ROLE OF THE NEUROPEPTIDE SUBSTANCE P IN BURN-INDUCED DISTANT ORGAN DAMAGE

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SUMMARY

The classical tachykinin substance P (SP) has numerous potent neuroimmunomodulatory effects in many inflammatory diseases. Belonging to a class of neuro-mediators targeting not only residential cells but also inflammatory cells, studying SP provides important information on the bidirectional linkage between how neural function affects inflammatory events and in turn, how inflammatory responses alter neural activity.

Burn injuries are one of the most common and devastating forms of trauma. One of the major causes of mortality in burn patients is respiratory failure, due to the development of acute lung injury (ALI), even without direct inhalational injury. Hence, much research interest is focused on understanding the role of mediators that contribute to the pathophysiology of burn-induced ALI. However, the role of SP in inducing inflammation in the lungs after burn injury is not known. Therefore, this study aimed to investigate whether SP instigates distant pulmonary SP release and ALI after severe local burn injury.

A 30% total body surface area full-thickness burn induced in male Balb/c wild-type (WT) mice showed heightened pulmonary SP production and SP-neurokinin-1-receptor (NK1R) signaling, a G protein coupled receptor, which SP binds preferentially to. Concurrently, elevated pro-inflammatory cytokines, chemokines, neutrophil infiltration, and increased microvascular permeability were observed. Furthermore, histological examination reveals higher alveolar congestion, interstitial inflammatory cellular

infiltrates and edema, all of which are evidences of severe ALI. Notably, these effects were abolished in burn-injured WT mice pre-treated with L703606, a specific NK1R antagonist, and in burn-injured preprotachykinin-A (*PPT-A*) gene deficient mice, which encodes for SP; while exogenous administration of SP to burn-injured *PPT-A*$^{-/-}$ mice restored the inflammatory response and ALI.

Results of the present study provide for the first time compelling evidence, that the enhanced release of SP levels in lung and blood could be a critical factor leading to the pathophysiology of remote ALI and disruption of breathing function early after severe local burn injury. Taken together, with an in-depth understanding of the early pro-inflammatory effects of SP, new approaches maybe achieved for the prevention of an acute inflammatory cascade and treatment of ALI in critically injured patients.

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ABBREVIATIONS

ALI	Acute lung injury
ARDS	Acute Respiratory Distress Syndrome
COX	Cyclooxygenase
ERK	Extracellular signal regulated kinase
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
ICAM-1	Intercellular adhesion molecule-1
IL-6	Interleukin-6
IL-1β	Interleukin-1β
MAPK	Mitogen-activated protein kinase
MIP-2	Macrophage inflammatory protein-2
MIP-1α	Macrophage inflammatory protein-1α
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NEP	Neutral endopeptidase
NKA	Neurokinin A
NF-κB	Nuclear factor-kappa B
NK1R	Neurokinin-1 receptor
PMN	Polymorphonuclear
PPT-A	Preprotachykinin-A
PGE_2	Prostaglandin E_2
RT-PCR	Reverse transcription-polymerase chain reaction
SP	Substance P
TNF-α	Tumor necrosis factor- α
TRPV1	Transient receptor potential vanillod receptor-1
VCAM-1	Vascular cell adhesion molecule-1

PUBLICATIONS

ORIGINAL ARTICLES

Sio S.W.S., Moochhala S., Lu J., Bhatia M. 2010. Early Protection from Burn-Induced Acute Lung Injury by Deletion of Preprotachykinin-A Gene. ***American Journal of Respiratory and Critical Care Medicine***. 181(1):36-46.

Sio S.W.S., Puthia M.K., Lu J., Moochhala S., Bhatia M. 2008. The Neuropeptide Substance P Is a Critical Mediator of Burn-Induced Acute Lung Injury. ***Journal of Immunology***. 180(12):8333-41.

Zhang J., **Sio S.W.S.**, Moochhala S., Bhatia M. 2010. Role of Hydrogen Sulfide in Severe Burn Injury Induced Inflammation in the Mouse. ***Molecular Medicine***. Epub ahead of print. 28 April 2010.

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ABSTRACTS

Sio S.W.S., Lu J., Moochhala S., Bhatia M. 2009. A Key Role of Substance P in Acute Lung Injury after Burn [abstract]. ***Clinical Immunology.*** 131:S114.

CONFERENCE PAPERS

Sio S.W.S., Lu J., Moochhala S., Bhatia M. 2009. A key role of Substance P in acute lung injury after burn. 9th Annual Meeting of the Federation of Clinical Immunology Societies (FOCIS 2009), 11th-14th June, San Francisco Marriott Hotel, San Francisco, California, USA.

Sio S.W.S., Puthia M.K., Lu J., Moochhala S., Bhatia M. 2008. The neuropeptide Substance P is a key mediator in the development of systemic inflammatory response syndrome and lung damage following burn injury. 1st International Singapore Symposium in Immunology, 14th-16th January. Breakthrough Theatrette, Biopolis, Singapore.

Moochhala S., **Sio S.W.S.**, Puthia M.K., Lu J., Bhatia M. 2009. The neuropeptide Substance P is a critical mediator of burn-induced acute lung injury.13th Congress of the European Shock Society, 24th-26th September 2009, Lisbon, Portugal.

Bhatia M., **Sio S.W.S.**, Puthia M.K., Lu J., Moochhala S. 2008. Substance P is a key mediator in systemic inflammatory response syndrome and lung damage following burn injury. 6th Congress of the International Federation of Shock Societies and 31st Annual Conference on Shock, 28th June – 2nd July 2008, Cologne, Germany.

Chapter I Introduction

1.1 General overview

Communication between the nervous system and the process of inflammation are interwoven. The intricate networks of the nervous system made up of the brain and its central and peripheral divisions can set off or impede activities of the innate immune system that is directly involved in inflammatory responses. Likewise, cells of the immune system, through the secretion of signaling messengers such as cytokines, can influence activities of the nervous system (Nguyen et al., 1996; Steinman, 2004; Tracey et al., 1986). Thereby, it is critical to understand the messengers involved in such mechanisms of cross talk between major systems. One such key link is the neuropeptide connection.

The nervous system has a number of characteristics that make it a perfect partner with the innate immune system in coordinating immediate inflammatory responses to injury or pathogens. It reacts right away, in a matter of milliseconds to minutes, to numerous non-specific insults (Sternberg, 2006). Neurotransmitters and neuropeptides, released by nerve cells, typically activate the same signaling transduction pathways as those activated by immune mediators via binding to G-protein-coupled receptors which result in stimulation of cyclic AMPs and protein kinases such as mitogen-activated protein kinases (Sternberg, 2006). Furthermore, neuropeptides, particularly, the well-known classical tachykinin, substance P (SP), stimulates the production of pro-inflammatory mediators such as histamine that contribute to inflammatory responses. While in-turn, numerous

immune mediators express or interact with receptors of neuropeptides and neurotransmitters, thereby regulating neural activities that are fundamental to the acute phase response (Grimm et al., 1998; Milligan et al., 2005; Watkins and Maier, 1999). Taken together, these characteristics of the nervous system, specifically, the peripheral nervous system, position it to provide a first line of defense at sites of injury via the secretion of neuropeptides that generally elevate inflammatory responses (Sternberg, 2006).

Burn-induced acute lung injury is a common clinical disorder associated with high morbidity and mortality in medical and surgical intensive care units (Dancey et al., 1999). The primary etiological factor of death after severe burns is multiple organ failure with the lungs often being the first organ to fail (Turnage et al., 2002). Once respiratory failure occurs, therapeutic interventions are limited (Wheeler and Bernard, 2007). Therefore, intense research on elucidating mechanisms of burn induced pulmonary pathophysiology and potential preventive strategies are of great interest.

Even in the absence of inhalational injury, the ongoing local burn wound inflammation is the triggering source of systemic inflammatory response and multiple organ failure (Arturson, 1996; Bone, 1992). The underlying mechanism is thought to be a network combination of burn-induced liberation of inflammatory mediators, such as cytokines, chemokines, complement factors, leukocytes and neutrophil trafficking (Arturson, 1996). However, the exact role of neuropeptides, such as SP, in regulating acute lung injury after severe burns still remains unknown. Therefore, in the present study, we have investigated

the potential role of SP in instigating remote acute lung injury in a mouse model of severe local burns. Additionally, we have explored the molecular mechanisms by which SP would modulate the inflammatory responses in lungs after burn.

1.2 Substance P (SP)

1.2.1 Physical properties, sources, distribution and biosynthesis of SP

SP was discovered by von Euler and Gaddum as an 11 amino acid peptide, with a structure comprising of: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (Von Euler US and Gaddum JH, 1931). SP belongs to the tachykinin family of bioactive neuropeptides defined by their common carboxyl-terminal sequence Phe-X-Gly-Leu-Met-NH_2, where X is an aromatic (Tyr or Phe) or hydrophobic (Val or Ile) amino acid (Chang et al., 1971; Uddman R et al., 1997). This conserved sequence is essential for the tachykinin's binding and activation to its receptor. In mammals, five tachykinin peptides have been identified: SP, neurokinin-A and B, neuropeptide K and γ, all of which are encoded by the preprotachykinin-A (*PPT-A*) gene through alternative splicing (Nawa et al., 1984).

SP is produced by both neuronal and non-neuronal sources. Neuronal sources represent the primary source of SP release by non-myelinated C-fiber sensory nociceptive neurons that respond to heat, cold, chemical or other noxious stimuli (O'Connor et al., 2004). Additionally, SP is also produced by numerous important immune cells (O'Connor et al.,

2004) including neutrophils, macrophages, eosinophils, monocytes, lymphocytes, mast cells, dendritic cells and platelets (Graham et al., 2004). Its synthesis is widely distributed in the central and peripheral nervous systems and in all major organs forming the respiratory, gastrointestinal, musculoskeletal and cardiovascular systems (O'Connor et al., 2004). Particularly, for the airways, non-myelinated C-fibers is the most abundant vagal afferents that innervate the lungs (Jia and Lee, 2007; Takemura et al., 2008). The afferent activity arising from C-fiber endings exerts an important influence in regulating airway functions under both normal and pathophysiological conditions.

Biosynthesis of SP initially occurs as a larger protein in the ribosomes of neuronal cell bodies of the trigeminal, jugular and nodose ganglia. It is then packaged into storage vesicles and exported to the terminal endings of the central and peripheral branches of sensory neurons by a mechanism of axonal transport, where it is enzymatically converted to the undecapeptide and stored (Brimijoin et al., 1980; Harmar et al., 1980). Immunohistochemical and biochemical studies show that the accumulation of SP in the peripheral branches is four times greater compared to the dorsal root (Harmar et al., 1980). Upon stimulation of C-fibers, a multi-modal, non-selective cation channel, known as the transient receptor potential vanilloid1 (TRPV1), which is predominantly found in sensory C-fibers are further activated and are responsible for the release of SP. This event initiates the onset of a phenomenon termed ‘neurogenic inflammation’ which refers to the inflammatory responses that result from the release of molecules from primary sensory nerve fibers (Clapham, 2003).

