemokine (C-C motif) receptor 2 (CCR-2), chemokine (C-C motif) receptor 5 (CCR-5) and 18S gene, optimal annealing temperature, and optimal cycles are shown in **Table 6.1.**

Table 6.1 Primer sequences and optimal conditions used in PCR analysis

Gene name	Sense primer sequence (5'-3')	Antisense primer sequence (5'-3')	Amplification conditions	No. of amplification cycles
SAA3	AGC CTT CCA TTG CCA TCA TTC TT	AGT ATC TTT TAG GCA GGC CAG CA	94°C 1min; 58°C 1min; 74°C 1min	25
IP10	GTGTTGACATCATTGCCACG	GCTTACAGTACAGAGCTAGG	95°C 30s; 60°C 30s; 45°C 45s	30
MCP1	CCCCACTCACCTGCTGCTACT	CACTGTCACACTGGTCACTCC	95°C 50s; 64°C 50s; 72°C 1min	36
MIP-1α	ACTGCCCTTGCTGTTCTTCTCT	AGGCATTCAGTTCCAGGTCAGTGA	95°C 30s; 61°C 30s; 72°C 30s	33
MIP-1β	CCCTCTCTCTCCTCTTGCTCGT	TTCAACTCCAAGTCACTCATGTACTCA	94°C 30s; 55°C 30s; 72°C 1min	32
MIP-2	GCTGTCAATGCCTGAAGACC	TAGTTCCCAACTCACCCTCTC	95°C 50s; 65°C 50s; 72°C 1min	36
IL-1β	AAGGAGAACCAAGCAACGAC	GAGATTGAGCTGTCTGCTCA	95°C 50s; 63°C 50s; 72°C 1min	34
CCR-2	CACGAAGTATCCAAGAGCTT	CATGCTCTTCAGCTTTTTAC	94°C 30s; 58°C 45s; 72°C 70s	35
CCR-5	TTCCCTGTCATCGCTTGCTCT	CGGATGGAGATGCCGATTTT	94°C 1min; 60°C 1min; 72°C 2min	40
IL-1ra	GACCCTGCAAGATGCAAGCC	CAGGACGGTCAGCCTCTAGT	95°C 20s; 51°C 20s; 72°C 20s	36
18S	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG	95°C 30s; 59°C 30s; 72°C 30s	24

6.2.7 ELISA analysis

For the measurement of cytokine IL-1ra, ELISA kit from R&D Systems (Minneapolis, MN, USA) was used employing a quantitative sandwich enzyme immunoassay as explained in **Section 2.5** with slight modification. Briefly, standards and samples were pipetted into the microplate wells pre-coated with mouse IL-1ra-specific polyclonal antibody and incubated for 2 h. After washing away unbound substances, an enzyme-linked mouse IL-1ra-specific polyclonal antibody was added to the wells and incubated for 2 h. Following a wash, a substrate solution was added to the wells and incubated for 30 min to yield a blue product that turned yellow when the Stop solution was added. The optical density of each well was determined using a microplate reader set to 450 nm. The intensity of the color measured was proportional to the amount of mouse IL-1ra bound in the initial step. The sample values were then read off the standard curve and corrected for the DNA content of the lung tissue. Lung IL-1ra was expressed as pg/µg of DNA and plasma as pg/ml. The lower limit of detection was 31.25 pg/ml.

6.2.8 Statistics

Differentially expressed genes were analyzed by One-way ANOVA ($p < 0.05$) for array data to compare differences between the medians of the groups. For RT-PCR and ELISA data, statistical analysis was performed as mentioned in **Section 2.17**.

6.3 Results

6.3.1 Microarray quality control

Each of the Affymetrix high-density oligonucleotide arrays that I used for lung expression profiling had 45000 probe sets to analyze the expression level of over

39000 transcripts and variants from about 34000 well characterized mouse genes. As the high cost of genomic investigations was a limitation, I used three arrays for each group according to MIAME guidelines (Brazma *et al.,* 2001). All the twelve arrays passed the quality controls included within the arrays such as hybridization controls (*bioB*, *bioC*, *bioD*, and *cre*), Poly-A controls (*dap*, *lys*, *phe*, and *thr*), normalization control set and housekeeping/control genes (GAPDH, beta-Actin, transferrin receptor, pyruvate carboxylase). Illustration of the box-whisker plot was done to examine the distribution of data and to ensure proper dynamic range **(Fig. 6.1)**. Data distributions were assessed based on the highest intensity value at saturation. Appropriately similar distribution of data was considered to be of high-technical quality for further analysis. The expression of genes was compared between sham controls and CLP-induced sepsis groups among wild-type and $PPTA^{-/-}$ mice. I focused on genes that were either consistently increased or decreased in all sets of experiments. Only those genes whose expressions changed by 2-fold or greater in at least one pair-wise comparison were taken as significant ($p < 0.05$). These differentially expressed genes were further annotated based on their known biological functions using the Database for Annotation, Visualization, and Integrated Discovery (DAVID). Many of the genes were present in more than one functional class.

6.3.2 Inflammatory gene profile of wild-type septic mice

Table 6.2 lists majority of the genes altered by CLP in wild-type and $PPTA^{-/-}$ mice, grouped under the biological process gene ontology classes of inflammatory response, chemotaxis, leukocyte activation, response to bacterium, regulation of cellular process, signal transduction, cytokine and chemokine mediated signaling pathway. Few others

Fig. 6.1

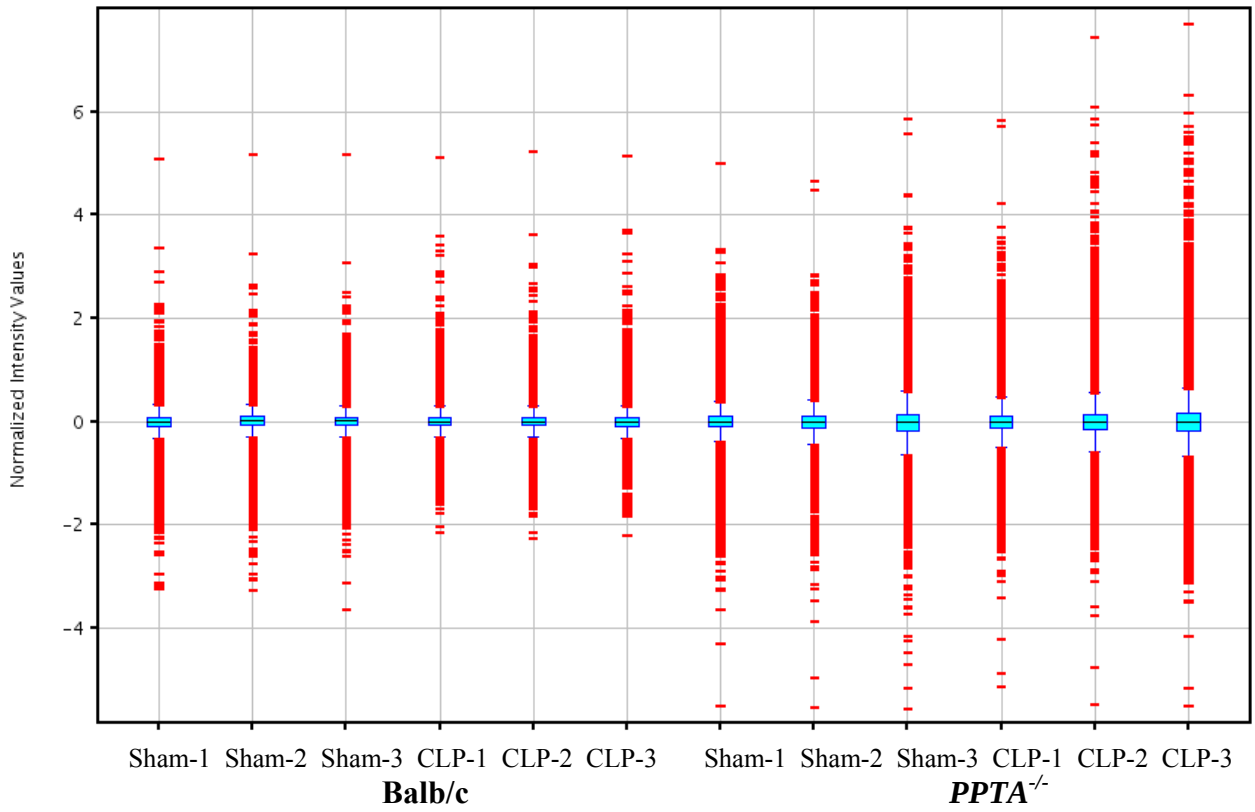


Figure 6.1 The box-whisker plot of distribution of normalized intensity values for each sample. The boxes represent interquartile range, with 75th percentile at the top and 25th percentile at the bottom. The line in the middle of the box represents the 50th percentile (median). Whiskers represent the rest of the distribution, with intensity values beyond 1.5 times the inter-quartile range shown in red. The *x*-axis represents individual microarray, while the *y*-axis represents the normalized intensity values. CLP – cecal ligation and puncture; *PPTA*$^{-/-}$- *preprotachykinin-A* knockout.

Table 6.2 Differentially expressed genes in wild-type and *PPTA*$^{-/-}$ mice 8 h after cecal ligation and puncture (CLP)-induced sepsis

Gene name	Fold increase/ decrease [b]	
	wild-type CLP (compared to sham)	*PPTA*$^{-/-}$ CLP (compared to sham)
inflammatory Response		
serum amyloid a 3 [a]	4.21	54.52
chemokine (c-c motif) ligand 2 [a]	5.52	54.37
fc receptor, ige, high affinity i, gamma polypeptide	2.30	7.15
chemokine (c-x-c motif) ligand 10 [a]	5.04	12.58
chemokine (c-c motif) receptor 1	4.66	15.52
chemokine (c-x-c motif) ligand 1	5.35	37.74
interleukin 1 receptor antagonist [a]	6.23	125.25
chemokine (c-x-c motif) ligand 2 [a]	107.41	114.76
cd14 antigen	7.93	13.70
toll-like receptor 13	3.72	5.52
chemokine (c-c motif) ligand 4 [a]	3.55	17.59
interleukin 1 beta [a]	6.77	8.85
selectin, platelet	2.57	12.03
toll-like receptor 2	4.09	6.09
complement component 5a receptor 1	3.19	6.50
tumor necrosis factor receptor superfamily, member 1b	2.15	8.14
cd28 antigen	1.87	2.56
chemokine (c-c motif) ligand 3 [a]	13.05	73.16
toll-like receptor 6	2.18	2.68
tumor necrosis factor receptor superfamily, member 1a	- [c]	2.09
mitogen activated protein kinase kinase 3	-	2.12
oxidized low density lipoprotein (lectin-like) receptor 1	-	5.51
orosomucoid 1	-	34.00
signal transducer and activator of transcription 3	-	2.63
chemokine (c-c motif) receptor 2 [a]	-	5.26
chemokine (c-c motif) ligand 22	-	2.83
Fc receptor, IgG, low affinity iib	-	9.90
Fc receptor, IgG, low affinity iii	-	6.59
toll-like receptor 5	-	-2.24 [d]
toll-like receptor 1	-	3.53
cd44 antigen	-	3.62
signal transducer and activator of transcription 5a	-	2.44
phospholipase a2, group vii (platelet-activating factor acetylhydrolase, plasma)	-	7.59

Table 6.2 (*Continued*)

Gene name	Fold increase/ decrease	
	wild-type CLP (compared to sham)	*PPTA*$^{-/-}$ CLP (compared to sham)
Chemotaxis		
chemokine (c-c motif) ligand 9	2.27	10.09
s100 calcium binding protein a8 (calgranulin a)	2.68	-
formyl peptide receptor 1	9.01	48.18
chemokine (c-c motif) ligand 17	-	8.49
chemokine (c-c motif) receptor 5 [a]	-	14.45
cysteine rich protein 61	-	-5.25
integrin beta 2	-	3.68
interleukin 16	-	3.71
chemokine (c-c motif) ligand 6	-	3.63
leukocyte activation		
cd80 antigen	3.19	2.60
cd52 antigen	1.92	2.27
interleukin 12a	-	2.61
growth arrest and dna-damage-inducible 45 gamma	-	4.21
fas (tnf receptor superfamily member)	-	4.82
cd40 antigen	-	2.10
response to wounding		
coagulation factor xiii, a1 subunit	2.12	4.17
gap junction membrane channel protein alpha 1	-	3.25
coagulation factor x	-	17.55
coagulation factor vii	-	2.43
fibrinogen, gamma polypeptide	-	11.57
response to bacterium		
cathelicidin antimicrobial peptide	5.33	40.93
peptidoglycan recognition protein 1	4.44	2.26
lipopolysaccharide binding protein	-	2.46
regulation of cellular process		
protein tyrosine phosphatase, non-receptor type 11	-2.25	-
matrix metallopeptidase 9	3.48	1.95
mitogen activated protein kinase kinase kinase 8	4.01	8.26
caspase 4, apoptosis-related cysteine peptidase	-	8.62
heat shock protein 1A	-	-7.87
interferon regulatory factor 1	-	3.25
interferon regulatory factor 7	-	2.63

Table 6.2 (*Continued*)

Gene name	Fold increase/ decrease wild-type CLP (compared to sham)	Fold increase/ decrease *PPTA*$^{-/-}$ CLP (compared to sham)
signal Transduction		
interleukin 7 receptor	-	9.81
g protein-coupled receptor 35	2.77	6.49
rho family gtpase 1	3.24	8.83
interleukin 1 receptor, type ii	8.97	18.83
g protein-coupled receptor 27	2.24	3.49
interleukin 10 receptor, alpha	2.06	2.53
regulator of g-protein signaling 1	4.64	8.55
suppressor of cytokine signaling 3	4.73	10.67
regulator of g-protein signaling 2	2.36	-
janus kinase 2	-	2.41
interleukin 17 receptor	-	2.61
mitogen activated protein kinase kinase kinase kinase 2	-	-15.93
phospholipase c, delta 3	-	-2.55
chemokine (c-x-c motif) receptor 6	-	2.29
peroxisome proliferator activator receptor delta	-	2.27
sphingosine kinase 1	-	6.67
suppressor of cytokine signaling 2	-	2.27
interleukin 4 receptor, alpha	-	4.38
interleukin 8 receptor, beta	-	3.05
neuropilin 1	-	-2.64
metallothionein 1	2.68	5.58
plasminogen activator, urokinase receptor	2.47	4.42
phosphatidylinositol 3-kinase catalytic delta polypeptide	-	3.39
mitogen-activated protein kinase kinase kinase kinase 5	-	-2.69
mitogen activated protein kinase kinase kinase 1	-	2.83
mitogen-activated protein kinase kinase kinase 6	-	9.08
mitogen activated protein kinase 8	-	-4.66
response to Other Organism		
neutrophilic granule protein	4.70	27.44
response to fungus		
pentraxin related gene	-	11.74
cytokine and chemokine mediated signaling pathway		
colony stimulating factor 2 receptor, beta 1, low-affinity (granulocyte-macrophage)	2.86	10.45
leukocyte immunoglobulin-like receptor, subfamily b (with tm and itim domains), member 3	2.57	6.62
colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	-	4.57