1.2.2 Neurokinin-1 receptor

The biological actions of SP are mediated by neurokinin receptors, which belong to a family of ubiquitous G protein-coupled receptors, comprising of neurokinin-1 receptor (NK1R), NK2R and NK3R. Of these three receptors, the membrane bound NK1R has the highest affinity for SP. The relative affinity of NK1R for SP is 500 and 100 folds higher than its affinity for neurokinin-A and B (Elling et al., 1995; Gerard et al., 1991; Holst et al., 2001; Macdonald et al., 1996). The distribution of NK1R is found in all major organs of the body including the lungs (O'Connor et al., 2004), and has been shown to be expressed in numerous immune cells including neutrophils (Iwamoto et al., 1993), eosinophils (Iwamoto et al., 1993), lymphocytes (Guo et al., 2002), monocytes (Ho et al., 1997), macrophages (Ho et al., 1997) and mast cells (Ansel et al., 1993).

1.2.3 Neural-immune bi-directional communication

The same neuropeptides produced in the nervous system is also produced in the immune system. Release of neuropeptides was thought to be facilitated via anterograde and retrograde peptide transport since the primary and secondary lymphoid organs are well-innervated (Rameshwar, 1997). However, studies reported molecular mechanisms by which the nervous and immune systems communicate in order to address specific questions, the rationale being the synthesis of neuropeptides and expression of their receptors on resident cells and cells within lymphoid organs, which result in functional responses when activated (Kavelaars et al., 1994; O'Connor et al., 2004; Payan and

Goetzl, 1985; Savino and Dardenne, 1995). Thereby, rendering continual transportation of neuropeptides from a distant neural source unnecessary and hence raising questions of the extent and kinetics of anterograde and retrograde transports. This phenomenon of inter-system crosstalk between the nervous and immune system is termed the neuro-immune axis and is mediated via a common biochemical language of shared molecules involving mainly neuropeptides such as SP, cytokines, and their receptors (Brogden et al., 2005; Steinman, 2004; Sternberg, 2006). Peptidergic mechanisms and inflammatory mediators earlier thought to occur and be secreted solely by the nervous system are now known to also be synthesized by immune cells, and vice versa. Thereby, enabling a widespread network of complex communication between peptidergic nerves and immunocytes present in all organs of the body, including the respiratory system.

1.2.4 Pro-inflammatory effects of SP

SP serves as one of the major mediators in neuro-immune interactions. Activation of the SP-NK1 receptor complex produces a variety of neuro-immune responses in mammalian airways including increased microvascular permeability and plasma extravasation, immune cell influx, increased edema, vasodilation and glandular secretions, thereby contributing to heightened inflammation (Groneberg et al., 2006; O'Connor et al., 2004). SP also stimulates lymphocyte proliferation and immunoglobin production, elicits activation of pro-inflammatory transcription factors and activates inflammatory cells such as neutrophils, lymphocytes, monocytes, macrophages and mast cells to produce cytokines, oxygen free radicals, arachidonic acid derivatives and histamine, all of which

exacerbate tissue injury, amplifying the inflammatory response in many inflammatory diseases of the respiratory, gastrointestinal, and musculoskeletal systems (Groneberg et al., 2006; Groneberg et al., 2004; O'Connor et al., 2004).

The diverse availability of SP and NK1R expression in the body positions SP to influence many physiological and pathological conditions. Its effects are evidently pro-inflammatory and hence plays a critical role in a variety of immune and inflammatory disorders, including polymicrobial sepsis (Puneet et al., 2006), asthma (Van Rensen et al., 2002), endotoxemia (Takemura et al., 2008), inflammatory bowel disease (Koon et al., 2006), acute pancreatitis (Bhatia et al., 1998a; Bhatia et al., 2003) and rheumatoid arthritis (Grimsholm et al., 2007). Therefore, it is obvious that an extensive cross-talk exists between SP and the inflammatory response to injury.

1.2.5 SP and immunoregulation

SP exerts a diverse spectrum of immunoregulatory effects on numerous immuno-inflammatory cells by intricately interacting with them (Zhang and Dong, 2005). An inflammatory response is initiated by invasion of polymorphonuclear leukocytes followed by recruitment of macrophages and T-cells to the injured site. The process of launching an inflammatory response relies on a tightly regulated multi-step signaling cascade typically involving neutrophils, macrophages, monocytes, eosinophils, mast cells, lymphocytes, dendritic cells, adhesion molecules, eicosanoids and an intricate network of cytokines, all of which SP has been implicated to be involved in,

leading to the pathophysiology of a variety of acute and chronic inflammatory diseases (O'Connor et al., 2004).

1.2.5.1 SP and immunoregulation: neutrophils

SP significantly enhances the migratory and cytotoxic functions of neutrophils. Studies show that SP can specifically stimulate the chemotaxis of neutrophils and induce the expression of the leukocyte integrin CD11b on human neutrophils (O'Connor et al., 2004). SP induces increases in the adherence of neutrophils to lung epithelial cells *in vivo*, induces the degranulation of human neutrophils, stimulates neutrophil respiratory burst, hydrogen peroxide production and secretion of granular constituents (DeRose et al., 1994; Kuo et al., 2000; Serra et al., 1988). The augmented adherence of neutrophils to epithelial cells is mediated by NK1R, present on the surface of neutrophils and is due to the effects of the C terminus in SP (O'Connor et al., 2004).

1.2.5.2 SP and immunoregulation: cytokines

SP induces the production of IL-1, IL-6, and TNF-α in monocytes, macrophages (Ho et al., 1996; Lotz et al., 1988) and in lung cells (Veronesi et al., 1999); IL-2, IL-6, IL-1β, and TNF-α release in neutrophils (Delgado et al., 2003); IL-2 in human T cells (Calvo et al., 1992); IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) in bone marrow mononuclear cells (Rameshwar et al., 1994); IFN-γ in peripheral blood mononuclear cells (Wagner et al., 1987); and CD117 or c-kit and IL-7 in bone marrow

stromal cells (Manske et al., 1995). The same cytokines which are up-regulated by SP can also induce NK1R expression and regulation by these cytokines may occur in an autocrine and paracrine manner. For example, SP stimulates IL-1 and stem cell factor in bone marrow stroma of mice, while these cytokines in turn modulate stromal expression of NK1R (Rameshwar and Gascon, 1995). This shows that the cytokines and SP can induce the synthesis of each other, suggesting that non-neuronal sources play an important role in an acute inflammatory response.

1.2.5.3 SP and immunoregulation: lung epithelium

In the human respiratory tract, an extensive and continuous pathway of SP-immuno-reactive sensory nerves are located throughout the epithelium to arterioles in bronchial mucosa, around submucosal bronchial glands and blood vessels, providing structural support for a local axon reflex. Significant levels of SP are found in central and peripheral airway tissues, bronchoalveolar lavage fluid and sputum.

1.2.6 SP in respiratory tract diseases

Numerous clinical and animal studies have implicated SP to exert numerous pro-inflammatory effects in airway diseases (Groneberg et al., 2006). Induction of *PPT-A* gene expression and SP levels are up-regulated significantly in airway neurons during allergic airway inflammation, asthma and chronic bronchitis (Harrison and Geppetti, 2001; O'Connor et al., 2004). In the airways, prominent extravascular neurogenic

inflammatory effects include significant bronchoconstriction, vasodilation, oedema formation, mucus hypersecretion, inflammatory cell chemotaxis and stimulation and release of inflammatory mediators including prostaglandins and nitric oxide (Groneberg et al., 2006).

Asthmatic patients showed increased SP levels in bronchoalveolar lavage fluid and sputum (Tomaki et al., 1995). Further evidence from autopsy (Ollerenshaw et al., 1991), lobectomy (Lilly et al., 1995) and bronchoscopy (Tomaki et al., 1995) samples implicated SP to be involved in the development of bronchial hyperresponsiveness in asthmatic patients. The amount of SP detected in non-asthmatics was significantly lower than asthmatics, while expression of NK1R levels was likewise lower in non-asthmatics compared to asthmatics (Adcock et al., 1993; Howarth et al., 1991). Animal studies showed that administration of NK1R antagonist (Ichinose et al., 1992; Solway et al., 1993) induced abrogated bronchial hyperreactivity and plasma leakage compared to animal models induced with asthma. Ovalbumin-sensitized and challenged guinea pigs had evidence of increased SP (Fischer et al., 1996) and bronchoconstriction produced by NK1R activation (Bertrand et al., 1993); while mice deficient in NK1R showed abolished lung injury and neutrophil infiltration compared to mice induced with airway allergy (Bozic et al., 1996). Cigarette smoking exposure induces SP release from sensory nerves along with airway hyperresponsiveness and cough, adhesion of leukocytes to tracheal mucosa and increased neurogenic inflammatory responses (Baluk et al., 1996; Dusser et al., 1989; Kwong et al., 2001; Lundberg and Saria, 1983). Guinea pigs exposed to tobacco smoke demonstrated an early phase of bronchoconstriction induced by

cholinergic reflex and SP release and a late phase caused by arachodonic acid metabolites, which SP has been shown to stimulate (Hong and Lee, 1996). Increased SP and NK1R levels were also found in BALF of patients with idiopathic pulmonary fibrosis and sarcoidosis (Takeyama et al., 1996). Airway rapidly adapting afferent nerves were shown to be involved in cough reflex. In normal subjects, no SP is present in these nerves, however, upon induction of allergic inflammation and viral infection, the nerves start to produce SP (Carr et al., 2002; Hunter et al., 2000). Patients displaying non-asthmatic chronic cough have elevated SP levels, IL-8 and neutrophilia (Pizzichini et al., 1999). Nasal allergic reactions also results in SP release and plasma leakage in the nose of patients with allergic rhinitis (Braunstein et al., 1991).

1.2.7 Metabolism of SP

The biological actions of SP is terminated by mainly two enzymes: neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE) (Di Maria et al., 1998). However, NEP is abundantly expressed in the airways and has been shown to be more important than ACE in degrading SP in the lungs (Martins et al., 1991). NEP is a cell surface enzyme, located in the nerves, epithelium, smooth muscles, and glands of the respiratory trachea, where it is ideal to degrade SP upon released from sensory nerves and where SP acts. Following inactivation of SP by proteolysis, the fragments are cleared by peptide transporters which are expressed throughout the airways (Groneberg et al., 2002; Groneberg et al., 2001). NEP activity has been shown to significantly affect SP responsiveness in the lungs. Inhibition of NEP potentiates airway smooth muscle

contraction, plasma extravasation and airway mast cell activation (Nadel and Borson, 1991; Roques et al., 1993). Targeted deletion of NEP expression in mice by disruption of the NEP gene resulted in augmented inflammation, greater susceptibility to septic shock, hypotension and widespread plasma leakage from postcapillary venules that is mediated by NK1R activation (Lu et al., 1997; Lu et al., 1995; Sturiale et al., 1999). Studies have also demonstrated that stimuli such as cigarette smoke, hypochlorous acid, toluene diisocyanate and viral infections such as influenza virus A/Taiwan, the Sendai virus and Mycoplasma decrease NEP activity, amplifying the neurogenic inflammatory response to SP (Borson, 1991; Di Maria et al., 1998; Nadel, 1991).

1.2.8 SP signaling pathways

Binding of SP to its high affinity NK1R, a GPCR, is known to activate intracellular effectors through both the G_q and G_s families of G proteins (Holst et al., 2001; Nakajima et al., 1992), followed by induction of the classical mitogen-activated protein kinases (MAPK) pathway and I-kappa B (IκB) dependent nuclear factor-kappa B (NF-κB) activation. This subsequently results in stimulation of NF-κB-regulated release of numerous inflammatory mediators such as pro-inflammatory cytokines and chemokines, arachidonic acid derivatives such as prostaglandins via enzymatic conversion by cyclooxgenase-2 (COX-2), oxygen free radicals, and histamine, in many cell types such as colonic epithelial cells (Koon et al., 2005), macrophages (Marriott et al., 2000; Simeonidis et al., 2003), mast cells (Azzolina et al., 2003), T lymphocytes (Guo et al., 2002), astrocytoma cells (Lieb et al., 1997) and lung epithelial cells (Williams et al.,

2007). Therefore, in the following section, signaling pathways with particular focus on molecules such as MAPKs and NF-κB would be discussed.

1.2.8.1 Mitogen-activated protein kinases

MAPKs are one of the most ancient signal transduction pathways which are conserved through evolution and are involved in numerous physiological processes. They are a family of proline-directed protein serine/threonine kinases activated via numerous intracellular phosphorylation and signaling transduction mechanisms from the cell surface to the nucleus (Chang and Karin, 2001; Pearson et al., 2001). MAPKs play important roles in all aspects of cell development, ranging from embryogenesis, cell differentiation, cell proliferation to cell death (Pearson et al., 2001). Likewise in immune responses, MAPKs form major components, starting from the early stage of innate immunity and subsequent induction of adaptive immunity and cell death when immune function is complete (Zhang and Dong, 2005). MAPKs consist of three well characterized subfamilies that culminate towards activation of a multi-cascade of MAPKs. They are the extracellular signal-regulated protein kinases (ERK1/2); the p38 MAP kinases; and the c-Jun NH_2-terminal kinases or stress-activated protein kinases (JNK/SAPK). Downstream pathways in each of the MAPK subfamilies include central three-tiered core signaling molecules. A wide variety of upstream signals feed into the core MAPK-kinase-kinase (MAP3K), MAPK-kinase (MEK), and MAPK, each one activating the next by phosphorylation. Their substrates which are found both in the cytoplasm and nucleus

involve phospholipases, other kinases, transcription factors and membrane and cytoskeletal proteins (Kyriakis and Avruch, 2001; Roux and Blenis, 2004).

The first mammalian MAPK pathway to be identified was the ERK1/2 pathway. This signaling cascade is largely regulated by monomeric GTPase, Ras, which recruits MAPKKKs of the Ras family (A-Raf, B-Raf, and Raf-1), to activate two MAPKKs (MEK1 and MEK2), which in turn activates the MAPKs (ERK1 and ERK2) (Pearson et al., 2001; Roux and Blenis, 2004). Put together, this cascade is in short known as the Raf/MEK/ERK cascade. ERK1 and ERK2 are approximately 83% identical in amino acid sequence with significant similarities in the core regions for binding substrates (Pearson et al., 2001). They are strongly activated by serum, growth factors, cytokines, stress, ligands for GPCRs such as SP binding to NK1R and transforming agents (Pearson et al., 2001). Notably, it has been shown that an activation of just 5% of Ras molecules through the signaling cascade is sufficient to lead to the amplification and full activation of ERK1/2 (Hallberg et al., 1994). Generally, ERK1/2 are involved in anabolic processes such as cell division, differentiation and growth.

The p38 MAPKs are the second member of the MAPK pathway in mammalian cells (Han et al., 1994; Lee et al., 1994). The p38 signaling cascade comprise of several MAPKKKs (MEKK 1 to 4, MLK2 and 3, DLK, ASK1, Tpl2, and Tak1), MAPKKs (MEK3 and MEK6), and four p38 isoforms (α, β, γ and δ) (Kyriakis and Avruch, 2001). p38 is about 50% identical in amino acid sequence to ERK2 and is activated by cellular responses to stress and release of inflammatory cytokines (Lee et al., 1994).

The JNK/SAPK subfamily consist of JNK1, 2 and 3 which exist in at least 10 different forms due to alternative splicing and are widely distributed in the body. They are induced by cytokines, stress stimuli such as UV radiation and DNA damaging agents, and when there is a lack of growth factors (Kyriakis and Avruch, 2001).

Taken together, it is now known that mammalian MAPK pathways that work in conjunction with NF-κB are significantly important for the onset of stress and inflammatory responses rather than to mitogen responses. Hence, as more is unraveled about MAPK signaling cascades, it is clear that these transduction pathways will be essential targets for novel anti-inflammatory therapeutic interventions.

1.2.8.2 Nuclear factor-kappa B

The mammalian NF-κB consist of five members that are structurally related and highly conserved through evolution: Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50), and NF-κB2 (p52) (Ghosh and Karin, 2002). Notably, the p65-p50 heterodimer make up the prototypical complex of NF-κB. Stimulation of transcription is regulated by p65/RelA, RelB and c-Rel, while p50 and p52 serve to enhance DNA binding (Sizemore et al., 1999). Numerous animal models of disease and studies on human diseases have established the pivotal role of NF-κB in instigating inflammation (Ghosh and Hayden, 2008). Upon induction of inflammatory stimuli, epithelial cells at the injured site or tissue resident haematopoietic cells including mast cells or dendritic cells initiate the

inflammatory response by stimulating pro-inflammatory signaling transduction mechanisms leading to activation of NF-κB (Hayden et al., 2006). This activation induces an increase in adhesion molecules expression and chemokine release by vascular endothelial cells and within the tissue, which subsequently results in the recruitment of neutrophils followed by macrophages and other leukocytes at the early phase (Ghosh and Hayden, 2008). Additionally, NF-κB is essential for the release of effector molecules targeted against microbial invasion and for the prolonged existence of leukocytes at the site of injury or infection (Ghosh and Hayden, 2008). Therefore, it is evident that NF-κB is a central regulator for the expression of genes involved in all classical components of the inflammation process, by instigating transcriptional up-regulation of inflammatory mediators in tissue epithelial cells, vascular endothelial and haematopoietic cells and stromal cells (Ghosh and Hayden, 2008).

The central paradigm for the activation of NF-κB, particularly the p65-p50 heterodimer, relies on a key regulatory event, the phosphorylation of IκB, which functions to sequester and inactivate NF-κB in the cytoplasm, mainly by IκB kinases (IKKs) (Baeuerle and Baltimore, 1988). This then leads to IκB ubiquitination and proteasomal degradation, thereby liberating the cytoplasmic NF-κB dimer and masking the nuclear localizing signals on the NF-κB subunits, which translocate to the nucleus to activate expression of specific target genes (Baeuerle and Baltimore, 1988).

Studies show that a number of posttranslational events further contribute to the activation of NF-κB (Chen et al., 2001; Kiernan et al., 2003). For example, increased

phosphorylation of p65/RelA subunit at particular serine residues significantly enhanced the transactivation potential of NF-κB (Bird et al., 1997; Bohuslav et al., 2004). While phosphorylated p65 subunit is able to recruit coactivators including histone acetyltranferases CREB binding protein (CBP) and p300 which augment the transcriptional activity of NF-κB (Sheppard et al., 1999; Zhong et al., 2002). Additionally, posttranscriptional acetylation of p65 was found to modulate NF-κB activity (Chen and Greene, 2003).

1.2.9 Clinical significance of SP: Implications for drug discovery

Neuropeptides have been extensively studied for over 30 years. Since evidence emerged that peptides are important messengers in the nervous system and the establishment that intercellular communication in the CNS is chemically mediated (Carlsson, 2001; Greengard, 2001; Kandel, 2001), much interest has been focused on the potential of neuropeptides for various indications in clinical trials. Of particular interest was the identification of pharmacological intervention against neuropeptide receptors, GPCRs, such as neurokinin-1 receptor, which have been the target of drug therapy for decades (Humphrey, 2003; Jones and Gibbins, 2008). In fact, more than 50% of all current drugs act on receptors (Drews, 2000). Furthermore, the human genome consists of about 550 GPCR genes and neuropeptides are ligands for about 20% of them (Hokfelt et al., 2003). Therefore, finally after seven decades since the discovery of SP and after 3 decades of research on neuropeptides, the first ‘peptide’ drug, a NK1R antagonist aprepitant, MK 869, marketed as ‘Emend’, has been clinically tested and was approved by the U.S Food

and Drug Administration in 2003 for the treatment of emesis after chemotherapy (Patel and Lindley, 2003). It also has clinical efficacy for the treatment of severe depression. Furthermore, MK 869 has fewer side effects and is as efficacious as selective serotonin reuptake inhibitors (Hokfelt et al., 2003). Nevertheless, the rate of discovery and specific problems to drug development such as peptide degradation when ingested orally pose challenges to these opportunities for therapeutic interventions. Additionally, despite encouraging clinical results, many NK1R antagonists became relatively ineffective in follow up studies (Rost et al., 2006). Therefore, further research is needed to realize the full potential of SP as a therapeutic target.

1.3 Burn Injury

1.3.1 Etiology of burn injury

A burn injury occurs when some or all layers of skin and other tissues are destroyed by hot liquid, hot contact, flame, radiation, electricity or chemicals (Kramer et al., 2002). The majority of burns are due to flame related injuries, while scald burns resulting from contact with hot liquid are the next most common, making up approximately 40% of all causes of burns (Hettiaratchy and Dziewulski, 2004a; Pruitt et al., 2002). Electrical or chemical burns comprise of the smallest percentage.