Table 6.2 (*Continued*)

Gene name	Fold increase/ decrease	
	wild-type CLP (compared to sham)	***PPTA*$^{-/-}$ CLP** (compared to sham)
other		
lipocalin 2	2.66	11.97
arginase type II	2.10	5.74
interleukin 1 family, member 9	5.18	19.02
matrix metallopeptidase 8	9.61	12.30
interferon induced transmembrane protein 6	5.61	34.46
s100 calcium binding protein a9 (calgranulin b)	3.19	2.92
inhibitor of dna binding 2	-	3.91
cytokine inducible sh2-containing protein	-	2.01
secretory leukocyte peptidase inhibitor	-	3.76
amphiregulin	-	3.59
interferon induced transmembrane protein 2	-	2.51
mitogen activated protein kinase kinase kinase 4	-	-3.23
mitogen-activated protein kinase-activated protein kinase 3	-	2.36
mitogen-activated protein kinase kinase kinase 14	-	2.60
natriuretic peptide receptor 3	-	3.12

[a] The genes validated by RT-PCR.
[b] Values represent fold-changes in gene expression in wild-type and *PPTA*$^{-/-}$ mice subjected to CLP-induced sepsis, over corresponding sham-operated mice. All the fold-changes analyzed by GeneSpring™ 7.3 software were statistically significant ($P < 0.05$) compared to sham control.
[c] Absence of a value (-) indicates that the fold-change was less than 2.
[d] Negative values represent decreased mRNA levels compared to respective sham group values.
PPTA$^{-/-}$, *preprotachykinin-A.*

of mixed or unknown functions are grouped separately as they are also reported in the literature to be differentially expressed in sepsis. As expected, CLP-induced sepsis in wild-type mice resulted in a significant increase in many of the inflammatory response and chemotaxis genes compared to sham surgery. Some of the chemokine superfamily members that were up-regulated significantly include C-C chemokines (CCL-2, CCL-3, CCL-4, CCL-9), and C-X-C chemokines (CXCL-1, CXCL-2, CXCL-10). In addition, SAA3, IL-1β, CCR-1, TLR-2, s100 calcium binding protein a8 (S100A8) and matrix metallopeptidase (MMP) 8 and 9 were also increased by more than 2-fold in wild-type septic mice compared to sham. It was interesting to note that the anti-inflammatory cytokine, IL-1ra also showed a greater than six-fold increase after CLP in these mice.

6.3.3 Inflammatory gene profile of *PPTA*$^{-/-}$ septic mice

However, *PPTA*$^{-/-}$ mice also exhibited high expression of inflammatory response and chemotaxis genes 8 h after CLP compared to sham mice. The fold increase was significantly higher compared to that in wild-type mice, especially for the chemokines CCL-2, CCL-3, CCL-4, CCL-9, CXCL-1, CXCL-2 and CXCL-10. Similarly, the expression of SAA3, IL-1β, CCR-1, TLR-2, and MMP-8 increased significantly in *PPTA*$^{-/-}$ mice 8 h after CLP compared to the corresponding fold increase in wild-type mice. Unlike in wild-type mice, S100A8 and MMP-9 in *PPTA*$^{-/-}$ mice did not show more than 2-fold increase after CLP. Further, CCL-6, CCL-17, CCL-22, CCR-2, CCR-5, TLR-1, and phosphatidylinositol 3-kinase catalytic delta polypeptide were expressed more in *PPTA*$^{-/-}$ mice after the induction of sepsis and this was not seen in wild-type mice after CLP. I also observed much higher increase of IL-1ra gene in *PPTA*$^{-/-}$ mice 8 h h after CLP compared to the wild-type mice. Lastly there was a significant down-

regulation of TLR-5 and heat-shock protein 1A only in *PPTA*$^{-/-}$ mice after the induction of sepsis.

6.3.4 Semiquantitative RT-PCR data

To further validate the microarray results, RT-PCR was carried out. Ten genes were selected whose expression altered by more than 2-fold in septic mice. RNA from individual mouse lung was evaluated for these genes by RT-PCR. 8 h after CLP-induced sepsis, pulmonary gene expression levels of MCP-1 (CCL-2), MIP-1α (CCL-3), MIP-1β (CCL-4), MIP-2 (CXCL-2), IP-10 (CXCL-10), IL-1 β, SAA3 and IL-1ra were increased compared to the corresponding levels in sham operated mice **(Fig. 6.2a-h)**. The elevated mRNA levels were observed in both the wild-type and *PPTA*$^{-/-}$ mice after sepsis, with a significantly higher increase in the latter. There was no significant change in the expression levels of chemokine receptors, CCR-2 and CCR-5, in septic wild-type mice compared to sham mice **(Fig. 6.2i and j)**. However, a significant up-regulation was observed for both CCR-2 and CCR-5 in *PPTA*$^{-/-}$ mice 8 h after CLP (CCR-2 $p < 0.01$ and CCR-5 $p < 0.05$ vs sham) **(Fig. 6.2i and j)**. The mRNA levels evaluated by RT-PCR showed a similar trend to the microarray gene expression data for the chosen 10 genes.

6.3.5 IL-1ra protein levels after sepsis

Further I also measured the protein levels of anti-inflammatory cytokine IL-1ra in lung and plasma by ELISA. IL-1ra level was up-regulated after CLP-induced sepsis with similar pattern as the gene expression **(Fig. 6.3)**. The increase in lung **(Fig. 6.3a)** and plasma **(Fig. 6.3b)** IL-1ra level was significantly higher in *PPTA*$^{-/-}$ mice after the induction of sepsis (lung: $p < 0.001$ vs sham; plasma: $p < 0.05$ vs sham) compared to the corresponding increase in wild-type mice.

Fig. 6.2a

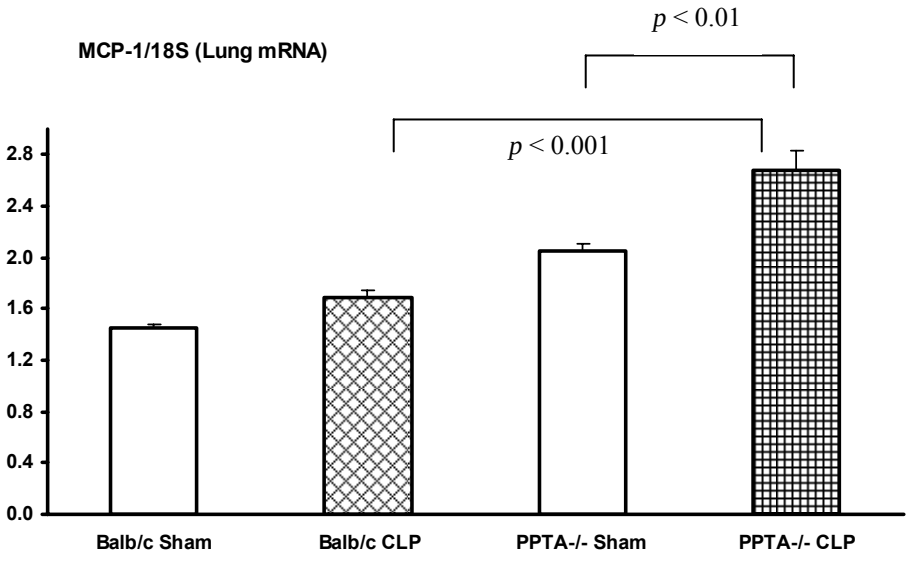


Fig. 6.2b

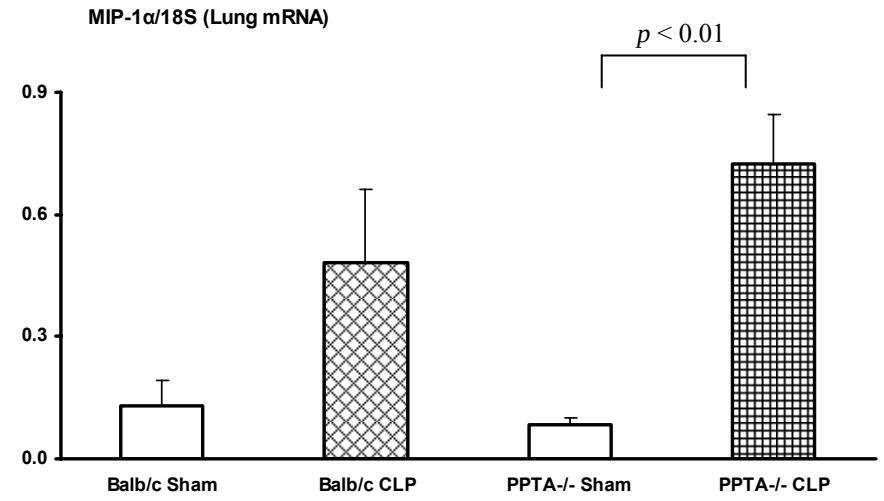


Fig. 6.2c

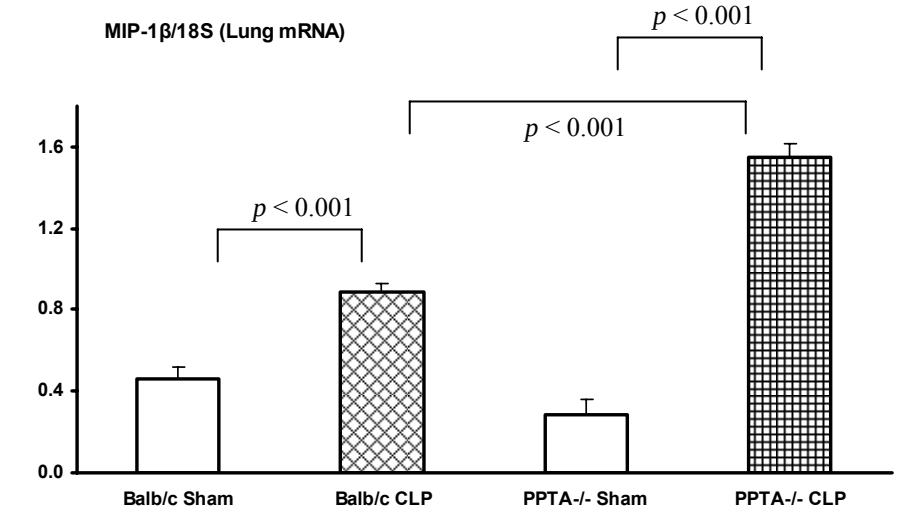


Fig. 6.2d

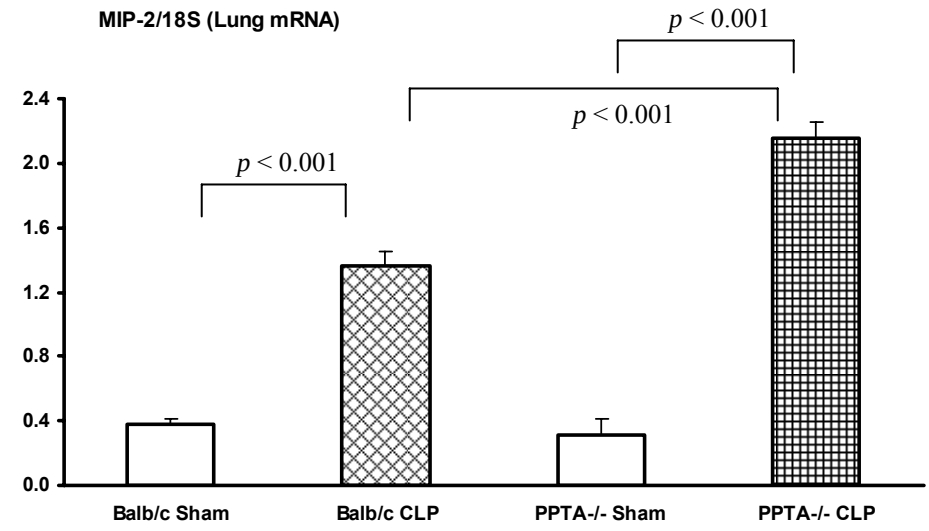


Fig. 6.2e

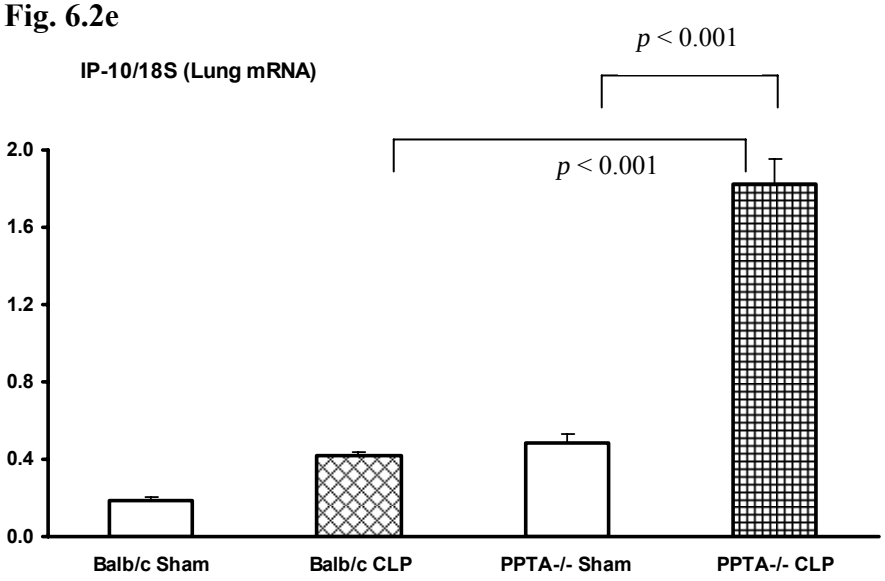


Fig. 6.2f

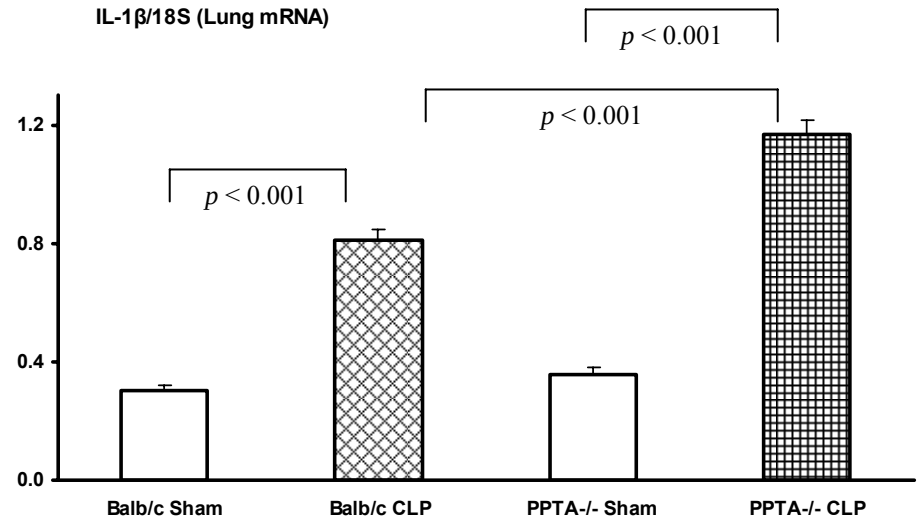


Fig. 6.2g

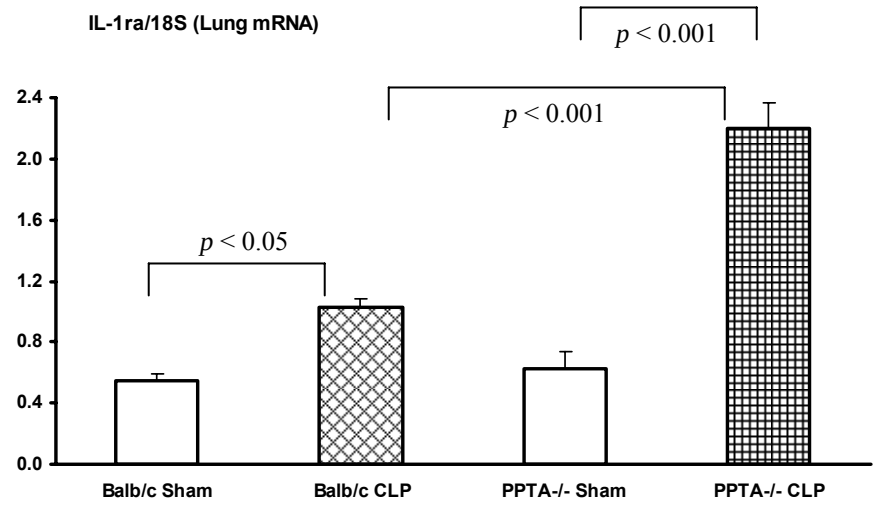


Fig. 6.2h

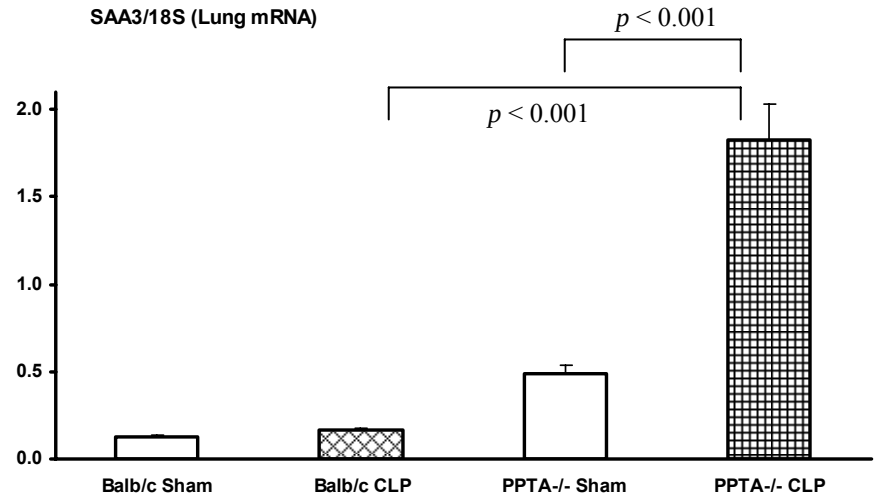


Fig. 6.2i

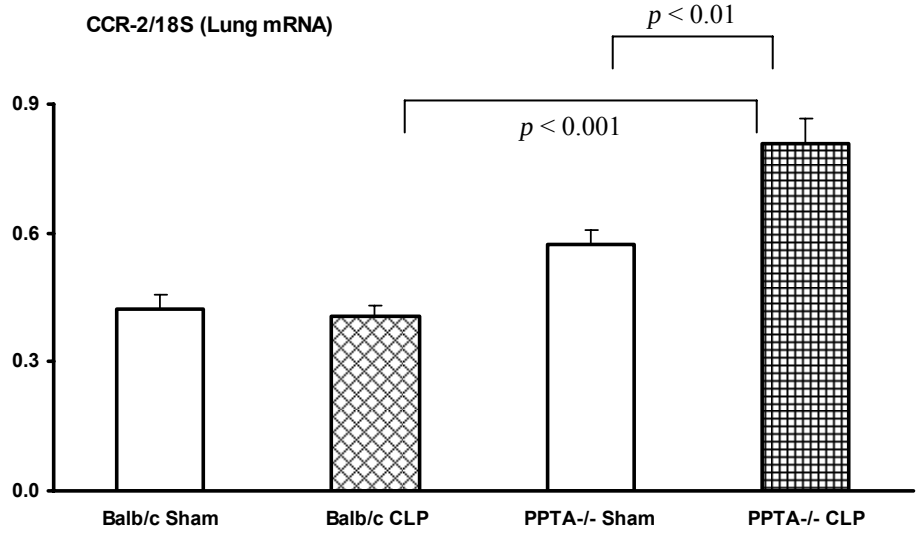


Fig. 6.2j

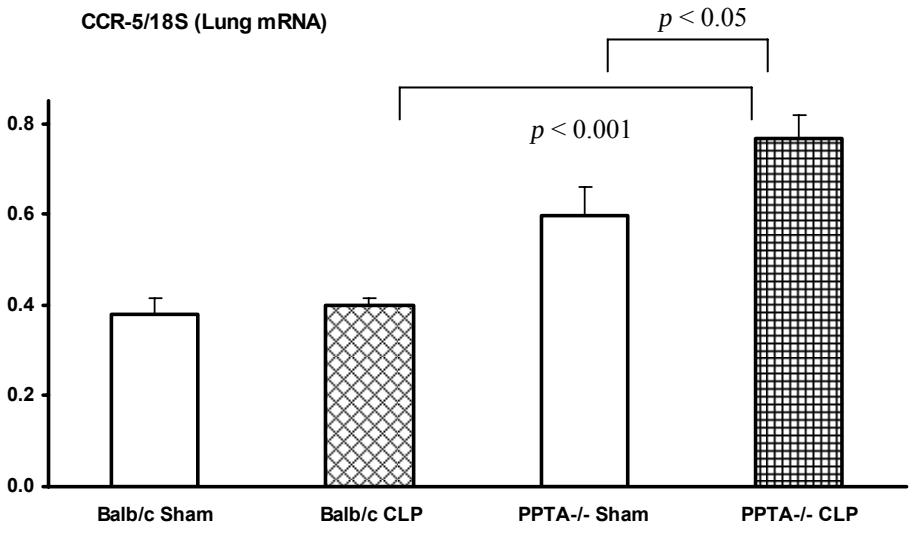


Figure 6.2 Pulmonary inflammatory and chemotaxis gene transcripts in wild-type and *PPTA*$^{-/-}$ mice. a. MCP-1, b. MIP-1α, c. MIP-1β, d. MIP-2, e. IP-10, f. IL-1β, g. IL-1ra, h. SAA3, i. CCR-2, j. CCR-5. mRNA levels of these genes in lung were evaluated 8 h after CLP or sham surgery in wild-type and *PPTA*$^{-/-}$ mice by semiquantitative RT-PCR analysis (expressed as a ratio of band density of the gene to 18S). Mouse 18S was used as a control. Results were expressed as mean ± SEM (n = 6-9 mice per group). *p* values < 0.05 were considered to be significant. CCR - chemokine (C-C motif) receptor; CLP – cecal ligation and puncture; IL – interleukin; IL-1ra – interleukin-1 receptor antagonist; IP-10 - IFN-γ-inducible protein 10; MCP-1 - monocyte chemoattractant protein-1; MIP – macrophage inflammatory protein; *PPTA*$^{-/-}$- *preprotachykinin-A* knockout; SAA3 - serum amyloid A3.

Fig. 6.3a

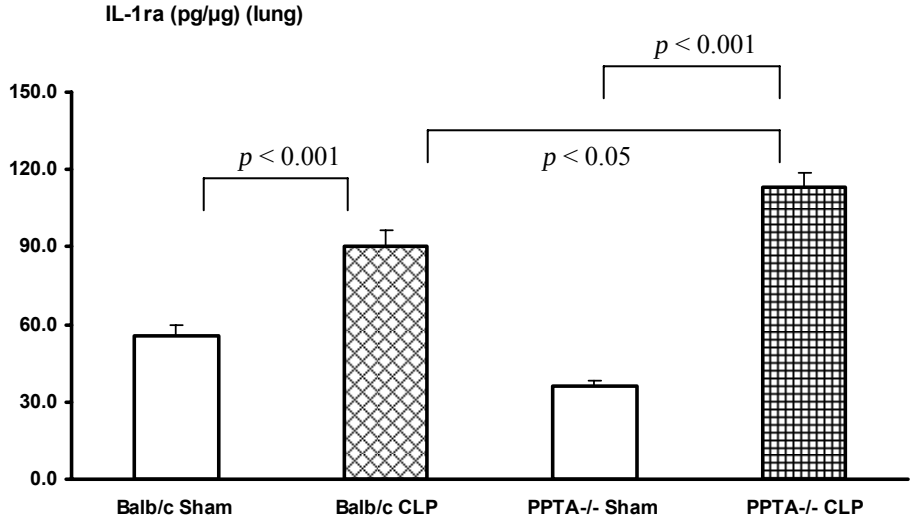


Fig. 6.3b

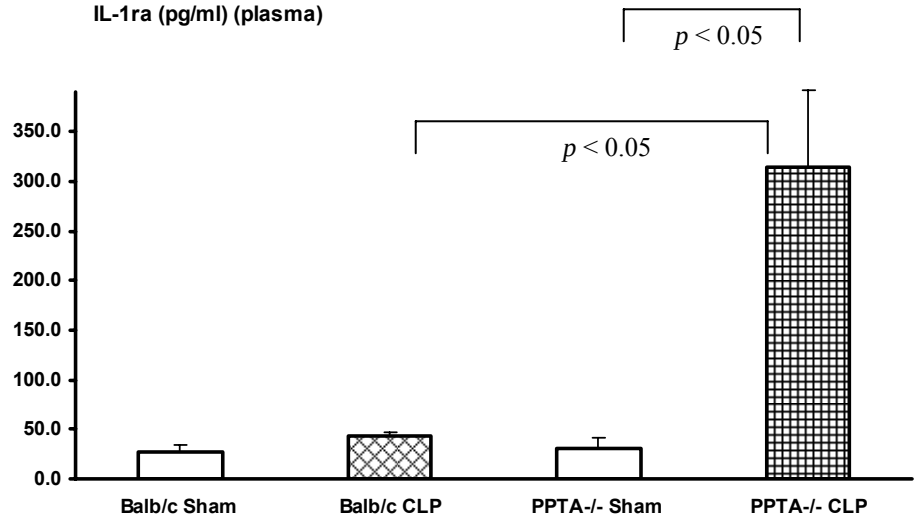


Figure 6.3 Protein levels of IL-1ra in wild-type and *PPTA*$^{-/-}$ mice. a. Lung, b. Plasma. IL-1ra levels in lung and plasma were measured 8 h after CLP or sham surgery in wild-type and *PPTA*$^{-/-}$ mice by ELISA. Results were expressed as mean ± SEM (n = 6-12 mice per group). *p* values < 0.05 were considered to be significant. CLP – cecal ligation and puncture; IL-1ra – interleukin-1 receptor antagonist; *PPTA*$^{-/-}$-*preprotachykinin-A* knockout.

6.3.6 Pathway analysis of differentially expressed genes

Pathway Studio software was used to identify a possible gene network in wild-type and *PPTA*$^{-/-}$ mice subjected to CLP-induced sepsis using the genes listed in **Table 6.2**. Genes were linked to each other based on the published literature **(Fig. 6.4a and b)**. Most of the differentially expressed genes were found to be connected and the few unconnected genes were excluded from the figure. The pathway analysis highlighted significantly higher number of links and interactions and higher expressions in *PPTA*$^{-/-}$ mice among known inflammatory and immune response genes compared to the wild-type mice **(Fig. 6.4a and b)**.

6.4 Discussion

It is well known that inflammatory response is an important part of sepsis. During the course of polymicrobial sepsis a range of pro- and anti-inflammatory cytokine and chemokine genes is up-regulated which is evident from the Pathway analysis. Although the inflammatory mediators activate leukocyte trafficking to the site of infection to fight the invading pathogens, excessive inflammation could be damaging (Sriskandan and Altmann 2008). At the same time, the host also produces counter-balancing anti-inflammatory mediators (Sriskandan and Altmann 2008) which produced in excess can cause immunosuppression and fatalities (Ashare *et al.,* 2005). Concurrent overexpression of both pro-inflammatory as well as anti-inflammatory cytokines has been reported in the early phase of lethal sepsis (Osuchowski *et al.,* 2006). So far there is a lack of consensus on whether to suppress or boost immunity or to do both at different times as clinical trials of various immunomodulators aimed at the inflammatory axis in severe sepsis have failed (Sriskandan and Altmann 2008).