1.3.2 Epidemiology of burn injury

Burn injury represents one of the most widespread and devastating forms of trauma (Church et al., 2006; Pruitt et al., 2002). It is ranked among the top leading causes of morbidity and mortality worldwide, with one of the highest burn death rates occurring in the United States of all developed nations (Church et al., 2006). Each year, more than 2 million patients in the United States alone seek care for burn injuries. Out of these 2 million, 20% require hospitalization, and as many as 7,000 die because of multiple organ failure (MOF) (Ogle et al., 1994). In the United Kingdom, approximately 250 000 people are burnt annually, half of which are children below 12 years old, with an average of 300 deaths occurring yearly (Hettiaratchy and Dziewulski, 2004a). These statistics from the United Kingdom provide a rough estimate for the incidences of burn injury in most developed countries, despite a higher occurrence of burn injuries in the United States (Hettiaratchy and Dziewulski, 2004a). In developing countries such as India, out of a population of 500 million, over 2 million people are thought to be burnt every year (Hettiaratchy and Dziewulski, 2004a). Additionally, the mortality rate in developing nations is significantly higher than that in developed nations. For instance, out of 20 million people in Nepal, 1700 deaths occur due to burn injuries annually which is about 17 times higher compared to Britain (Hettiaratchy and Dziewulski, 2004a).

1.3.3 Demographics of burn injury

People with significantly greater risk to burn injury are children and the elderly compared to people of other age groups (Cadier and Shakespeare, 1995; Hunt and Purdue, 1992; Pruitt et al., 2002). Elderly burn patients over 65 years show a greater number of fatalities particularly those with pre-existing medical conditions or disabilities. They make up about 10% of people with burn injuries (Hettiaratchy and Dziewulski, 2004a; Pruitt et al., 2002). Children, particularly those aged 1 to 4 years old, constitute 20% of burn patients, notably with about 70% of the burn injuries originating from scalds due to exposure to hot fluids, which have the potential to cause large area burns (Hettiaratchy and Dziewulski, 2004a; Pruitt et al., 2002). In 2001 to 2002, statistics show that 92 500 children, aged 14 years and below in the United States, were admitted for emergency treatment due to burn injuries with as many as 500 of them pronounced dead (Church et al., 2006). Out of all these children, two-thirds experienced sustained thermal injury, and those below 4 years old were found to be more like to encounter scald injury (Church et al., 2006). Working adults are considered to be at least risk to burn injury, nevertheless this age group of 15 to 64 years, comprise of the majority of burn injuries, making up 60% of all the burn patients (Hettiaratchy and Dziewulski, 2004a).

1.3.4 Assessment of burn injury severity

Upon admission to a hospital, a burn patient's breathing function and circulation are first examined with a thorough assessment for other major injuries apart from the burn injury

itself (Church et al., 2006). Particularly, management of the airways is a top priority (Marko et al., 2003). After which, factors such as the etiology of burn, burn size, burn depth, injury location, age and presence of other injuries or previous medical history are determined, which affect the extent of morbidity and mortality (Gueugniaud et al., 2000). Burn depth and burn size are the two most important assessment criteria. Areas of partial thickness burn, which constitute first and second degree burns; and areas of full thickness burn, which constitute third degree burns are described (Monafo and Bessey, 2002). For burn size, body diagrams, such as the "rule of nines" are used to calculate the approximate percentage of the total body surface area (% TBSA) (Herndon and Spies, 2001; Roth and Hughes, 2004). The initial determination of burn size enables a forecast of the initial fluid resuscitation requirements (Gueugniaud et al., 2000). Additionally, burn depth information helps to determine treatment requirements including the amount of excision and grafting needed (Church et al., 2006). Burn depth is traditionally assessed by trained personnel but more recently by Laser Doppler Imaging (LDI), which has been shown to facilitate a more objective assessment (Banwell et al., 1999; Hemington-Gorse, 2005; Jeng et al., 2003).

1.3.5 Pathophysiology of burn injury

The body's reaction to a severe burn wound injury is much more complex than the launch of a local inflammatory response at the burn site. In severe cases of burn injury, even in the absence of inhalational injury (Turnage et al., 2002) and infection (Barton, 2008; Wolfe et al., 1982), the ongoing local burn wound inflammation is a sufficient triggering

source to create systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (Kramer et al., 2002; Rodriguez et al., 1993; Turnage et al., 2002). The underlying mechanism is thought to be a network combination of burn-induced liberation of inflammatory mediators, such as cytokines, chemokines, complement factors, leukocytes and neutrophil trafficking (Arturson, 1996). As such, this section will highlight the respiratory, cardiovascular, metabolic, inflammatory and immunological responses to burn injury. Additionally, important mediators that contribute to the pathophysiology of burns will be covered.

1.3.5.1 Respiratory responses to burn injury

In the first 24h, generalized edema in remote tissue is frequently encountered, it is most rapid in the first 6 to 8 hours and continues for 18 to 24 hours (Marko et al., 2003; Monafo, 1996). Within 48 hours following burn injury, the body usually remains infection free but virtually all cell-mediated and humoral immune functions are deranged (Kramer et al., 2002; Marko et al., 2003). Notably, impaired gas exchange, decreased lung and chest compliance and increased pulmonary vascular resistance are more obvious clinical consequences (Turnage et al., 2002). Eventually, these inflammatory events develop to acute lung injury (ALI) and its more severe form of acute respiratory distress syndrome (ARDS) (Dancey et al., 1999; Ware and Matthay, 2000). Mortality resulting from ARDS has remained the primary cause of death in burn-injured patients, with the lungs frequently being the first organ to fail (Marshall and Dimick, 1983; Saffle et al., 1993). Patients with severe local burn injury (more than 30% TBSA) are particularly at

significant risk of respiratory failure even when the lungs have not sustained a direct burn or inhalational injury (Kramer et al., 2002; Turnage et al., 2002).

Results of clinical and animal studies have established that the persistence of SIRS further leads to ARDS (Arturson, 1996; Wolfe et al., 1982). ARDS being an eventual consequence of SIRS have brought attention to strategies that hasten the resolution of the immediate SIRS following major trauma, particularly those that alleviate early inflammatory lung injury (Ware and Matthay, 2000). By doing so, preventing the onset of early ALI will prove to have more practical benefit to the patient than efforts to treat ARDS once it has ensued (Wolfe et al., 1982). Once respiratory failure occurs, therapeutic interventions are limited. Therefore, intense research on elucidating mechanisms of burn induced pulmonary pathophysiology and potential preventive strategies are of great interest.

1.3.5.2 Cardiovascular responses to burn injury

Cardiac dysfunction is a critical factor affecting morbidity and mortality following burn injury (Hoesel et al., 2009; Hoesel et al., 2007; Horton, 2004; Niederbichler et al., 2006). Typically, capillary leakage is increased significantly, causing a marked loss of plasma proteins and fluids into the interstitial compartments. Concomitantly, peripheral and splanchnic vasoconstriction takes places, along with reduced myocardial contractility. Together these cardiovascular changes result in impaired peripheral microcirculation, systemic hypotension and organ hypoperfusion (Hettiaratchy and Dziewulski, 2004b).

Notably, the mechanism leading to hypoperfusion was described in clinical and experimental studies, which showed that severe burn injury with TBSA greater than 47%, showed marked changes in contraction and relaxation of the left ventricle (Adams et al., 1984; Adams et al., 1982; Adams et al., 1981). Specifically, significantly lowered isovolumic relaxation, marked reduction in diastolic compliance and dysfunction of left ventricular contractility was found. This directly affected the filling ability of the left ventricle and lowered ejection fraction, thereby causing an overall drop in cardiac output following burn injury.

Additionally, burn injury stimulates myocardial release of numerous inflammatory mediators, particularly, TNF-α, which exerts negative inotropic effects on the myocardium. Treatment strategies targeted against TNF-α prevented myocardial contraction and improved outcome (Giroir et al., 1994). Experimental animal models also showed that administration of recombinant TNF-α resulted in cardiac contractile depression (Eichenholz et al., 1992; Pagani et al., 1992; Tracey et al., 1986); while isolated cardiac myocytes and ventricular muscle tissue resulted in negative inotropic effects when treated with TNF-α in a concentration dependent manner (Heard et al., 1992; Horton et al., 2001; Yokoyama et al., 1993). Furthermore, the effect of oxygen free radicals on cardiac injury is also well studied due to the persistence of oxygen debt and tissue ischemia regardless of consistent resuscitation efforts. Studies demonstrate that levels of $_{L}$-arginine are reduced significantly after burn injury, resulting in nitric oxide synthase mediated production of superoxides, which is also destructive to tissues (Enkhbaatar and Traber, 2004). Administration of exogenous arginine attenuated lung

injury after burn in sheep (Enkhbaatar and Traber, 2004; Murakami et al., 2007). Therefore, it is clear that cardiovascular changes contribute extensively to the pathophysiology of burn injury.

1.3.5.3 Metabolic responses to burn injury

Severe burn injury causes major increments in metabolic rates, increasing to two times as high as that of a normal person (Saffle et al., 1993), thereby constituting a huge challenge for effective treatment in burn patients. A large amount of wasting of lean body mass can result within weeks of the injury and failure to satisfy this hypermetabolic state of increased energy and protein requirements causes severe cellular and immune dysfunction, delayed wound healing, lowered resistance to infection and even death (Hartmannsgruber et al., 2000; Marko et al., 2003; Saffle et al., 1993). Therefore, strategic steps taken to ensure effective treatment include immediate wound closure by debridement and grafting, and adequate nutritional support (Marko et al., 2003). Tube feeding via the duodenojejunal and non-bolus enteral feeding should be provided as early as possible. This is important as not only are nutritional demands met, but the intestinal barrier integrity kept intact, which is essential for preventing bacterial translocation (Wischmeyer et al., 2001). Together, these measures can lower morbidity and mortality, enhance wound healing and minimize hypermetabolism and subsequent catabolism.

The reasons behind hypermetabolism are because of hormonal imbalances after major burns (Demling and Seigne, 2000). For example, catabolic hormones such as epinephrine,

cortisol and glucagon are elevated which increase gluconeogenesis, glycogenolysis and muscle proteolysis (Bessey et al., 1989). Higher levels of catabolic hormones impair the effects of insulin and consequently blood sugar levels are elevated, while protein synthesis, lipogenesis, and glycogenesis are subdued (Saffle et al., 1993). The clinical outcome of these hormonal imbalances is an increased metabolic rate, with skeletal muscle being used as the energy source, while the effect of burn injury prevents the body from processing fat as the fuel source (Saffle et al., 1993).

1.3.5.4 Inflammatory responses to burn injury

Immediately after burn injury, the inflammatory cascade is initiated (Marko et al., 2003; Wolfe et al., 1982). Studies report that the immune status after thermal injury can generally be seen as two phases. Firstly, the initial immune response post-burn is pro-inflammatory which secondly, after prolonged post-burn eventually becomes predominantly anti-inflammatory (Church et al., 2006). Such an anti-inflammatory state or better described as immunosuppressed state predisposes patients to increased susceptibility to subsequent sepsis and multiple organ failure (Baue et al., 1998; Harris and Gelfand, 1995; Nguyen et al., 1996; Saffle et al., 1993; Still et al., 1993; Teodorczyk-Injeyan et al., 1986). These are major complications associated with burn trauma and interestingly, recent evidence suggests that the activation of a pro-inflammatory cascade after burn appears to be a predisposing factor for launching the development of subsequent immune suppression, dysfunction and susceptibility to septic shock and organ failure (Meakins, 1990).

Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 are important in mediating the acute phase response to injury. TNF-α is known to be one of the first initiating cytokines that stimulate a cascade of secondary cytokines and humoral factors leading to subsequent systemic response. Additionally, TNF-α has a potent role in inducing the shock-like state associated with thermal injury and sepsis (Marano et al., 1988). However, studies have found subsequent low levels of TNF-α post-burn and suggest it may contribute to an immunosuppressed state (Yamada et al., 1996). IL-1 is important in activating T- and B-cells, but like TNF-α, its levels also remain low after burn and therefore is also likely to play a role in the subsequent response to infection (Vindenes et al., 1998). Of these pro-inflammatory cytokines, circulating levels of IL-6 is the only cytokine consistently elevated systemically post-burn. Results of clinical and experimental studies have shown that the marked increase in IL-6 following burn injury and sepsis, actually correlates with suppressed cell-mediated immunity and significant mortality while Inhibiting IL-6 after burn and sepsis has shown an improved outcome (Biffl et al., 1996; Dinarello et al., 1986; Kowal-Vern et al., 1994). TGF-β is a potent chemo-attractant of monocytes and neutrophils and aids in tissue repair. Moreover, TGF-β is also immunosuppressive, as it has been shown to suppress maturation of B- and T-cells. Studies show that TGF-β plasma levels are elevated 6-8 days post-burn and suggest potential causative contributions to immunosuppressed state and increased susceptibility to sepsis (Varedi et al., 2001).

Addtionally, important enzymes such as iNOS, inducible nitric oxide synthase, have been shown to suppress splenic T-cell proliferation only until 4 days post

thermal injury (Schwacha and Somers, 1998a). Furthermore, it was demonstrated that this suppression by RNI was IFN-γ driven 10 days post-burn injury (Masson et al., 1998). Moreover, recent studies have implicated iNOS in loss of gut barrier function (Inoue et al., 2001) and increased vascular permeability (Chen et al., 1999), indicating its role in development of subsequent immune dysfunction and sepsis.

Macrophages are major producers of pro-inflammatory mediators. Studies by Schwacha and Sommers have shown that thermal injury dysregulates macrophage activity by increasing its productive capacity of pro-inflammatory factors, leading to a "hyperactive" phenotype, termed macrophage hyperactivity (Schwacha and Somers, 1998b). This is evidenced by increased pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, TGF-β (Ogle et al., 1994), RNI (Masson et al., 1998), and Prostaglandin E_2 (PGE_2) (Miller-Graziano et al., 1988). Therefore, studies have implicated macrophage hyperactivity to the subsequent increased susceptibility to sepsis following thermal injury due to its active role in producing IL-1, TNF-α, IL-6, TGF-β and RNI.

1.3.5.5 Immunological responses to burn injury

After burn injury, the number of T lymphocytes drops in the serum of burn patients, furthermore, as the severity of burn increases, a concomitant reduction in T cell dependent immune functions occurs (Heideman and Bengtsson, 1992; Wolfe et al., 1982). γ/δ T-cells which form part of the innate and adaptive immune system are seen as a "first line of defense". Studies demonstrated that 4–9 days post-burn, γ/δ T-cells appear in the

spleen, suppressing proliferation of normal splenocytes (Kobayashi et al., 1995; Kupper et al., 1985). Findings also demonstrated high mortality in mice deficient in γ/δ T-cells of 75% 48h post burn (Schwacha et al., 2000). Thereby, impaired T-cell function may be a crucial marker in the development of burn-induced immunosuppression.

1.3.6 Prognosis and criteria for hospital and burn unit admissions

The larger the percentage TBSA of the burn and the deeper the burn depth, the worse the prognosis (Hartford, 2002). Generally, partial thickness burns greater than 15 to 25% TBSA, or full thickness burns of approximately 2 to 10% TBSA or greater must be admitted to the hospital (Marko et al., 2003). Burn injuries that require quick admission to a burn unit generally include: partial thickness burns with more than 10% TBSA; all third degree full thickness burns regardless of age; electrical burns especially those originating from lightning, chemical and inhalational burns; patients with a history of medical conditions that can complicate the burn treatment, delay recovery or are fatal; and burn injuries occurring on specific parts of the body such as face, hands, feet, genitalia, perineum, across joints, and on areas where other injuries have occurred such as fractures (McCampbell et al., 2002). Additionally, young children should be put under observation for some time regardless of the size of the burn area.

1.3.7 Treatment and critical care management of burn patients

A patient with severe burns suffers from hypovolaemic shock, hypoxia, hypothermia and extreme pain, all of which should receive immediate attention while treatment of the burn

wound itself could be attended to after this (Gueugniaud et al., 2000). Initial care generally involves fluid resuscitation, airway management, pain control measures, followed by cooling, cleansing, closure and dressing up of the burn wound.

1.3.7.1 Fluid resuscitation

Fluid resuscitation is essential to reduce the severity of hypovolaemic shock. Any delay may result in death. The most widely used solution is lactated Ringer's solution (Monafo, 1996). If initial fluid resuscitation had been delayed, then greater volumes are required especially for large burn areas, this is because only approximately 20 to 30% of the fluid actually remains within the vascular system (Monafo, 1996; Sokawa et al., 1981). Concerns have been raised over the use of fluid resuscitation which may result in higher lung and tissue edema, following a reduction in tissue perfusion and the likelihood of compartment syndrome development (Marko et al., 2003). Reports have shown that during the first 48 hours of resuscitation, the incidence of elevated extravascular lung water is rare, even in patients who received greater than 400% over the accepted dose of Ringer's solution. However, it is important to note that close monitoring of hydrostatic forces, central venous and lung wedge pressures is essential. Additionally, right ventricular end diastolic volumes must be kept within the acceptable limits (Marko et al., 2003).

1.3.7.2 Airway management

The next priority is to ensure adequate airway flow. Administration of oxygen by usage of an oxygen face mask is sufficient if the patient's breathing is not badly affected. Otherwise, the following cases would require endotracheal intubation: facial burns, fire in an enclosed area, burns over 30% TBSA, elevated carboxyhemoglobin levels of more than 10%, unconsciousness, and evidences of respiratory failure (Gueugniaud et al., 2000; Herndon and Spies, 2001; Yowler and Fratianne, 2000). Such mechanical ventilation lowers the risks of hypoxia and makes up for the higher need of oxygen due to increased efforts for breathing especially in cases of smoke inhalation (Demling et al., 1994). Additionally, lung injury should be determined by laryngoscopy once the patient is intubated (Gueugniaud et al., 2000).

1.3.7.3 Pain control measures

Burn wounds which cause significant pain are those with partial thickness wounds devoid of the epithelium layer. The pain is increased greatly when wound care and dressing changes are performed. Typically, narcotics are used as a first line of treatment while intravenous doses of morphine are used in an emergency setting (Hartford, 2002). Non-steroidal anti-inflammatory drugs (NSAIDS) may be useful while analgesics may be supplemented for sedation (Hartford, 2002). Clearance of these drugs is higher particularly for patients with a history of alcohol or drug abuse, thereby; large quantities

of analgesics may be required (Jaffe, 1990). Anesthetics which are topically applied or injected locally are also not suggested.

1.3.7.4 Infection control measures

Application of antimicrobial agents is not just important for reducing the risk of infection, but lowering the conversion of partial to full thickness burn wounds and for early excision treatment (Heggers et al., 2002; Monafo and West, 1990). Choice of the antimicrobial agent should be based on the agent's selective inhibition against the microorganism found from cultures in the burn wound and any nosocomial infections acquired in the burn unit (Church et al., 2006). Topical ointments or creams and dressing most widely used are based on the antibacterial effects of the silver ion (Falcone and Spadaro, 1986; Heggers et al., 2002). Studies show that the mechanism is due to its strong binding to thiol groups in respiratory enzymes of the bacteria and its ability to prevent cell division by binding to DNA and structural proteins (Lansdown, 2002; Lansdown et al., 2005). Examples of topical agents include: silver nitrate solution, silver sulfadiazine and mafenide acetate creams, and nanocrystalline silver dressings (Church et al., 2006).

1.3.7.5 Burn wound cooling, cleansing, closure and dressing

It is important to first cool down the burn wound. Studies show that immediate cooling by tap water or saline of about 8ºC is effective (Jandera et al., 2000; Saranto et al., 1983), while application of ice may be turn out to be more harmful than beneficial (Sawada et al.,

1997). Apart from heat dissipation, cooling stabilizes mast cells in the skin, lowering production of histamine, and reduces wound edema, with additionally alleviation of pain (Hartford, 2002; King and Zimmerman, 1965; Ofeigsson, 1965). Cleaning and change of dressing of the burn wound should be performed everyday. Washing is done usually with room temperature water, with povidone iodine, and a mild soap. Chlorhexidine gluconate is highly recommended due to its antiseptic properties against microorganisms in skin (Demling, 1985).

Closure of a burn wound by primary healing is the most desirable; however, when this is not possible for some shallow to deep burns, then a reconstructive procedure using skin grafts or flaps is required (Robson, 2002). Treatments of shallow burns by using skin allograft, xenograft or commercially available synthetic skin membranes are effective (Gerding et al., 1990; Poulsen et al., 1991; Robson, 2002). These materials do not require the need for dressing changes and lower pain. Surgical treatment of full thickness deep burns, with about 25% TBSA involve closing the burn wound with split-thickness skin grafts (Monafo, 1996). The excision of the eschar and skin grafting procedure should be performed immediately after resuscitation without delay. Furthermore, sufficient amounts of autologous skin grafts should be present with adequate amounts of matching blood type to the patient.

1.3.8 Social and economical impact of burn injury: long term consequences

Most survivors of burn injury gradually resume well to their lives of daily work and self-confidence (Blakeney et al., 2002). The first year is reported to be the most stressful however, in some cases the psychological effects can linger on for months to years (Blakeney et al., 2002). Disfiguring, scarring, deformity and functional disability are some of the serious long term effects of burn injury (Marko et al., 2003). Additionally, burn patients tend to undergo depression, which studies show that the severity is proportional to the burn area, well being and anxiety of the patient (Ptacek et al., 2002). 54% of burn patients displayed moderate to severe depression one month following discharge from the hospital, while 43% continued to remain depressed after two years (Wiechman et al., 2001). Additionally, about 50 to 60% of burn patients have reported a required change in employment (Browne et al., 1985). Furthermore, cost of treatment and particularly materials required such as skin grafts and blood can be very expensive (Barret, 2002).