Fig. 6.4a

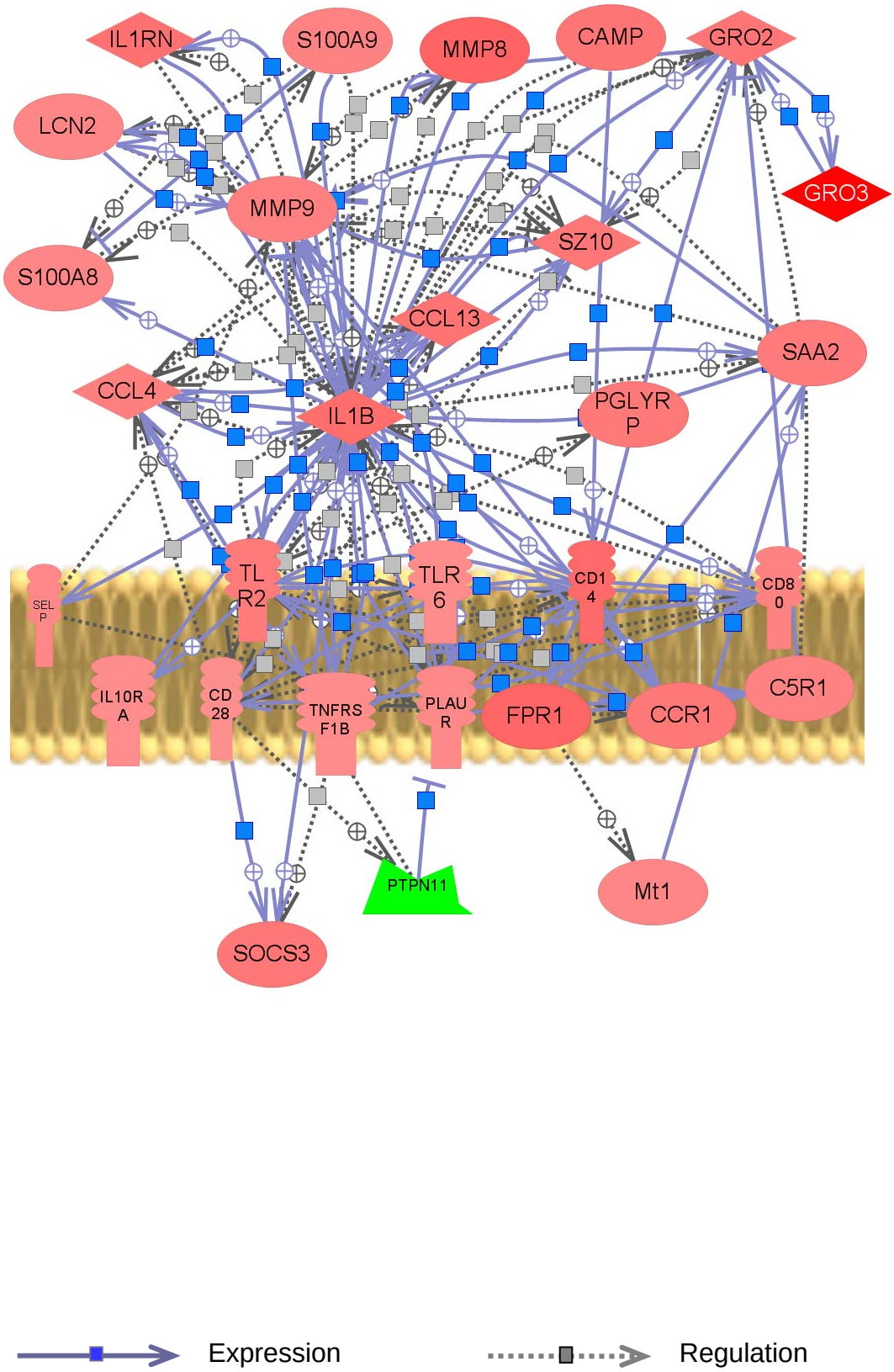
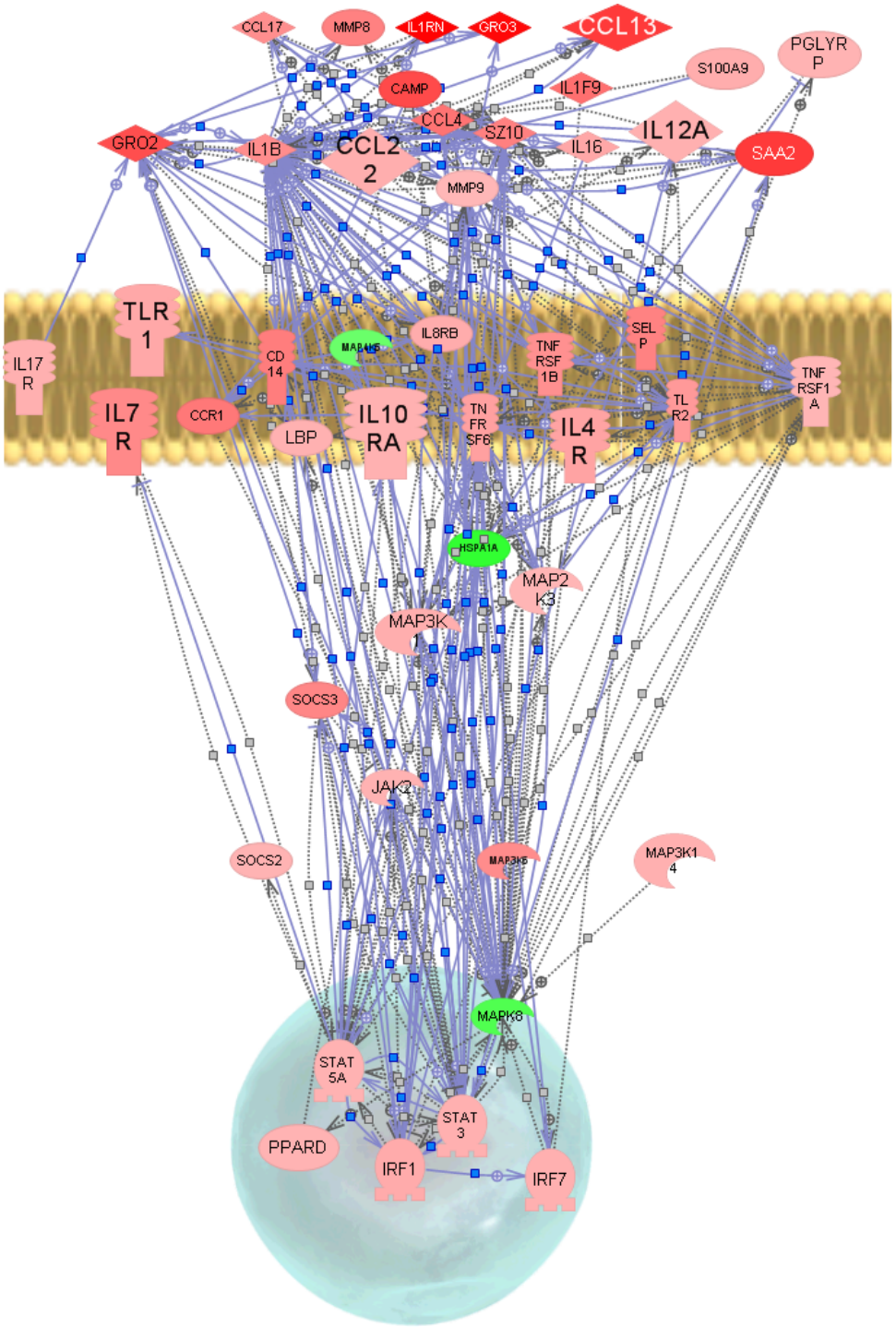

IL1RN
S100A9
MMP8
CAMP
GRO2
LCN2
GRO3
MMP9
SZ10
S100A8
CCL13
SAA2
CCL4
IL1B
PGLYR
P
SEL
P
TL
R2
TLR
6
CD1
4
CD8
0
IL10R
A
CD
28
TNFRS
F1B
PLAU
R
FPR1
CCR1
C5R1
PTPN11
Mt1
SOCS3
Expression
Regulation

Fig. 6.4b


CCL17
MMP8
IL1RN
GRO3
CCL13
PGLYRP
S100A9
CAMP
IL1F9
CCL4
SZ10
IL12A
GRO2
IL1B
CCL22
IL16
SAA2
MMP9
TLR1
IL8RB
SELP
IL17R
CD14
TNFRSF1B
TNFRSF1A
IL7R
CCR1
IL10RA
TNFRSF6
TLR2
LBP
IL4R
HSPA1A
MAP2K3
MAP3K1
SOCS3
JAK2
SOCS2
MAP3K14
MAPK8
STAT5A
STAT3
PPARD
IRF1
IRF7

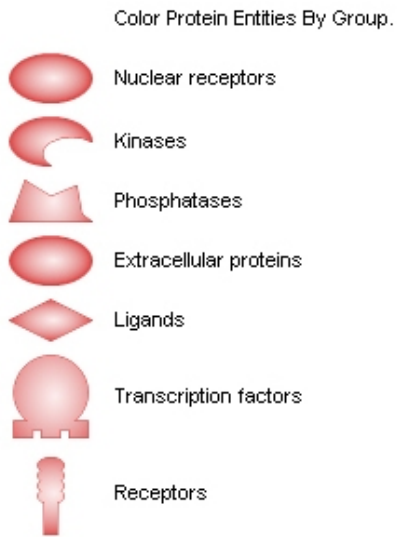


Figure 6.4 Proposed biological network of differentially expressed genes in sepsis. a. wild-type septic mice, b. *PPTA*$^{-/-}$ septic mice. Using Pathway Studio, a pathway network was built for microarray data based on previously reported interactions in the literature. The links and nodes represent literature based findings. Some of the prominent, differentially expressed genes shared in inflammatory and immune response in sepsis were used to create the network. ''+'' depicts positive regulation/expression; line ending with ''⊣'' depicts negative regulation/ expression. Up- and down-regulated genes are indicated in red and green, respectively. IL1RN – interleukin-1 receptor antagonist; S100A9 – S100 calcium binding protein A9 (calgranulin B); MMP-8 - Matrix metalloproteinase 8; CAMP – cathelicidin antimicrobial peptide; GRO2 – growth related oncogene 2 (CXCL2); GRO3 - growth related oncogene 3 (CXCL3); MMP-9 - Matrix metalloproteinase 9; S100A8 – S100 calcium binding protein A8 (calgranulin A); CCL4 – chemokine (c-c motif) ligand 4; IL1B – interleukin-1β; PGLYRP – peptidoglycan recognition protein; CCR1 – chemokine (c-c motif) receptor 1; TLR – toll-like receptor; CCL17 – chemokine (c-c motif) ligand 17; LBP – lipopolysaccharide binding protein; HSP1A – heat shock protein 1A; *PPTA*$^{-/-}$- *preprotachykinin-A* knockout.

Chemokines such as MIP-2, MIP-1α and MCP-1 levels are reported to be increased 8 h after CLP-induced severe sepsis in mice (Walley *et al.,* 1997). These chemokines are chemoattractants to neutrophils and contribute to the host defense (Smith 1994), but in severe sepsis, may also cause mortality (Walley *et al.,* 1997). Elevated serum level of CCL3 was also found in septic patients (Cavaillon *et al.,* 2003). Further, peritoneal CCL6 (Ness *et al.,* 2004) and CCL22 (Matsukawa *et al.,* 2000; Ness *et al.,* 2003) concentrations were elevated in the CLP mouse model of sepsis. The present microarray analysis showed a significantly higher expression of few of the inflammatory genes in *PPTA*$^{-/-}$ mice after sepsis compared to the wild-type mice. Although gene expression of CCL-2, CCL-3, CCL-4, CCL-9, CXCL-1, CXCL-2 and CXCL-10 were increased in wild-type mice after CLP, which was as expected, the increase was more significant in *PPTA*$^{-/-}$ mice 8 h after sepsis. In spite of the elevated gene expression of pro-inflammatory chemokines, *PPTA*$^{-/-}$ mice are protected against CLP induced sepsis (Puneet *et al.,* 2006). The precise mechanism of protection is not clear yet.

Interestingly, CCL3, CCL6 and CXCL10 have been demonstrated to be protective in sepsis-induced injury and mortality in a murine CLP model (Ness *et al.,* 2004; Ness *et al.,* 2003; Takahashi *et al.,* 2002). CXCL10 (IFN-γ-inducible protein 10, IP-10) is a potent antimicrobial chemokine (Yang *et al.,* 2003) but the mechanism of protection is unknown (Ness *et al.,* 2004). However, CCL6 is reported to augment peritoneal macrophage activity and reduce bacterial leak from the gut (Ness *et al.,* 2004). Macrophage-derived chemokine (MDC) (CCL22) also protected mice against CLP-induced death (Matsukawa *et al.,* 2000). CCL17, a Th2 lymphocyte chemoattractant, is thought to regulate the pro-inflammatory type 1 response in CCR1$^{-/-}$ mice after CLP-

induced sepsis (Ness *et al.,* 2004). I found a significant increase in CCL-6, CCL-17 and CCL-22 gene exclusively in *PPTA*$^{-/-}$ mice after the induction of sepsis. It is possible that these chemokines provide some protection against pathogens and damage in *PPTA*$^{-/-}$ mice after CLP. Pathway analysis further provides an insight into the complex network of interactions among these pro-inflammatory chemokines.

The role of chemokine receptors in sepsis is not very clear (Ness *et al.,* 2004). Mice lacking CXCR-2 or CCR-1 are reportedly less susceptible to CLP-induced sepsis (Ness *et al.,* 2004; Ness *et al.,* 2003). On the contrary, macrophages expressing CX3CR-1, CCR-2 or CCR-4 have been shown to protect against pathogens due to their bactericidal functions (Ishida *et al.,* 2008). The present microarray data showed a significant increase of more than 2-fold in CCR-2 and CCR-5 gene, only in *PPTA*$^{-/-}$ septic mice compared to sham, implying bactericidal functions. However, CCR-1 expression in the wild-type and *PPTA*$^{-/-}$ mice also increased after CLP and the increase was more in the latter. CCL-3 is a known ligand of CCR-1 and CCL-2, CCL-3 and CCL-22 along with their receptors have been observed to enhance antibacterial activities of macrophages (Matsukawa *et al.,* 2000; Takahashi *et al.,* 2002; Matsukawa *et al.,* 1999). Thus the elevated CCL-3-CCR-1 expression seen in the present data could be beneficial in *PPTA*$^{-/-}$ mice.

Cytokines play an important but complex role in sepsis. Activated CD4 T cells secrete cytokines with either inflammatory (type 1 helper T-cell [Th1]) properties (TNF-α, interferon-γ, IL-2), or anti-inflammatory (type 2 helper T-cell [Th2]) properties (IL-4 and IL-10) (Abbas *et al.,* 1996; Opal and DePalo 2000) influenced by the type, size and the site of infection (Abbas *et al.,* 1996). Cytokines are considered both friend and foe in sepsis (Hotchkiss and Karl 2003). Although normal cytokine reactions help to

defend host and eliminate bacteria, overwhelming invasion induces systemic cytokinemia. Successful treatment of sepsis involves cytokine modulation and restriction of the systemic reactions only to the inflammatory foci. However it should be noted that only a small amount of cytokines can defend host and a complete blockade may damage the host (Kato *et al.,* 1995).

The microarray analysis showed that IL-1β increased significantly in both wild-type and *PPTA*$^{-/-}$septic mice compared to sham. However, the anti-inflammatory cytokine, IL-1ra also showed a significant increase after CLP in these mice and the expression was much higher in *PPTA*$^{-/-}$ mice compared to wild-type mice. Elevated IL-1ra gene expression in wild-type mice after sepsis is consistent with a literature report that IL-1ra mRNA level increased (by 10-fold) 3 to 6 h after CLP and sustained for 18 h (Salkowski *et al.,* 1998). IL-1ra competitively inhibits the binding of IL-1α and IL-1β to their receptors and thus neutralizing their effects (Bresnihan and Cunnane 1998). Recent clinical trial of neonatal-onset multisystem inflammatory disease showed a promising improvement with IL-1ra treatment (Remick 2007; Goldbach-Mansky *et al.,* 2006). It is believed that anti-inflammatory strategies applied early in patients with a hyper-inflammatory immune response may be life-saving (Hotchkiss and Karl 2003). *PPTA*$^{-/-}$ mice exhibited high expression of IL-1ra early in sepsis along with the hyper-inflammatory state, which could partly explain the improvement in survival. High levels of blood IL-1ra are found in septic patients and in animal models of sepsis although the role of IL-1ra in the immunosuppression is not clearly defined (Reddy *et al.,* 2001).