1.4 Acute Lung Injury (ALI) and the Acute Respiratory Distress Syndrome (ARDS)

1.4.1 Definition and diagnosis of ALI/ARDS

The problem of excessive systemic inflammatory responses, which is a direct consequence of SIRS leading to MODS, continues to be a major healthcare challenge (Bone, 1992; Brun-Buisson, 2000). ALI manifests clinically as ARDS which is a major

component of MODS, arising from a variety of predisposing conditions such as burns, sepsis, haemorrhagic shock, acute pancreatitis, pneumonia and trauma (Bhatia and Moochhala, 2004; Wheeler and Bernard, 2007). Globally, hundreds of thousands of people develop this syndrome annually along with significant morbility, mortality and costs incurred (Wheeler and Bernard, 2007). Various studies report that burn injury accounts for about 2 to 9% of patients with ARDS (Dancey et al., 1999).

ALI/ARDS was first reported about 40 years ago, in a group of patients who displayed significant hypoxemia with diffuse alveolar infiltrates on chest X-ray (Ashbaugh et al., 1967). These infiltrates were found to be edema in lung tissue resulting from increased microvascular permeability. The syndrome was then known as the *adult* respiratory distress syndrome. In 1994, the American-European Consensus Conference Committee proposed that since the syndrome was not confined to only adults, the term *acute* respiratory distress syndrome should be used instead (Bernard et al., 1994). Additionally, ARDS was defined as "a severe form of ALI and a syndrome of acute pulmonary inflammation and resultant increased capillary endothelial permeability (Bernard et al., 1994)." It was also stated that "ARDS is characterized by a constellation of clinical, radiological and physiological abnormalities that cannot be explained by but may coexist with left atrial or pulmonary capillary hypertension (Bernard et al., 1994)." This definition provides the platform for researchers and doctors to speak on common grounds when discussing ALI/ARDS.

1.4.2 Pathogenesis of ALI/ARDS

The development of ALI/ARDS occurs in three stages: acute or exudative, proliferative and fibrotic with each one displaying its own distinguishing clinical, histological and radiological indications (Ware and Matthay, 2000). The first phase is an acute or exudative phase, which is distinguished by the rapid invasion of inflammatory cells in the first 24 to 48 hours (Tasaka et al., 2002). One prominent feature is arterial hypoxemia (Ware and Matthay, 2000). Radiographic analysis shows that the syndrome reflects that of cardiogenic pulmonary edema (Aberle et al., 1988); while pathological results show severe disruption of alveolar epithelium and vascular endothelium, producing an influx of inflammatory cells such as neutrophils, macrophages, erythrocytes and protein rich edema into the interstitium and the alveolar spaces (Wheeler and Bernard, 2007). These changes are mediated by a complex interplay of pro-inflammatory mediators. It is possible that following the acute phase, ALI/ARDS may fully resolve in some patients, however, in others it can enter the second proliferative phase, taking place between 2 to 7 days whereby fibroblast infiltrate the inflamed lungs (Tasaka et al., 2002). Permanent injury to the type I alveolar cells would prompt the deposition of unnecessary cellular proteins, fibrin, and debris, resulting in hyaline membranes, while damage to the surfactant producing type II cells leads to alveolar collapse. At this stage, it is again possible that the type II cell divide with some epithelial cell regeneration and remodeling (Ware and Matthay, 2000). However, in some patients, this may progress to an irreversible third phase, the fibrotic phase which persists for over 7 days resulting in severe collagen deposition in alveolar, vascular and interstitial beds of the pulmonary

parenchyma, with further development of microcysts, exacerbated hypoxemia, pulmonary compliance and alveolar dead space (Tasaka et al., 2002; Tomashefski, 1990).

1.4.3 Role of inflammatory mediators in ALI/ARDS

Risk factors for the development of ALI/ARDS can be broadly categorized into those that cause direct or indirect injury to the lungs (Ware and Matthay, 2000). Examples of direct injury insults include pneumonia, aspiration of gastric contents, toxic inhalational, near drowning, pulmonary contusion and fat embolism (Ware and Matthay, 2000). Examples of indirect injury to the lungs in the setting of a systemic process are such as severe trauma and shock, sepsis, acute pancreatitis, cardiopulmonary bypass, numerous transfusions and overdose of drug abuse (Ware and Matthay, 2000). The severity of ARDS depends significantly on the extent of SIRS which is directly affected by the magnitude of inflammatory mediators from the site of injury spilling over into the general circulation. Thereby, the following section will focus on some of the prominent mediators known to contribute to ALI/ARDS such as TNF-α, IL-1β, IL-6, ICAM-1, IL-8, SP, cyclooxygenases and prostaglandins.

1.4.3.1 TNF-α and IL-1β

Activated macrophages are the main source of TNF-α and IL-1β, both of which are significantly elevated in the bronchoalveolar lavage fluid of patients with ARDS, suggesting that these cytokines are produced locally (Goodman et al., 1996; Meduri et al.,

1995; Park et al., 2001; Siler et al., 1989). TNF-α is known to be a major inducer of shock (Cohen, 2002; Norman et al., 1995; Putensen and Wrigge, 2000). This was further demonstrated in animal models administered with exogenous TNF-α which displayed a syndrome indistinguishable from septic shock. IL-1β has been found to also induce a shock-like state in experimental animals. Additionally, IL-1β increases neutrophil activation and stimulates adhesion molecules expression on leukocytes and the vascular endothelium (Cohen, 2002; Dinarello et al., 1989; Norman et al., 1995). Numerous attempts have shown that specific antagonist to TNF-α and IL-1β, such as anti-TNF-α antibody, soluble type 1 TNF receptor and IL-1 receptor antagonists were successful in animal models of lung injury (Denis et al., 1994; Leff et al., 1994), but not in clinical trials (Abraham et al., 2001; Opal et al., 1997), although, it has been suggested that they may hold promise in situations where prophylactic intervention is required.

1.4.3.2 IL-6

The release of IL-6 is triggered by numerous cell types such as monocytes, macrophages, endothelial cells and fibroblasts in response to endotoxin, TNF-α and IL-1β (Bhatia et al., 2000a; Bhatia et al., 2001; Cohen, 2002). In the clinic, concentration levels of IL-6 in the blood have been demonstrated to be an outstanding indicator of the severity of ARDS for patients with acute pancreatitis and sepsis (Leser et al., 1991; Remick et al., 2002). Furthermore, during the acute phage of ARDS, elevated levels of IL-6 have been correlated with increased production of acute phase proteins, such as C reactive protein *in vitro* and *in vivo* (Castell et al., 1989; Geiger et al., 1988).

1.4.3.3 ICAM-1

Under normal conditions, ICAM-1 expression levels on the surface of vascular endothelial cells are low, however, in inflammatory conditions, such as ALI/ARDS, its levels are highly up-regulated (Roebuck and Finnegan, 1999). Administration of neutralizing antibodies or gene deletion of ICAM-1 has been found to confer significant protection from lung injury in diseases of acute pancreatitis, sepsis, diesel exhaust particle induced lung injury and trauma (Bhatia et al., 1998b; Giannoudis et al., 1998; Takano et al., 2002). A reduction in ICAM-1 activation has a direct effect on neutrophil sequestration to the injured site, thereby, antagonizing important adhesion molecules such as ICAM-1 maybe useful for strategies targeted against lung injury primarily mediated by neutrophils.

1.4.3.4 IL-8

Chemokines such as IL-8 play a major role in the development of ARDS (Strieter, 2002). Particularly, IL-8 belongs to the CXC family of chemokines, secreted mainly by macrophages and epithelial cells. The chemoattractant effects of IL-8 influence a wide number of leukocytes in lung inflammatory diseases including macrophages, neutrophils, mast cells and endothelial cells (Pease and Sabroe, 2002). Furthermore, in inflammatory diseases such as sepsis and acute pancreatitis, elevated IL-8 levels detected in the blood serve as a major predictor of morbidity and mortality (Hack et al., 1992; Makhija and Kingsnorth, 2002).

1.4.3.5 SP

Numerous studies have shown clear evidences for the regulatory role of SP in multiple respiratory diseases (Groneberg et al., 2006; O'Connor et al., 2004). For example, the pro-inflammatory role of SP signaling via NK1R has been demonstrated in acute pancreatitis and associated lung injury (Bhatia et al., 1998a). Furthermore, mice deficient in the *PPT-A* gene, which encodes for SP, are protected against acute pancreatitis and associated lung injury (Bhatia et al., 2003). Similarly, significant protection in the lungs of mice lacking the *PPT-A* gene was found in an experimental model of cecal ligation and puncture-induced sepsis associated lung injury (Puneet et al., 2006). Additional reports showed increased levels of SP in pulmonary oedema fluid of patients with ARDS (Espiritu et al., 1992). The significance of NK1R in instigating lung injury and mortality was also demonstrated in rats injected with neurotoxic venom (Matos et al., 1999); while SP was shown to lead to the development of ARDS following fire smoke inhalation (Wong et al., 2004). Therefore, the blockade of SP or NK1K for the treatment of patients with ALI/ARDS may hold promise, and deserves further studies.

1.4.3.6 Prostaglandins and cyclooxygenases

Prostaglandins are known to be potent lipid mediators of inflammation (FitzGerald and Patrono, 2001). The most abundant in the human body is prostaglandin E_2 (PGE_2), which is produced and regulated in nearly every cell type (Smith, 1989). It is also the most prominent prostanoid in exerting inflammation, as it increases vasodilation, vascular

permeability and edema (Dubois et al., 1998). Furthermore, studies show that PGE_2 exerts its pathologic effects in numerous systems including the cardiovascular, respiratory, gastrointestinal and reproductive systems (Robertson, 1995; Versteeg et al., 1999). Hence, it is important to note that tight regulation of PGE_2 is essential to avoid unnecessary cellular and biological processes. Metabolism and degradation of PGE_2 takes places within the local cell environment. However, specific organs, notably the lungs, are particularly designed not just for blood gas exchange but to metabolize bioactive molecules such as PGE_2 to ensure that it does not escape into the circulation (Schuster, 1998). Interestingly, studies have reported that the structure and specific location of the lungs enables it by means of a PGE_2 transporter to eliminate unnecessary PGE_2 from the circulation and further degrade it into inactive components (Schuster, 1998). ALI/ARDS is a common consequence in severely burn-injured trauma patients. As such, it is likely to result in these burn-injured patients, significant disruption of lung PGE_2 metabolic processes (Hahn and Gamelli, 2000). Thereby, rendering leakage of excess PGE_2 into the blood where it exerts its pro-inflammatory effects resulting in dysfunction of the immune system.

PGE_2 is synthesized from arachidonic acid via the actions of cyclooxygenase (COX) enzymes, COX-1 and COX-2 (Funk, 2001). Both enzymes have about 61% sequence homology. However, the main differences that set them apart are the sequences at the promoter regions (Tanabe and Tohnai, 2002). The human COX-1 promoter does not contain a TATA or CAAT box, thereby making it constitutively expressed in many cells (Tanabe and Tohnai, 2002). On the other hand, the promoter region of COX-2 has many

inducible gene sequences such as a TATA box, NF-IL6 and CRE motif, sites for AP-2, SP1, NF-κB binding, and an E-box (Morita, 2002). The expression of COX-2 is induced by numerous cytokines and growth factors, via binding to proteins or transcriptional factors that stimulate the gene promoter regions (Morita, 2002). Therefore, this renders COX-2 to be the primary enzyme accounting for synthesis of PGE_2 in response to inflammatory stimuli (Krakauer, 2004). In many diseases, including lung inflammatory diseases, the elevated concentrations of PGE_2 are known to be well correlated with higher levels of COX-2 expression (Funk, 2001). Additionally, the transcriptional gene expression of COX-2 and many other proteins has been shown to occur after burn injury, thus, making it an important molecule of burn-induced inflammation (Hahn and Gamelli, 2000). Therefore, in the present study, we were also interested to investigate the effect of SP on pulmonary COX-2 and PGE_2 expression levels following burn injury.

1.4.4 Treatment of ALI/ARDS

None of the potential therapeutic agents have been successful for treatment of ALI/ARDS in randomized controlled clinical trials (Bhatia and Moochhala, 2004). There is no specific treatment for ALI/ARDS but generally, the basis of treatment for ALI/ARDS is limited to excellent supportive care to ensure adequate provision of oxygen and to prevent respiratory complications (Wheeler and Bernard, 2007). Majority of patients with ALI/ARDS require positive-pressure ventilation with oxygen while a cuffed tracheal tube is supplied where more support is needed. Also, the use of tracheal tube delivered

ventilation has been shown to be more beneficial over non-invasive ventilation in such patients (Antonelli et al., 1998; Kramer et al., 1995).

1.5 Research Rationale

1.5.1 Question of interest:

What is the role of SP in burn-induced acute lung injury?

The focus of this study is on 3 major areas: SP, burn injury and ALI/ARDS. The hallmarks of these areas have been well characterized with respect to their pathologic, biochemical and immunologic manifestations (Bhatia and Moochhala, 2004; Parker and Townsley, 2004; Ware and Matthay, 2000; Wheeler and Bernard, 2007).

For example, with regard to ALI/ARDS, it is known that the release of pro-inflammatory cytokine cascade, chemokines, complement factors, eicosanoids and other inflammatory mediators such as reactive oxygen species by cells of the innate immune system contributes to heightened lung inflammation (Bhatia and Moochhala, 2004; Deitch, 1992; Mulligan et al., 1993). But despite this, the triggering mechanisms, signaling events involved in inducing pulmonary inflammation and other mediators that can initiate inflammation still remain unclear.

As such, many investigators have focused their work on identifying and elucidating the role of potential mediators in ALI. Results from different animal models of lung injury

have implicated emerging mediators of lung inflammation. Studies show increased toll-like receptor-4 reactivity in contributing to intense lung inflammation following burn injury and lipopolysaccharide administration (Paterson et al., 2003). Other investigators have reported the emerging role of platelets in inflammatory lung disease (Kuebler, 2006); while it was also reported that a deficiency in platelets played a significant role the pathophysiology of burn injury. Our earlier work has also shown that preprotachykinin-A (*PPT-A)* gene products, which encodes SP, are critical inflammatory mediators in acute pancreatitis and cecal ligation and puncture-induced sepsis associated lung injury (Puneet et al., 2006). However, none have investigated the neurogenic inflammatory effects of neuropeptides, such as SP, in mediating remote ALI after local burn injury.

Additionally, with regard to previous studies of neurogenic inflammation after burn injury, the effect of SP was only studied in the local cutaneous wound site or hind paw (Dunnick et al., 1996; Papp and Valtonen, 2006; Pinter et al., 1999; Saria, 1984; Siney and Brain, 1996; Sulpizio et al., 2004). Again, none have addressed the role of SP in inducing distant lung injury after burn.

Knowledge on characteristics of individual immune mediators is undoubtedly a central research theme, but in depth understanding of the effect of these mediators at the molecular, cellular and physiological level is important in identifying potential targets for future pharmacological manipulation. Therefore, the purpose of the present study is to determine the exact role of SP in mediating remote ALI after severe local burns and the precise mechanisms by which it works.

1.5.2 Approach

Identification of early endogenous triggers that activate inflammatory cells leading to the early onset of SIRS and ALI represents an important goal but remains poorly defined. This is important because it could direct therapeutic strategies to hasten the resolution of immediate inflammation; hence, providing greater benefit to burn patients than efforts to treat ALI once it has ensued. For these reasons, we have focused our study on investigating the early inflammatory response in lungs following severe burn injury.

We investigated our hypothesis using several approaches. Pharmacological inhibition of NK1R and the genetic deletion of SP by removal of the *PPT-A* gene in mice was employed. Additionally, WT mice and mice lacking the *PPT-A* gene were also challenged with exogenous SP or an analogue peptide form to see whether there is a reversal of effects observed. Uses of such mice models were excellent for analysis of neuropeptide function in affecting *in vivo* pathologic conditions.

1.5.3 Objectives

The primary aim of the present study is to investigate the potential role of SP in mediating remote ALI after severe local burns. More specifically, the aims of the study sought to examine:

1. The role of SP in mediating ALI after severe local burns
2. The role of SP in regulating the levels of pulmonary cytokines, chemokines, and zinc metalloproteinases following burn injury
3. The effect of SP on inflammatory cells in the circulation after burn injury
4. The effect of SP on respiratory function after burn injury
5. The mechanisms by which SP regulates remote ALI after burns

Chapter II Role of SP in burn-induced acute lung injury

2.1 Introduction

Substance P (SP) is an 11 amino acid neuropeptide, encoded by the preprotachykinin-A (*PPT-A*) gene, and is released from peripheral nerve endings in all major organs (Mantyh, 1991). Particularly, in patients with airway diseases, significant amounts of SP have been found to be up-regulated in the lung tissues, bronchoalveolar lavage fluid and sputum (Joos and Pauwels, 2000; Lamb and Sparrow, 2002; Nieber et al., 1992). SP signals via membrane bound neurokinin-1 receptors (NK1R), to elicit its pro-inflammatory effects. Studies have shown that it plays a pathogenic role in a variety of immune and inflammatory disorders, including polymicrobial sepsis, asthma, endotoxemia, inflammatory bowel disease, acute pancreatitis and rheumatoid arthritis (Bhatia et al., 2003; Grimsholm et al., 2007; Koon et al., 2006; Puneet et al., 2006; Takemura et al., 2008; Van Rensen et al., 2002).

Acute lung injury (ALI) is characterized by increased vasodilation, elevated microvascular plasma leakage and increased edema, significant neutrophil infiltration, and up-regulation of inflammatory mediators following an insult (Matthay and Zimmerman, 2005). The mechanism underlying the severity of ALI is thought to be determined by the extent of the resulting systemic inflammatory response. Excessive activation of leukocytes caused by the release of a cascade of pro-inflammatory cytokines, in particular neutrophils, are stimulated in the general circulation and are recruited to

lodge in the pulmonary microvasculature, directly contributing to systemic inflammation (Matthay and Zimmerman, 2005). The prominent occurrence of ALI is a major problem in burn patients even in the absence of inhalational injury (Turnage et al., 2002). However, the early triggers of remote ALI following severe local burn injury are not well understood. Additionally, the potential role of neuropeptides, such as SP, in ALI after burns has not been investigated. Therefore, the aim of this part of the study was to evaluate the possible role of SP in triggering early ALI after burns.

2.2 Materials and methods

2.2.1 Mouse burn injury model

All experiments were approved by Institutional Animal Care and Use Committee of DSO National Laboratories and conducted in accordance with their established guidelines. Burn injury was performed as previously described (Sio et al., 2010; Stevenson et al., 2003). Briefly, male BALB/c mice, 6-8 weeks old, were anesthetized with ketamine (160mg/kg) + xylazine (4mg/kg) and the dorsal hair clipped. Mice were placed in an insulating mold device with an opening calculated to expose 30% total body surface area. The exposed skin was immersed in 95ºC water for 8 seconds. This has been shown to produce an anesthetic full thickness burn (Stevenson et al., 2003). Sham mice, which served as controls, were anesthesized, shaved and exposed to 24ºC room temperature water. After sham or burn injury, mice were resuscitated with 1ml of 0.9% sterile normal saline solution (i.p.) and were individually housed. 8h or 24h after sham or burn-injury,

animals were sacrificed by a lethal dose of pentobartitone (90mg/kg, i.p.). Samples of lung and blood were collected. Plasma was prepared from anti-coagulated blood samples by centrifugation (13000rpm, 10min, 4°C). Samples were then stored at -80ºC for subsequent analysis.

In one group of mice, L703606, oxalate salt {cis-2-(diphenylmethyl)-N-[(2-iodophenyl)-methyl]-1-azabicyclo[2.2.2]octan-3amineoxalate} (Sigma-Aldrich), or saline was administered i.p. to mice at a dose of 12mg/kg 1h before sham or burn-injury. L703606 binds with high affinity to NK1R and has been shown to be a potent antagonist to it (Cascieri et al., 1992; Fong et al., 1992; Francis et al., 1994) . L703606 works as a non-peptide, competitive antagonist of NK1R by displacing the binding of SP to NK1R (Cascieri et al., 1992). Additionally, L703606 has improved stability and greater bioavailability compared to peptide antagonist of NK1R developed earlier. In preliminary experiments, dose dependent assays on lung neutrophil infiltration were carried out using 4 to12mg/kg of L703606 administered 1h before burn injury. A dose of 12mg/kg was found to significantly inhibit lung neutrophil accumulation (data not shown), and was used for all future experiments in this study.

PPT-A$^{-/-}$ mice were a gift from Prof. A. Basbaum (University of California, San Francisco, CA) and bred as described previously (Cao et al., 1998). Groups of male *PPT-A*$^{-/-}$ mice with BALB/c background and its wild-type (WT), *PPT-A*$^{+/+}$ BALB/c male mice, were randomly selected for sham or burn injury. In a separate group, SP (Bachem; Peninsula Laboratories) dissolved in 0.9% sterile normal saline, was administered i.v. to

PPT-A$^{-/-}$ mice. Mice received doses of 0, 0.012, 0.12 or 1.2µg/kg. A final dose of 0.12µg/kg SP was chosen for all future experiments. Similarly, SP analogue peptide, (D-Arg^{1}, D-Trp$^{7,\ 9}$, Leu^{11})-SP (Bachem; Peninsula Laboratories), was dissolved in 0.9% sterile normal saline and 0.12µg/kg i.v. was administered 15min before (pre-treatment) and 15min after (post-treatment) sham or burn injury to WT mice. *PPT-A*$^{-/-}$ mice were administered 0.12µg/kg i.v. SP analogue peptide immediately after sham or burn injury. The function of this analogue is known to specifically and competitively antagonize NK1R (Folkers et al., 1984; Maggi et al., 1991).