Serum amyloid A3, a major acute phase protein with a role in antibacterial immunity, has been reported to be enhanced by up to 1000-fold in sepsis (Sriskandan and

Altmann 2008). SAA3 is released at the site of injury and LPS-stimulated macrophages show enhanced expression of SAA3 (Meek *et al.,* 1992). In the present data, SAA3 was observed to increase in wild-type and *PPTA*$^{-/-}$ septic mice compared to sham, with a higher increase in the knock-out mice, thus providing more antibacterial immunity. S100A8 is a new group of pro-inflammatory proteins expressed by phagocytes in inflammatory diseases (Roth *et al.,* 2003). S100A8 was reported to be up-regulated in the blood samples of severe sepsis patients (Prucha *et al.,* 2004). I found a similar increase in S100A8 in wild-type mice after sepsis. Interestingly, this increase was absent in *PPTA*$^{-/-}$ septic mice. Surfactants containing antibacterial substances such as cathelicidins can destroy both Gram-positive and Gram-negative pathogens (Sriskandan and Altmann 2008) and orchestrate a variety of inflammatory and immune responses (Zanetti 2004). *PPTA*$^{-/-}$ mice showed much higher fold-increase of cathelicidins after sepsis compared to the corresponding wild-type mice.

MMP-8, secreted mainly by neutrophils, is known to contribute to inflammatory cell trafficking and inflammation in asthma (Gueders *et al.,* 2005). However, MMP-8 is also suspected to regulate protective immune functions as it is found to be anti-inflammatory in allergic asthma (Gueders *et al.,* 2005). As expected, the microarray data showed a significant up-regulation of MMP-8 gene after sepsis in wild-type mice. But the increase was more prominent in *PPTA*$^{-/-}$ mice, thus possibly providing anti-inflammatory protection and neutrophil apoptosis. MMP-9 is a matrix degrading enzyme which is highly expressed at inflammation sites (Grimm *et al.,* 2006). MMP-9 plays a role in the pathogenesis of various chronic inflammatory diseases such as asthma where MMP-9 is up-regulated (Lee *et al.,* 2001; Kelly and Jarjour 2003;

Wenzel *et al.,* 2003). MMP-9 is implicated in the recruitment of eosinophils and neutrophils (Lee *et al.,* 2001). Reduced MMP-9 secretion on a molecular level might cause an anti-inflammatory effect (Grimm *et al.,* 2006). *PPTA*$^{-/-}$ mice did not show more than 2-fold increase in MMP-9 after CLP unlike the wild-type mice implying some beneficial anti-inflammatory effect.

Although TLRs and their downstream signal transduction via PI3K are important in sepsis, I excluded them from this discussion as it is beyond the scope of this thesis to consider all the mediators involved in sepsis. However, I would like to allude to the fact that PI3K activation can lead to survival in sepsis (Cinel and Opal 2009) and the *PPTA*$^{-/-}$ mice had elevated PI3K gene expression after CLP in the present experiment. Similarly, it is sufficient to mention that the significant down-regulation of heat shock protein 1A in *PPTA*$^{-/-}$ septic mice (Fig. 4 B) could be associated with beneficial effects as heat shock protein induction before a pro-inflammatory stimulus is reported to be protective but after a pro-inflammatory stimulus it is found to be cytotoxic (Chen *et al.,* 2007).

RT-PCR results for selected differentially expressed genes further validated the data from the microarray analysis. The RT-PCR data showed a similar expression of all the 10 genes evaluated when compared to microarray results. Although the fold-change between the sham control and CLP-induced sepsis mice determined by RT-PCR differed from that found on microarray, the trend of the response was similar between the two methods.

DNA microarray reflects the functional state of the cell, but it is the translated protein that executes the instructions of the genome (White and Salamonsen 2005). Reportedly, less than 50% of the changes at the mRNA level are conveyed to the

protein level and various post-translational modifications alter their function (Schulze and Downward 2001; White and Salamonsen 2005). To evaluate if transcription and translation are coordinately regulated, IL-1ra was quantified in plasma and tissue by ELISA. In line with literature reports of elevated circulating IL-1ra in sepsis (Cavaillon *et al.,* 2003), and consistent with the microarray and RT-PCR data, I found a significant increase in IL-1ra protein level in both wild-type and *PPTA*$^{-/-}$ mice 8 h after sepsis. It seems prudent to define the inflammatory status on the basis of complete plasma profile and genome data, even if grouping into traditional immunologic categories becomes difficult (Osuchowski *et al.,* 2006).

So far the scientific community does not have an answer for whether the septic patients are hyper-inflammatory or immuno-compromised (Remick 2007). Failed clinical trials have shown that simply blocking inflammatory response may be paid back by an impaired resolution of infection. Instead of targeting any individual mediator that reflects the functional status of sepsis, inflammation should be treated at the right time in the right place. In this perspective this study provides a valuable molecular fingerprint of *PPTA*$^{-/-}$ mice that are protected against mortality in CLP-induced sepsis. This is the first investigation exploring pulmonary gene expression profiles using microarray analysis in *PPTA*$^{-/-}$ mice subjected to CLP-induced sepsis. Elevated levels of pro- and anti-inflammatory gene expression observed in the early stages of sepsis may help in resolving the infection without excessive immunosuppression. Antimicrobial mediators such as CXCL10, SAA3, cathelicidins, that were observed in the present study would further support in maintaining this precarious balance of inflammatory forces. In conclusion, in this study I have shown the gene profile following sepsis and the effect of *PPTA* gene deletion. This study will

help define the mechanisms by which *PPTA* gene products contribute to lung injury in sepsis.

In addition to the gene expression, I also did simultaneous cytokine protein expression at various time points, as explained in the next chapter.

CHAPTER 7. PLASMA CYTOKINE PROFILE IN *PPTA*$^{-/-}$ MICE

7.1 Introduction

In sepsis, the nature of the insult, the cellular composition and the micro-environment of each organ, influences the extent of local tissue injury (Cavaillon and Annane 2006). Infection, injury and inflammation trigger the release of cytokines that act as immune mediators (Ray *et al.,* 2005; de Jager and Rijkers 2006). These inflammatory proteins are elevated in various disease states such as autoimmune diseases, bowel inflammatory disease and sepsis. It has been well established that cytokine cascades play a major role in the progression of sepsis. Large amounts of cytokines are produced mainly within tissues and released into the systemic circulation to mediate the inflammatory responses in sepsis. Anti-inflammatory mediators predominate systemically to avoid new inflammatory foci, but within the tissues their levels may not always be sufficient to prevent deleterious inflammatory response (Cavaillon and Annane 2006). Thus, in addition to the pathophysiological evaluation of lung tissue, it is important to analyze the cytokine profile in peripheral blood compartment in *PPTA*$^{-/-}$ septic mice.

Plasma is considered as one of the major sources for measurement of clinical markers in sepsis (Osuchowski *et al.,* 2006). As early diagnosis and treatment are critical in sepsis management, evaluation of plasma cytokines over a time course can provide a window towards a better understanding of the nature and severity of sepsis. Rather than analyzing individual cytokine levels by conventional ELISA, integration of data

from simultaneous measurement of multiple cytokines is more likely to be representative of biological processes. In this regard, I used Multiplexed bead-based suspension arrays for the measurement of a set of plasma cytokines in *PPTA*$^{-/-}$ mice subjected to polymicrobial sepsis.

Luminex Multianalyte Profiling (xMAP) technology is widely used for quantification of analytes such as proteins, ligands, DNA and RNA in multiplexed bead-based assays (Kingsmore 2006; Nolan and Mandy 2006). The beads bound with antibodies, oligonucleotides or peptides are run through a Luminex instrument and classified by laser excitation of the internal dyes (Arellano-Garcia *et al.,* 2008). The reporter dye is excited by another laser and the fluorescence proportional to the bound analyte is recorded (Vignali 2000; Ray *et al.,* 2005). High throughput, accuracy, efficiency, sensitivity, simultaneous analyte detection, low cost and time reduction are some of the pros of these liquichips (Vignali 2000; Dupont *et al.,* 2005; Prabhakar *et al.,* 2002; Linkov *et al.,* 2007). In addition, the data collected by xMAP technology has been reported to be comparable to that from ELISA (Dupont *et al.,* 2005).

7.2 Materials and Methods

7.2.1 Animal Ethics

Reference: **Section 2.2**

7.2.2 Induction of polymicrobial sepsis

PPTA$^{-/-}$ and wild-type Balb/c male mice (25-30 g) were randomly divided into sham or CLP experimental groups (n = more than 6 in each group). Polymicrobial sepsis was induced by CLP as explained in **Section 2.3**. The same surgical procedure except the cecal ligation and puncture was performed on sham-operated animals. The mice were

sacrificed at various time points (1, 5, 8 and 24 h; n = at least 6 for each time point) after surgery by an i.p. injection of pentobarbitone (**Fig. 7.1**). Blood was harvested through cardiac puncture, heparinized, centrifuged, plasma removed and stored at -80° C for subsequent measurement.

7.2.3 Plasma cytokine profile using bead array

Time-dependent plasma cytokine profile was obtained using Procarta™ Cytokine kits (Panomics, CA, USA) employing multiplex immunoassays based on xMAP™ detection technology developed by Luminex™ (Luminex Corporation) using Luminex™ bead array system. Fluorescently encoded antibody beads that were uniquely detected in a flow cytometer were used and 18 mouse cytokines were evaluated through a sandwich immunoassay **(Fig. 7.1)**. Briefly, 50 µl of the antibody beads were added to each well of the pre-wet 96-well filter bottom plate and washed with wash buffer. Assay buffer (75 µl/well), standard and sample (25 µl/well) were added to the pre-designated wells and incubated for 30 min at room temperature on a shaker (500 rpm). After washing, detection antibody (25 µl/well) was added and incubated for 30 min at room temperature on a shaker (500 rpm). Streptavidin-PE (50 µl/well) was added to the washed plate and incubated again for 30 min at room temperature on a shaker (500 rpm). Subsequent to another wash, 120 µl/well of the reading buffer was added, placed on a shaker (500 rpm) for 5 min at room temperature and analyzed on Luminex 100 instrument. The median fluorescence intensity of 100 beads per sample per cytokine was used to determine the intensity levels of cytokines. Standard curves were plotted and fitted using a 5-parameter logistic model, from which the sample cytokine concentrations were determined. The bead-analyte associations are given in **Table 7.1** (Procarta™ Mouse Cytokine Assay kit, Panomics,

Fig. 7.1

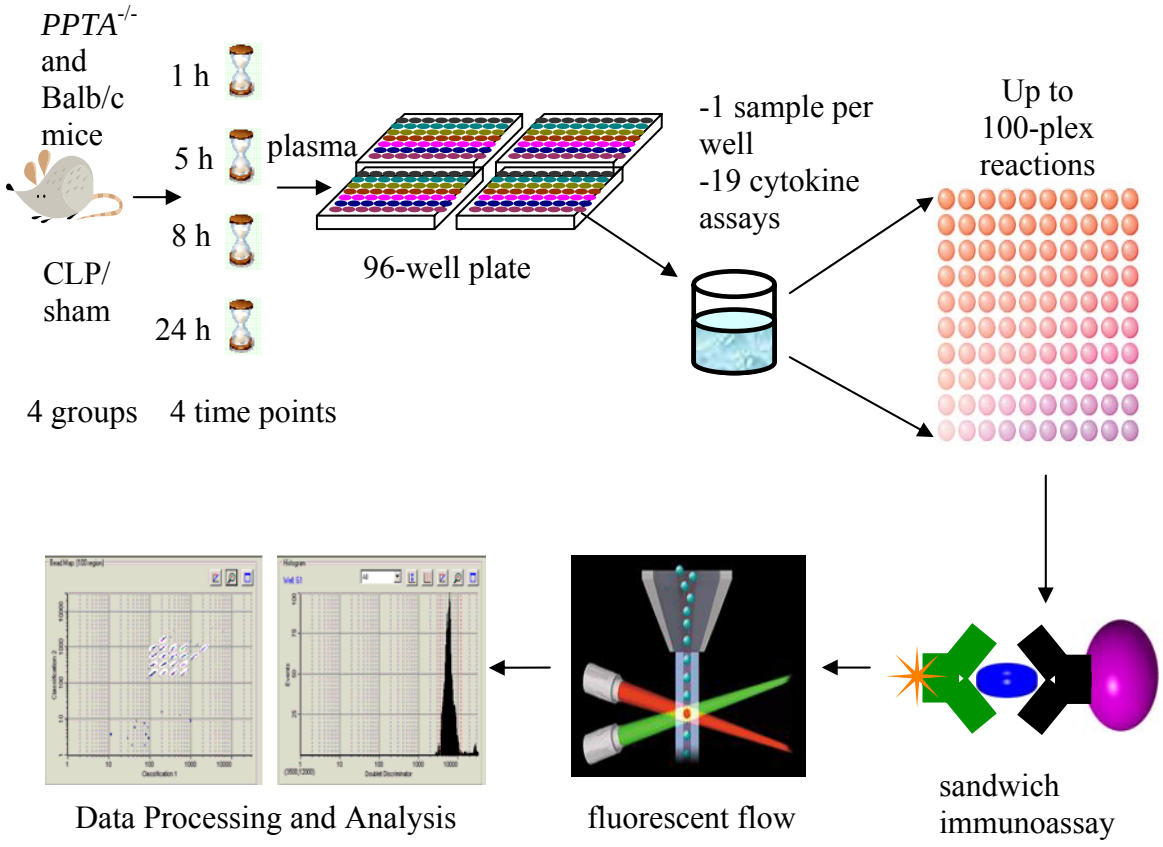


Fig. 7.1 Schematic workflow of multiplex immunoassay. *PPTA*$^{-/-}$ and wild-type Balb/c mice were randomly divided into sham or CLP experimental groups (n = 6-8 in each group). The mice were sacrificed at various time points (1, 5, 8 and 24 h) after surgery and plasma was analyzed for 18 mouse cytokines by a sandwich multiplex immunoassay. The sample cytokine concentrations were determined using standard curves. CLP – cecal ligation and puncture; *PPTA*$^{-/-}$- *preprotachykinin-A* knockout.

Table 7.1 Bead-analyte association-Procarta Mouse Cytokine Assay Kit[1]

Bead[2]	Analyte
18	IL-1α
19	IL-1β
20	IL-2
21	IL-3
25	IL-4
26	IL-5
27	IL-6
28	IL-10
33	IL-12(p40)
34	IL-12(p70)
35	IL-17
36	IL-13
37	KC
42	RANTES
43	IFN-γ
44	GM-CSF
45	TNF-α
47	MIP-1α
52	EOTAXIN

[1] Procarta Mouse Cytokine Assay Kit was used for the assay. Each of the beads was coated with a reagent specific to the analyte given in the table.
[2] Each of the colour-coded tiny beads (microspheres) was given a number to identify the cytokine analyte it represents.
GM-CSF - granulocyte macrophage-colony-stimulating factor; IFN-γ – interferon-γ; IL – interleukin; KC - keratinocyte-derived chemokine; MIP-1α - macrophage inflammatory protein-1α; RANTES - regulated upon activation normal T cell expressed and secreted; TNF-α - tumor necrosis factor-alpha.

CA, USA)**.** The cytokine concentrations obtained for each group at the different time points were averaged across each replicate set and expressed as pg/ml. The kit sensitivity (Limit of Detection, LOD) was 1 pg/ml/cytokine.

7.2.4 Statistics

Statistical analysis was performed as mentioned in **Section 2.17**.

7.3 Results

PPTA$^{-/-}$ and wild-type mice were sacrificed at 1, 5, 8 and 24 h after sham or CLP surgery and 18 plasma cytokines were analyzed. Among all the cytokines tested (namely CCL11, GM-CSF, IFN-γ, IL-10, IL-12, IL-13, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, CXCL1, CCL3, CCL5, TNF-α), levels of both pro- **(Fig. 7.2)** and anti-inflammatory (**Fig. 7.3**) cytokines were significantly elevated in the *PPTA*$^{-/-}$ sepsis mice compared to the wild-type mice. IL-2, IL-3, IL-4 and IL-17 levels were below the detection limit of the assay in all the samples.

7.3.1 Cytokine profile as a function of time for the sham groups

Mice subjected to sham surgery showed elevated levels of various cytokines at 1 and 5 h after the surgery ($P < 0.05$) **(Fig 7.2 and 7.3)**. IL-1β, GM-CSF, CCL11, IL-5, IFN-γ, IL-10 and IL-13 were increased in wild-type mice at the early time points studied **(Fig 7.2a,g,j,l,m and 7.3a,b** respectively). Similarly, *PPTA*$^{-/-}$ mice showed elevated levels of IL-1β, CCL5, GM-CSF, CCL11, IL-5, IFN-γ, IL-10 and IL-13 (**Fig 7.2a,f,g,j,l,m and 7.3a,b** respectively) at 1 and 5 h after sham surgery. However the levels were reduced in both *PPTA*$^{-/-}$ and wild-type sham groups at the later time points.

Fig. 7.2a

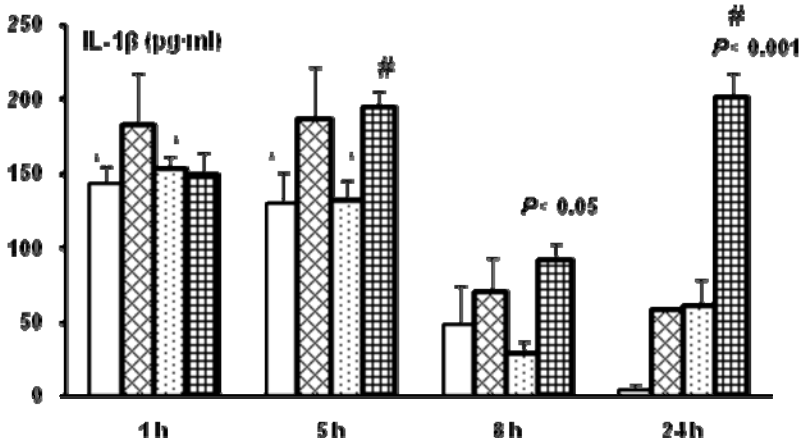


Fig. 7.2b

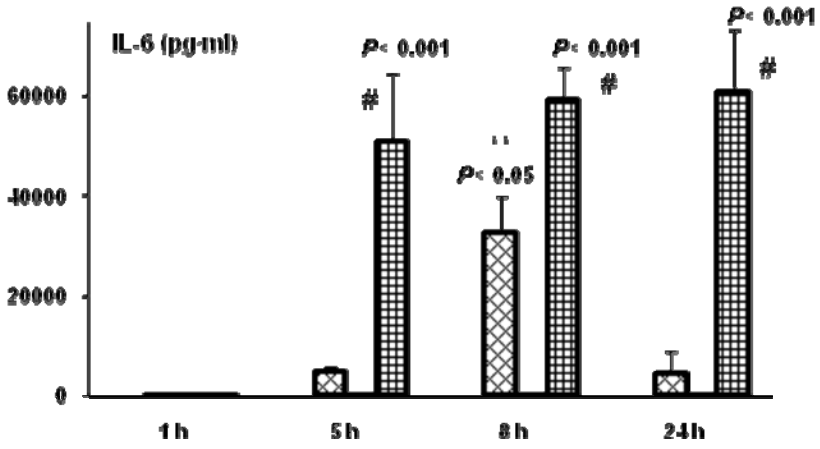


Fig. 7.2c

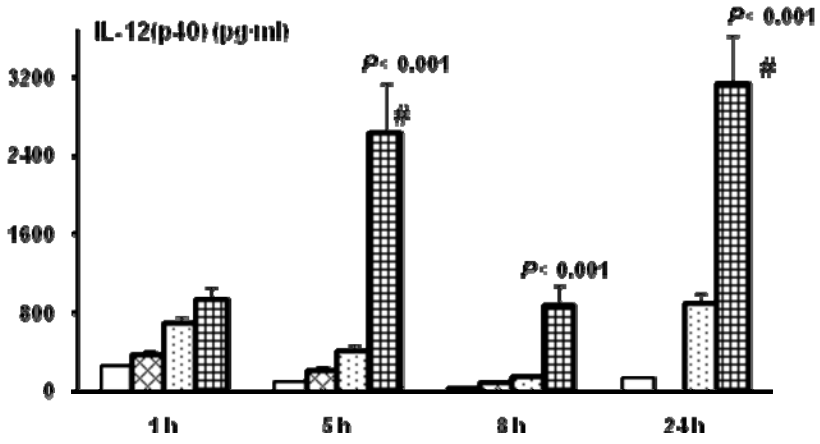


Fig. 7.2d

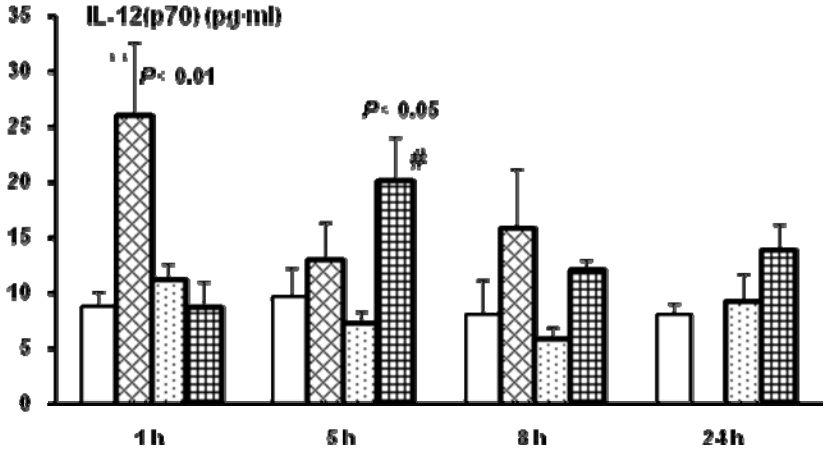


Fig. 7.2e

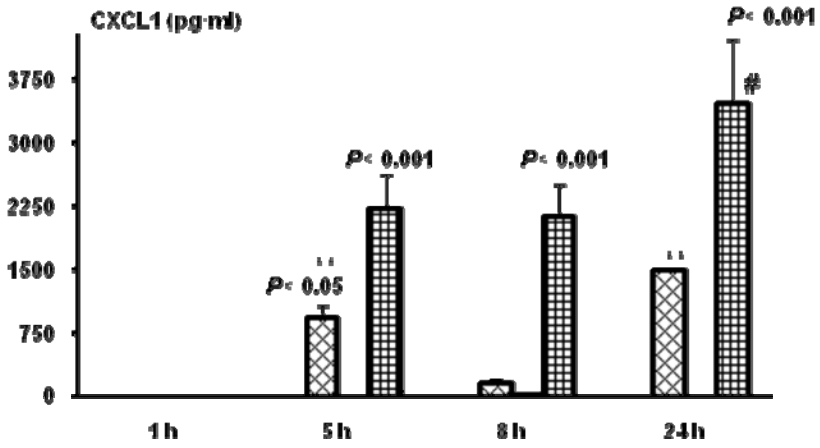


Fig. 7.2f

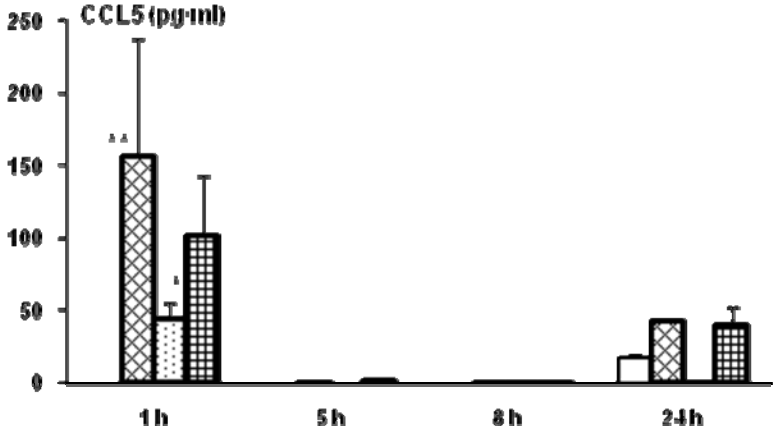


Fig. 7.2g

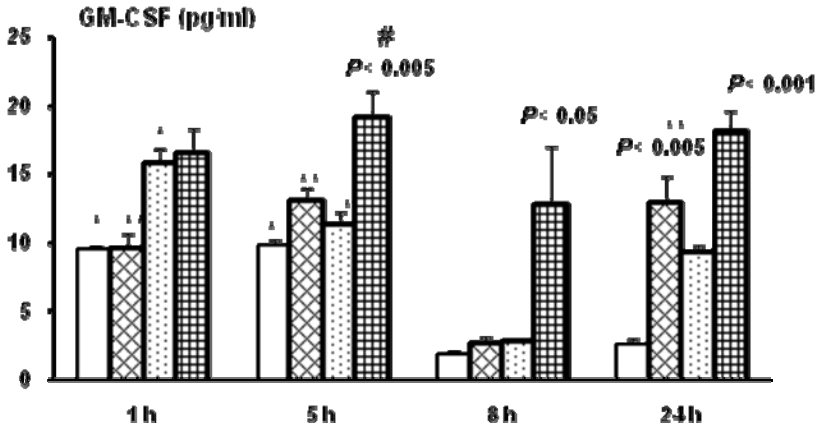


Fig. 7.2h

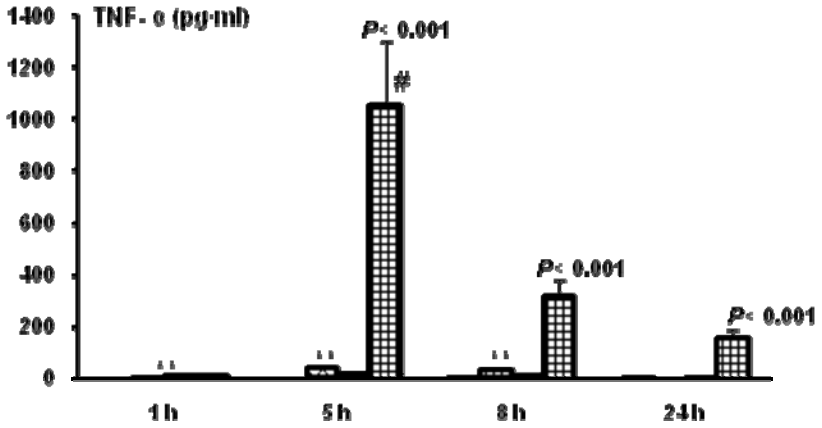


Fig. 7.2i

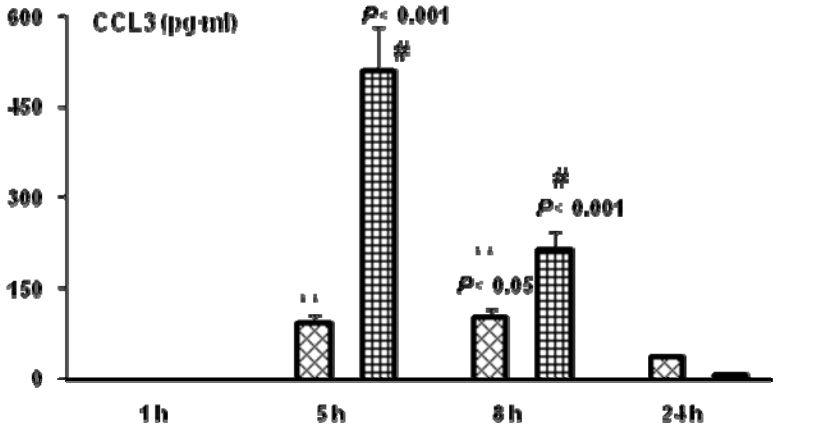


Fig. 7.2j

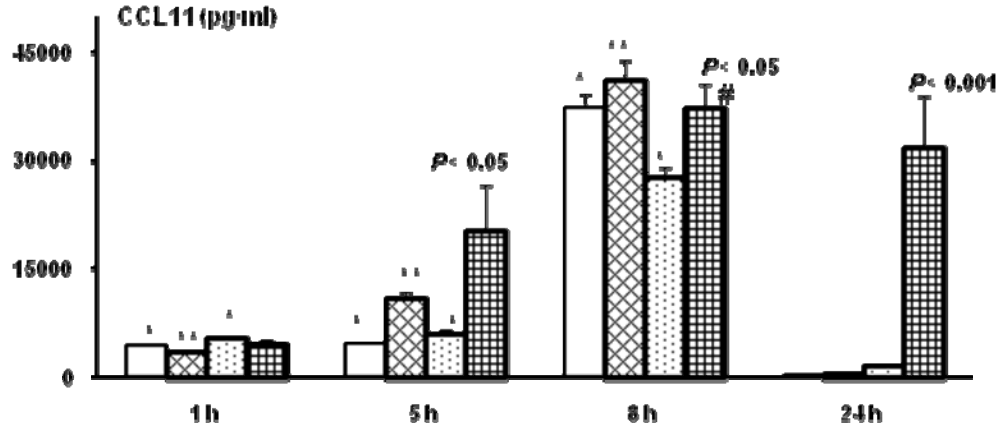


Fig. 7.2k

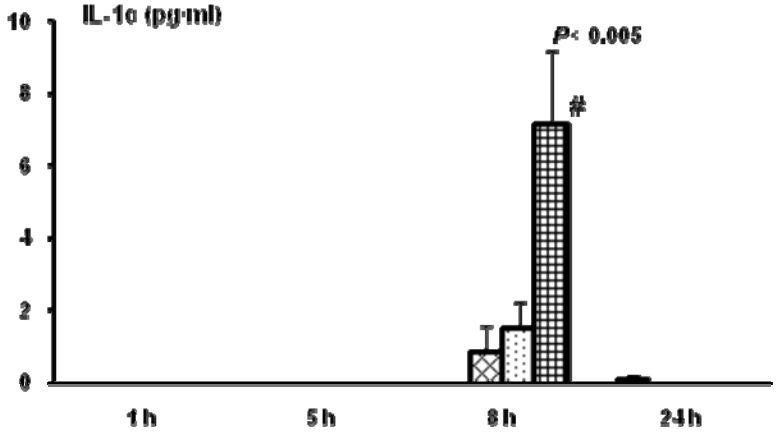


Fig. 7.2l

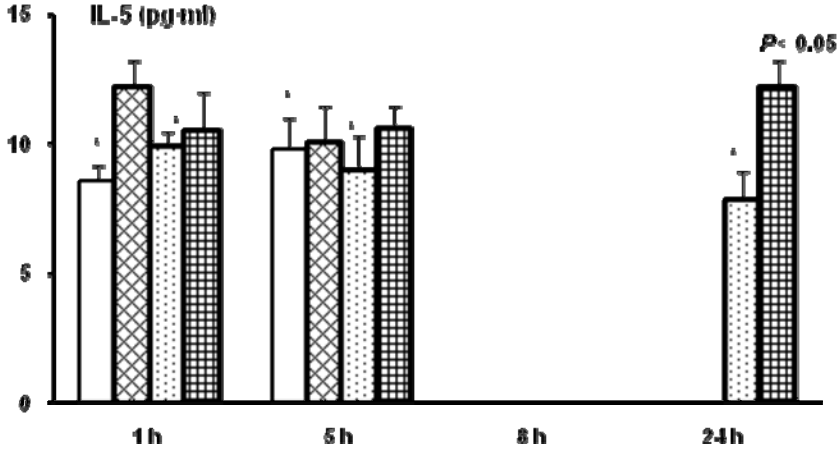


Fig. 7.2m

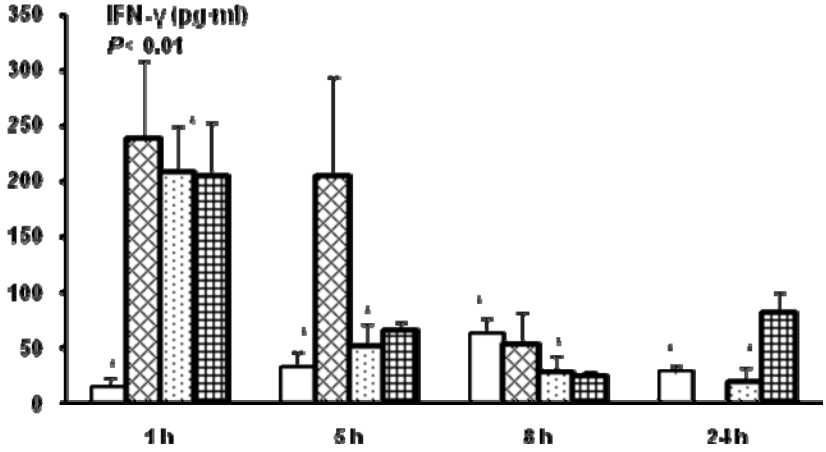


Figure 7.2 Plasma pro-inflammatory cytokine profile in wild-type and *PPTA*$^{-/-}$ septic mice. a. IL-1β; b. IL-6; c. IL-12(p40); d. IL-12(p70); e. CXCL1; f. CCL5; g. GM-CSF; h. TNF-α; i. CCL3; j. CCL11; k. IL-1α; l. IL-5; m. IFN-γ. Cytokine levels in plasma were measured 1, 5, 8 and 24 h after CLP or sham surgery in wild-type and *PPTA*$^{-/-}$ mice by multiplex immunoassay. Results were expressed as mean ± SEM (n = 6 mice per group). *P* values were shown for comparison with corresponding sham group. Symbols were used to denote significant differences between groups as a function of time. Key: Balb/c sham, open bars; Balb/c CLP, outlined diamond bars; *PPTA*$^{-/-}$ sham, dotted bars; *PPTA*$^{-/-}$ CLP, small grid bars. $^{*}P<0.001$ when compared to the corresponding normal value; $^{**}P<0.05$ when compared to the corresponding values of Balb/c septic mice at different time points; $^{\#}P<0.05$ when compared to the corresponding values of *PPTA*$^{-/-}$ septic mice at different time points; CLP, cecal ligation and puncture; GM-CSF, granulocyte macrophage-colony-stimulating factor; IL, interleukin; IFN-γ, interferon-γ; *PPTA*$^{-/-}$, *preprotachykinin A* knockout; TNF-α, tumor necrosis factor-α.

Fig. 7.3a

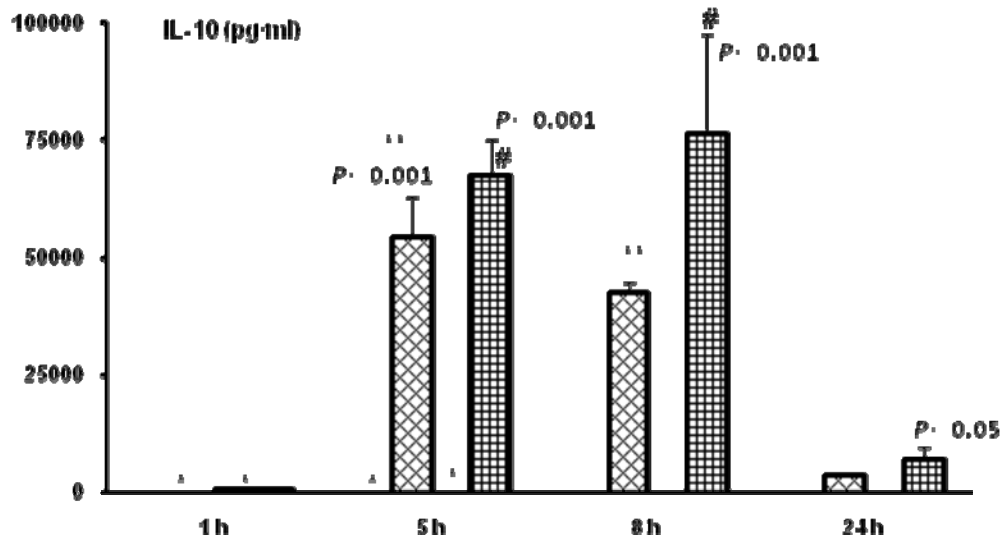


Fig. 7.3b

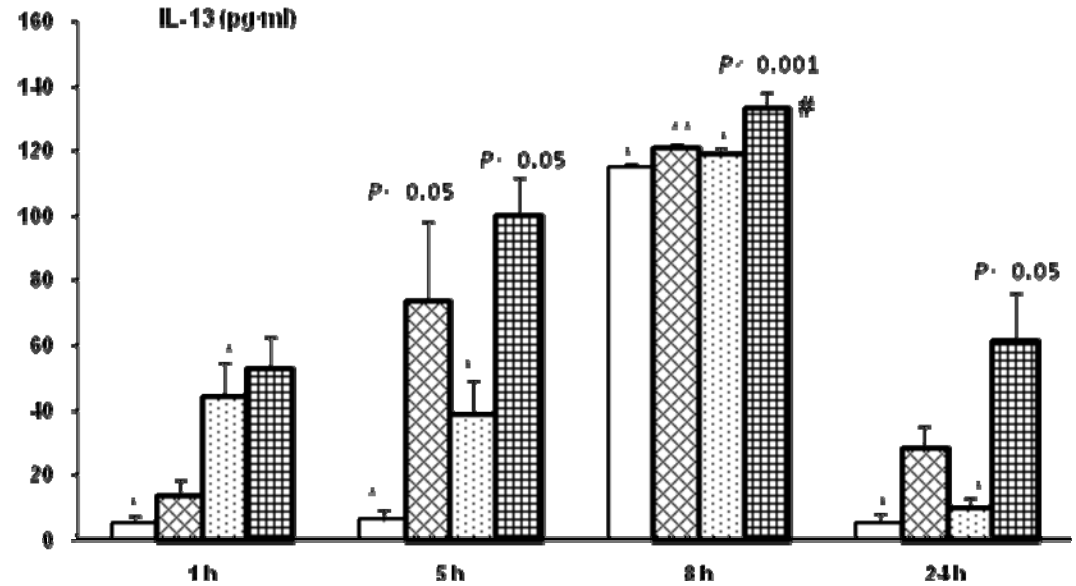


Figure 7.3 Plasma anti-inflammatory cytokine profile in wild-type and *PPTA*$^{-/-}$ septic mice. a. IL-10; b. IL-13. Cytokine levels in plasma were measured 1, 5, 8 and 24 h after CLP or sham surgery in wild-type and *PPTA*$^{-/-}$ mice by multiplex immunoassay. Results were expressed as mean ± SEM (n = 6 mice per group). *P* values were shown for comparison with corresponding sham group. Symbols were used to denote significant differences between groups as a function of time. Key: Balb/c sham, open bars; Balb/c CLP, outlined diamond bars; *PPTA*$^{-/-}$ sham, dotted

bars; *PPTA*$^{-/-}$ CLP, small grid bars. *P<0.001 when compared to the corresponding normal value; **P<0.05 when compared to the corresponding values of Balb/c septic mice at different time points; $^{\#}$P<0.05 when compared to the corresponding values of *PPTA*$^{-/-}$ septic mice at different time points; CLP, cecal ligation and puncture; IL, interleukin; *PPTA*$^{-/-}$, *preprotachykinin A* knockout.

7.3.2 Cytokine profile as a function of time for the Balb/c septic mice

Mice subjected to CLP have been reported to show elevated levels of pro-inflammatory cytokines such as IL-6, CXCL2 and TNF-α (Zhang *et al.,* 2007; Puneet *et al.,* 2006; Ertel *et al.,* 1991; Salkowski *et al.,* 1998). Consistently, I found increased plasma IL-6, IL-12(p70), CXCL1, CCL5, GM-CSF, TNF-α, CCL3 and CCL11 levels **(Fig. 7.2b,d-j** respectively**)** in Balb/c septic mice ($P < 0.05$). The elevated levels were apparent by 5 h after CLP for many of the cytokines and continued to remain high even at the 24 h time point **(Fig. 7.2)**. However, CXCL1 and GM-CSF levels showed a reduction at 8 h time point **(Fig. 7.2e,g)**.

Anti-inflammatory cytokine, IL-10 levels were increased in wild-type mice only at 5 and 8 h after CLP ($P < 0.001$) **(Fig. 7.3a)**. In addition, IL-13 levels showed significant increase only at 8 h after surgery ($P < 0.05$) **(Fig. 7.3b)**.

7.3.3 Cytokine profile as a function of time for the *PPTA*$^{-/-}$ septic mice

PPTA$^{-/-}$ septic mice also showed an increase in various cytokines over 24 h after induction of sepsis ($P < 0.05$). A significant elevation was observed for IL-1β, IL-6, IL-12(p40), IL-12(p70), CXCL1, GM-CSF, TNF-α, CCL3, CCL11, IL-1α, IL-5, IL-10 and IL-13 **(Fig. 7.2a-e,g-l and Fig. 7.3a,b** respectively). However, IL-1β and IL-5 levels were lowered at 8 h after CLP surgery **(Fig. 7.2e,g)**.

7.3.4 Comparative cytokine profiles for the *PPTA*$^{-/-}$ and wild-type septic mice

Several sets of cytokines showed significantly different patterns across the *PPTA*$^{-/-}$ and the wild-type septic mice compared to their corresponding sham control groups.

7.3.4.1 Pro-inflammatory cytokine profiles

Plasma IL-1β levels were elevated more significantly in *PPTA*$^{-/-}$ septic mice during the later phase of sepsis (8 and 24 h) ($P < 0.05$ and $P < 0.001$ respectively) compared to the corresponding wild-type mice **(Fig. 7.2a)**. Levels of IL-6, an important pro-inflammatory cytokine in sepsis, were increased significantly in wild-type mice at 8 h after CLP ($P < 0.05$), but the increase was significantly higher in *PPTA*$^{-/-}$ septic mice at 5, 8 and 24 h after CLP ($P < 0.001$) and also when compared to the corresponding increase in wild-type group **(Fig. 7.2b)**. Pro-inflammatory cytokine, IL-12(p70), is a heterodimer of IL-12(p40) and IL-12(p35) subunits connected by a disulphide bond that is essential for the biological activity (Cooper and Khader 2007). IL-12(p70) was significantly increased in wild-type mice only at 1 h after CLP ($P < 0.01$) but the difference was apparent in *PPTA*$^{-/-}$ septic mice at 5 h ($P < 0.05$) **(Fig. 7.2d)**. Levels of IL-12(p40), a component of cytokines IL-12 and IL-23, were higher in *PPTA*$^{-/-}$ septic mice at 5, 8 and 24 h after CLP ($P < 0.001$) **(Fig. 7.2c)**. CXCL1 and GM-CSF levels were also significantly elevated ($P < 0.001$ and $P < 0.05$ respectively) in *PPTA*$^{-/-}$ septic mice compared to the wild-type septic mice **(Fig. 7.2e,g)**.

Systemic levels of TNF-α were increased significantly more in *PPTA*$^{-/-}$ mice 5 h after CLP ($P < 0.001$) and the increase was apparent upto 24 h post-CLP ($P < 0.001$) **(Fig. 7.2h)**. CCL3 protein levels in plasma were also significantly elevated in *PPTA*$^{-/-}$ mice at 5 and 8 h after CLP ($P < 0.001$) compared to the increase in wild-type mice at 8 h ($P < 0.05$), but this increase was reversed by 24 h post-CLP **(Fig. 7.2i)**. In contrast, plasma CCL11 levels were elevated to a greater extent in *PPTA*$^{-/-}$ mice upto 24 h after CLP **(Fig. 7.2j)**. IL-1α levels were found to be increased in *PPTA*$^{-/-}$ septic mice compared to the other groups only at 8 h after CLP **(Fig. 7.2k)**. IL-5 plasma levels

were not significantly different between the wild-type and *PPTA*$^{-/-}$ septic mice at any of the time points studied except at 24 h ($P < 0.05$) **(Fig. 7.2l)**. Lastly, IFN-γ was found to be significantly increased in wild-type septic mice as early as 1 h ($P < 0.01$) and persisted upto 5 h after CLP, but the increase was not statistically significant in *PPTA*$^{-/-}$ septic mice **(Fig. 7.2m)**.

7.3.4.2 Anti-inflammatory cytokine profiles

Levels of IL-10 were increased after CLP in both *PPTA*$^{-/-}$ and wild-type mice, but the difference was significant in *PPTA*$^{-/-}$ mice at 5, 8 and 24 h ($P < 0.001$, $P < 0.001$ and $P < 0.05$ respectively) after the surgery **(Fig. 7.3a)**. Similarly, plasma levels of another anti-inflammatory cytokine, IL-13, were elevated in *PPTA*$^{-/-}$ and wild-type septic mice compared to the sham group, but the increase was more significant for the knock-out mice especially at 5, 8 and 24 h ($P < 0.05$, $P < 0.001$ and $P < 0.05$ respectively) after the induction of sepsis **(Fig. 7.3b)**.

7.4 Discussion

It is interesting to note that the *PPTA* gene deletion in mice contributed to a survival phenotype evidenced by a greater resilience to sepsis (Puneet *et al.,* 2006). However, the mechanism of tolerance and survival at elevated levels of systemic inflammatory cytokines has yet to be established. In the previous section, elevated levels of pulmonary cytokines were seen in *PPTA*$^{-/-}$ mice subjected to polymicrobial sepsis. Although tissue-associated cytokine levels represent the cytokine production more closely, systemic levels also provide a faster and reliable means of measurement, especially in clinical applications. Detectable plasma cytokines are likely to represent the excess of produced mediators which have not been contained within target tissues

or organs. Using a bead-array based platform coupled with a flow-cytometric fluorescent based reader, I performed simultaneous measurement of 18 mouse cytokines using a very small volume (25 µl) of plasma per assay. Multiplexed bead-based arrays have been shown earlier to be especially useful for detection of analytes in precious small volume (Liu *et al.,* 2005).

Plasma cytokine time-point data showed that *PPTA*$^{-/-}$ mice subjected to CLP-induced sepsis exhibited elevated levels of both pro- and anti-inflammatory cytokines. Indeed, early phase of lethal sepsis is reported to show over-expression of both pro- and anti-inflammatory cytokines (Osuchowski *et al.,* 2006). Plasma concentrations of TNF-α, IL-1β, IL-6, IL-8, soluble cytokine receptors, cytokine receptor antagonists and counter-inflammatory cytokines are known to be elevated in human sepsis (Blackwell and Christman 1996). I found significantly elevated levels of various pro-inflammatory cytokines such as TNF-α, CCL3, IL-1β, IL-6, CXCL1 and CCL11 in *PPTA*$^{-/-}$ mice compared to the wild-type mice, especially at later time points after induction of sepsis. TNF-α, IL-1 and IL-6 coordinate the initiation of acute phase response in sepsis (Sriskandan and Altmann 2008) that is triggered by the pathogen recognition and is important for survival in sepsis. CCL3, CCL6 and CXCL10 have been demonstrated to be protective in sepsis-induced injury and mortality in a murine CLP model (Ness *et al.,* 2004; Ness *et al.,* 2003; Takahashi *et al.,* 2002). CCL22 also protected mice against CLP-induced death (Matsukawa *et al.,* 2000). In our previous study using *PPTA*$^{-/-}$ septic mice, only CCL2 and CXCL2 levels in lung and plasma were analysed by ELISA (Puneet *et al.,* 2006). Although both the chemokines were elevated in *PPTA*$^{-/-}$ and wild-type septic mice, the increase was lower in the former group (Puneet *et al.,* 2006). These two chemokines were believed to act as chemoattractants to

leukocytes and play a role in tissue damage (Puneet *et al.,* 2006). We did not repeat these two chemokines in the present study, but the range of chemokines and cytokines studied showed a significant elevation upto 24 h after induction of sepsis. It is not clear yet as to why genetic deletion of SP, a product of *PPTA* gene, leads to significantly elevated cytokine levels, although it is possible that these pro-inflammatory cytokines are useful in countering the pathogenic invasion in the early phase of sepsis. A significant initial increase in IL-6 and subsequent reduction at a late stage has been reported to protect septic mice (Zhu *et al.,* 2009). Multiple mechanisms and mediators could be at play in this scenario which needs to be probed further.

Balance between pro-inflammatory and anti-inflammatory mediators plays an important role in the pathophysiology of sepsis. Anti-inflammatory cytokines such as IL-10 and IL-13 were also significantly elevated in $PPTA^{-/-}$ mice after sepsis. The increase was more significant in $PPTA^{-/-}$ septic mice compared to the corresponding wild-type mice. It has been reported that anti-inflammatory strategies applied early in patients with a hyper-inflammatory immune response may prove to be life-saving (Hotchkiss and Karl 2003). Inhibition of IL-10 12 h after CLP has been shown to improve survival in mice (Song *et al.,* 1999). Depending on the time of intervention, IL-10 has been reported to be protective or deleterious in sepsis (Latifi *et al.,* 2002). $PPTA^{-/-}$ septic mice showed elevated levels of IL-10 at 5 and 8 h after sepsis and a subsequent reduction, both of which could have proved beneficial against mortality.

IL-12(p80), a homodimer of IL-12(p40) has been reported to be an antagonist of pro-inflammatory IL-12 receptor β1 (Cooper and Khader 2007). IL-12(p40) is released from various inflammatory cells in response to pathogenic or inflammatory signals (Trinchieri *et al.,* 2003). IL-12(p40) is reported to show both protective and pathogenic

immune responses (Cooper and Khader 2007). Interestingly, we found significantly elevated levels of IL-12(p40) in *PPTA*$^{-/-}$ mice compared to the wild type mice after the induction of sepsis. The increase corresponded with the elevation in pro-inflammatory cytokine IL-12(p70) in *PPTA*$^{-/-}$ septic mice. However, the significance of this effect is not very clear. The observed levels of IL-12(p40) were in agreement with the reported 50-fold higher IL-12(p40) secretion compared with IL-12p70 in murine shock model (Wysocka *et al.,* 1995). In addition, in the previous section (**Chapter 6**), elevated levels of another anti-inflammatory cytokine, IL-1ra, were shown in *PPTA*$^{-/-}$ mice compared to wild-type mice after sepsis. Cytokine receptor antagonists are cytokine-like molecules binding to receptors but without signal transduction (Blackwell and Christman 1996). IL-1ra plasma levels are reported to be elevated both in human volunteers injected with endotoxin as well as patients with severe sepsis, although its function is not clear (Kuhns *et al.,* 1995; Gårdlund *et al.,* 1995).

Although the specific role of anti-inflammatory molecules in sepsis remains undefined, a complex interaction between cytokines and cytokine-neutralizing molecules is assumed to determine the clinical presentation and outcome of sepsis (Blackwell and Christman 1996). In patients with lethal septic shock, the level of secreted anti-inflammatory molecules is believed to be insufficient to counter the overwhelming pro-inflammatory mediators (Blackwell and Christman 1996). However, in *PPTA*$^{-/-}$ mice, elevated levels of both the pro- and anti-inflammatory mediators may act simultaneously and help to resolve the infectious assault at the early stages of sepsis without excessively damaging the host tissue; and thus prolong the survival in these mice. Overall data indicates that multiple factors play protective roles in polymicrobial sepsis in *PPTA*$^{-/-}$ mice and render them resistant to microbial infection. The current

time-dependent cytokine snapshot represents a rich source of information for further analysis and investigation.

Limited knowledge of the molecular mechanisms in sepsis has in the past led to the failure of various clinical trials of otherwise promising drug molecules from pre-clinical stages. Recent enabling ways to detect genetic signatures of sepsis and biomarker identification more rapidly and cost-effectively are beginning to provide added insight to both the research and clinical arenas (Cinel and Opal 2009). Finding a "magic bullet" is not more important than evaluating the complete immune response and inflammatory status and tailoring the treatment for individualized therapy in critically ill patients. Toward this end, our multiplexed approach of time-point analysis of cytokines, which are major mediators of sepsis, provides a relevant and valuable platform for further research and discovery and a better diagnostic tool to profile septic patients clinically.

CHAPTER 8. CONCLUSION AND FUTURE DIRECTIONS

8.1 Concluding remarks

It is evident from the overall data that the neuropeptide SP had a major role to play in polymicrobial sepsis and associated lung injury and its actions were mediated through NK-1R, as summarized in **Fig. 8.1**. As described in Chapter 3, I used the formal approach of blocking the SP actions through NK-1R with SR140333, a specific NK-1R antagonist. The results showed that SR140333 treatment in CLP surgery-induced polymicrobial sepsis in mice had a beneficial role observed 8 h later in terms of lung injury. Pulmonary damage that is generally associated with polymicrobial sepsis was alleviated by blocking the actions of SP via NK-1R. Also there was a lowering of leukocyte infiltration and lung levels of chemokines, cytokines and adhesion molecules that are involved in the propagation of inflammation.

The mechanistic studies in Chapter 4 revealed that the inhibition of SP actions mediated through NK-1R in polymicrobial sepsis, involved the downstream signaling cascade involving PKCα and NF-κB and AP-1 transcription factors. Pro-inflammatory signals of SP-NK-1R were carried from the cell surface to the nucleus by these messenger molecules leading to the modulation of various pro-inflammatory mediators in sepsis. There was also a significant down-regulation of NK-1R. Further, to rule out the possibility of SP actions through other NK receptors in sepsis, albeit marginal, the mice were treated with GR159897, a specific NK-2R antagonist as explained in Chapter 5. Inhibition of SP actions via NK-2R did not help in reducing the lung injury

Fig. 8.1

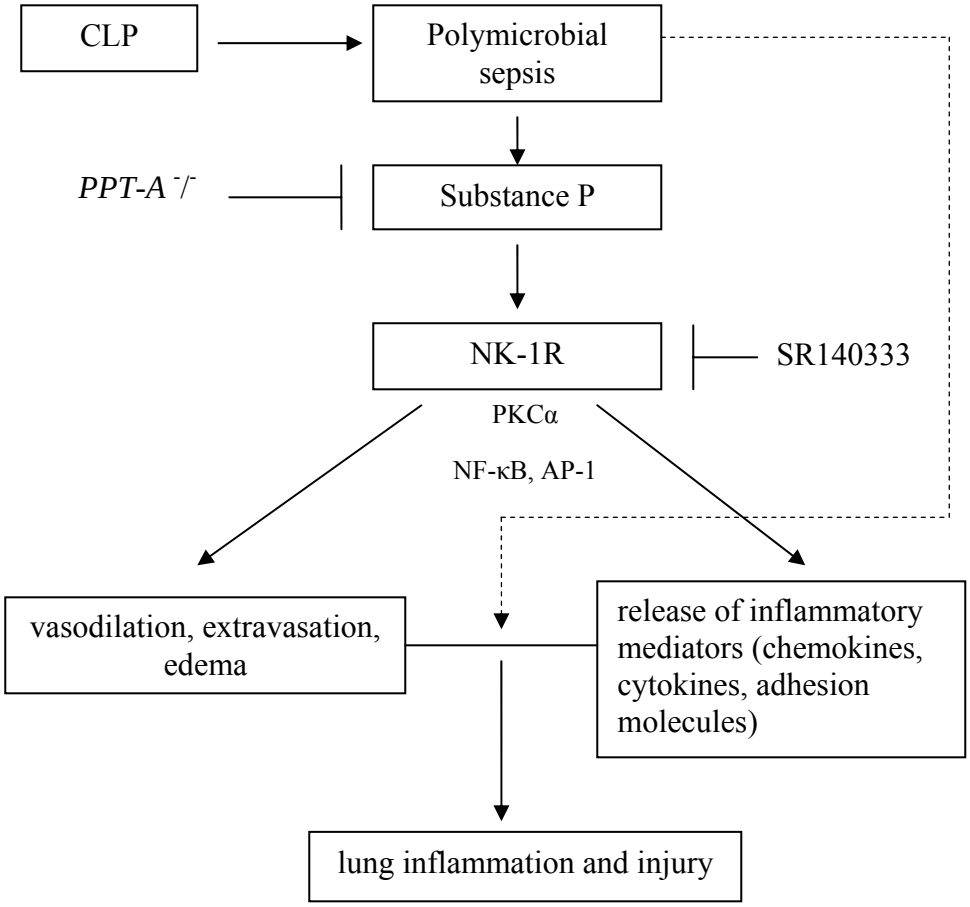


Figure 8.1 Schematic representation of role of SP in polymicrobial sepsis
AP-1 – activator protein-1; CLP – cecal ligation and puncture; NF-κB - nuclear factor kappa B; NK-1R – neurokinin-1 receptor; PKCα – protein kinase C alpha; *PPTA*$^{-/-}$- *preprotachykinin-A* knockout.

8 h after induction of polymicrobial sepsis. Thus it is clear that SP activation of NK-1R, but not NK-2R, is important in its pro-inflammatory actions in sepsis.

In addition to the use of pharmacological tools to block neurokinin receptors, gene knock-out mice subjected to polymicrobial sepsis were also studied, as described in Chapter 6, to understand the immunological basis of protection enjoyed by these mice lacking SP. *PPTA*$^{-/-}$ and wild type mice were subject to sham or CLP surgery. Pulmonary gene expression profiling showed interesting elevation of both pro- and anti-inflammatory gene in the early stages of sepsis in *PPTA*$^{-/-}$ mice. Semi-quantitative RT-PCR and ELISA were used to validate the results for selected differentially expressed genes. Consistent with recent literature reports that conventional division of pro- and anti-inflammatory status does not exist in sepsis, I found an active involvement of both the forces in providing protection to *PPTA*$^{-/-}$ mice in sepsis. Lastly, multiplexed bead-based suspension arrays were employed for the time-course measurement of a set of plasma cytokines as detailed in Chapter 7. Interesting changes in both pro- and anti-inflammatory cytokines in *PPTA*$^{-/-}$ mice in sepsis over time further substantiated the gene analysis data and conclusions.

The combined data implied that SP is one of the major players in polymicrobial sepsis, the immunological disorder which is known to be complicated with various mediators in action at any given time. The study may help in substantiating the therapeutic approach of resolving the infection without excessive immunosuppression as well searching for novel therapeutic interventions to modulate the pro-inflammatory actions of SP-NK-1R better.

8.2 Future directions

Potential scope of the future work in this area is immense as thorough understanding of this critical disorder and tailoring suitable therapy for individual patients may help conquer complications of sepsis and bring down the economic strain on the healthcare systems all over the world. Recommendations for future work are as follows:

1. Global genomic analysis of mice treated with SP receptor antagonists by microarray would be a valuable tool to compare the beneficial effects of receptor antagonism with gene deletion.
2. Charting out complete cytokine profile of septic mice treated with receptor inhibitors over a 24 – 48 h period can also be considered.
3. Study of another *PPTA* gene product NKA in polymicrobial sepsis might be carried out to see if it has any beneficial role in alleviating lung injury in addition to SP.
4. Enhancement of SP actions with inhibitors of neutral endopeptidase (NEP) could be a potential approach. NEP, a metalloprotease enzyme, degrades small secreted signaling peptides like SP, and thus inhibition of NEP enhances SP actions.

CHAPTER 9. BIBLIOGRAPHY

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