2.2.2 Measurement of SP levels

Samples of lung and plasma were collected from the animals. Lung samples were homogenized in 1ml of ice-cold SP assay buffer for 20s (Bachem; Peninsula Laboratories). The homogenates were centrifuged (13,000 x *g*, 20min, 4°C) and the supernatants collected. They were adsorbed on C_{18} cartridge columns (Bachem) as described (Bhatia et al., 1998b; Puneet et al., 2006). The adsorbed peptide was eluted with 1.5ml of 75% v/v acetonitrile. The samples were freeze-dried and reconstituted in the SP assay buffer (Bachem; Peninsula Laboratories). SP content in the samples was then determined with an ELISA kit (Bachem; Peninsula Laboratories) according to the manufacturer's instructions and expressed as nanograms per milliliter. Results were then corrected for the DNA content of the tissue samples fluorometrically using Hoechst dye 33256 (Labarca and Paigen, 1980) and were expressed as nanograms per microgram of DNA.

2.2.3 Measurement of myeloperoxidase (MPO) activity

Neutrophil sequestration in lung was quantified by measuring tissue MPO activity. Tissue samples were thawed, homogenized in 20mM phosphate buffer (pH 7.4), centrifuged (13,000 x *g*, 10min, 4°C), and the resulting pellet was resuspended in 50mM phosphate buffer (pH 6.0) containing 0.5% w/v hexadecyltrimethylammonium bromide (Sigma-Aldrich). The suspension was subjected to four cycles of freezing and thawing and was further disrupted by sonication (40s). The sample was then centrifuged (13,000 x *g*, 5min, 4°C) and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant (50µl), 1.6 mM tetramethylbenzidine (Sigma-Aldrich), 80mM sodium phosphate buffer (pH 5.4) and 0.3mM hydrogen peroxide (reagent volume: 50µl). This mixture was incubated at 37°C for 110s, the reaction terminated with 50µl of 0.18M H_2SO_4 and the absorbance measured at 450nm. This absorbance was then corrected for the DNA content of the tissue sample and results were expressed as fold increase over control (Labarca and Paigen, 1980).

2.2.4 Measurement of pulmonary microvascular permeability

Two hours before sacrifice, each animal received an i.v. bolus injection containing FITC-albumin (5mg/kg; Sigma-Aldrich) dissolved in normal saline. After mice were euthanized by an i.p. injection of pentobarbitone (90mg/kg), the blood was collected by cardiac puncture and plasma was separated. The tracheae were exposed and cannulated and the lungs were washed twice with 1ml of saline to provide 2ml of bronchoalveolar

lavage fluid. The lavage fluid was combined and FITC fluorescence was measured in the lavage fluid and plasma (excitation = 494nm; emission = 520nm). The ratio of fluorescence emission in lavage fluid to plasma was calculated and used as a measure of pulmonary microvascular permeability (Bhatia et al., 1998b; Puneet et al., 2006).

2.2.5 Histopathological examination

A small portion of lung was excised and fixed with 10% neutral buffered formaline (Sigma-Aldrich), then subsequently dehydrated through a graded ethanol series, embedded in paraffin wax, and sectioned as previously described (Bhatia et al., 2000b; Bhatia et al., 1998b). Sections of 5-µm thickness were stained with hematoxylin/eosin and examined by light microscopy using a Carl-Zeiss microscope (objective lens magnification of x20; eyepiece magnification of x10).

2.2.6 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA from lung was extracted with TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad) at 25°C for 5mins, 42°C for 30mins, followed by 85°C for 5mins. The cDNA was used as a template for PCR amplification by iQ Supermix (Bio-Rad). The primer sequences for detection of PPT-A, NK1R and 18S, optimal annealing temperature, optimal cycles, and product sizes are as shown in Table 2.1. PCR amplification was conducted in MyCycler (Bio-Rad). The

reaction mixture was first subjected to 95°C for 3mins, followed by an optimal cycle of amplifications, consisting of 95°C for 30s, optimal annealing temperature (Table 2.1) for 30s and 72°C for 30s. Final extension was at 72°C for 10min. PCR products were analyzed on 1.5% w/v agarose gels containing 0.5 µg/ml ethidium bromide.

2.2.7 Bronchoalveolar lavage fluid (BALF) and neutrophil counting

BALF and neutrophil counting was performed as previously described (Bao et al., 2009). Briefly, BALF was collected by performing two intratracheal instillations of 1ml of saline, followed by gentle aspiration. Total fluids from the two collections were pooled and centrifuged to pellet cells. Cells were re-suspended in PBS centrifuged to cytospin slides, fixed, and stained in a modified Wright stain. Differential cell count was performed on at least 500 cells in each cytospin slide.

2.2.8 Western immunoblot

Lungs were homogenized at 4°C in 0.75 ml radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitor cocktail (Roche, Switzerland) and phosphatase inhibitor cocktail (Sigma Chemical Co.). The tissue homogenates were centrifuged at 14,000 g for 10 min at 4°C. Protein concentration in the soluble fraction was determined by the method of Bradford. Protein samples (80 µg) were separated by SDS-PAGE on Novex® 10% Tris-glycine polyacrylamide gels (Invitrogen) and transferred onto polyvinylidene difluoride membranes (Invitrogen) by electroblotting in Novex® transfer

buffer (Invitrogen) containing 20% (v/v) methanol. Membranes were then washed, blocked, and probed overnight at 4°C with rabbit polyclonal anti-NK1R (Sigma; 1: 650 dilution), followed by secondary detection for 2h with a 1:1000 dilution of HRP-conjugated, goat anti-rabbit IgG (Santa Cruz Biotechnology). Membranes were washed and then incubated in SuperSignal™ West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) before exposure to X-ray films (CL-XPosure™, Pierce). Gels were calibrated by protein kaleidoscope standards (Bio-Rad). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Santa Cruz Biotechnology; 1:1000 dilution) was applied as an internal control to normalize protein loading. The intensity of bands was quantified using LabWorks™ Image Analysis software (UVP).

2.2.9 Immunohistochemical Analysis

Immunostaining for NK1R was performed in paraffin-embedded sections of 5-µm thickness. Sections were deparaffinated and rehydrated. Ag sites were retrieved by heating the sections on the slides in 10 mM citrate buffer in a microwave oven and cooling for 10 min at room temperature and incubation in 3% H_2O_2 in methanol for 10 min to inactivate endogenous peroxidase. Sections were again washed in PBS for 10 min before blocking with normal goat serum (Vector Laboratories) for 30 min. Primary Ab specific for NK1R, rabbit polyclonal to NK1R (anti-NK1R; Sigma) was then added at a dilution of 1/50 in 0.3% BSA for 60 min. The sections were then incubated with biotinylated goat anti-rabbit Ab (Vector Laboratories) for 30 min. Sections were again washed in PBS and incubated with AB reagent, avidin D-HRP (Vector Laboratories), in

PBS for 30 min. After washing in PBS, staining was visualized using diaminobenzidine (DAB) (DakoCytomation) and counterstained by methyl green. The sections were dehydrated, permanently mounted with mounting medium, and observed by light microscopy. The DAB substrate chromogen yields a brown/reddish brown reaction end product at the site of the target Ag. The staining was examined by light microscopy using a Carl-Zeiss microscope (objective lens magnification of ×20; eyepiece magnification of ×10).

2.2.10 Statistics

The data were expressed as mean ± SEM. The significance of difference among groups was evaluated by ANOVA with a post-hoc Tukey's test for multiple comparisons when comparing three or more groups. A value of $p < 0.05$ was regarded as statistically significant.

Table 2.1 PCR primer sequences, optimal conditions, and product sizes

Gene	Primer Sequence	Optimal Conditions	Size (bp)
r18S	Sense: 5'-GTAACCCGTTGAACCCCATT-3’ Antisense: 5'-CCATCCAATCGGTAGTAGCG-3'	28 cycles Annealing: 59ºC	150
NK1R	Sense: 5‘-CTTGCCTTTTGGAACCGTGTG-3' Antisense: 5'-CACTGTCCTCATTCTCTTGTGGG-3'	38 cycles Annealing: 59ºC	501
PPT-A	Sense: 5'-GCCAATGCAGAACTACGAAA-3' Antisense: 5'-GCTTGGACAGCTCCTTCATC-3'	38 cycles Annealing: 60ºC	282

2.3 Results

2.3.1 Burn injury significantly elevates endogenous SP levels in lung and plasma

The endogenous concentration of SP was increased significantly in both lung and plasma early after burn injury as compared with sham-injured mice at 1h (Fig. 2.1A and B) and 8h (Fig. 2.1C and D) in wild type (WT) mice.

2.3.2 Burn Injury markedly increased biological activity of SP-NK1R signaling

The levels of SP was significantly elevated early after burn injury as compared with sham injured mice at 1h and 8h in both lung (Fig. 2.2A) and plasma (Fig. 2.2B). Additionally, SP levels exhibited a trend of higher levels at 8h compared to 1h in both lung and plasma. Findings from densitometric analysis of PCR products on agarose gel demonstrated that *PPT-A* mRNA expression levels, at both 1h and 8h, correlated well with protein expression levels, with 8h showing a higher rise in levels compared to 1h (Fig. 2.3A). Additionally, mRNA levels of lung NK1R expression were concomitantly increased (Fig. 2.3B) as *PPT-A* mRNA was increased as early as 1h after burn injury. NK1R mRNA levels was significantly increased at 1h, however, the levels were lowered back to basal levels at 8h following burn injury.

Next, we were interested to know the association between SP and NK1R signaling at both the protein and transcriptional levels following burn injury. Pre-treatment with L703606, a specific NK1R antagonist at a dose of 12mg/kg, significantly suppressed the protein levels of SP in both lung (Fig. 2.2A) and plasma (Fig. 2.2B). Similarly, mRNA transcriptional levels of *PPT-A* and NK1R gene expression were significantly lowered with administration of L703606 (Fig. 2.3A and B respectively). A higher degree of attenuation in *PPT-A* and NK1R expression levels occurred at 1h in both cases but at 8h only *PPT-A* expression was reduced and there was no difference in NK1R mRNA expression by 8h. Collectively, these findings demonstrate that SP biological activity and transcriptional activity following burn injury is NK1R signaling dependent, in both lung tissue and plasma.

2.3.3 Increased SP-NK1R signaling response correlated with significant ALI following severe burn while disruption of SP-NK1R signaling by L703606 attenuated this effect

A prominent clinical pathology of ALI is the invasion of polymorphonuclear leukocytes (PMNs), which is also accompanied with augmented microvascular permeability and edema (Puneet et al., 2006). Therefore, to confirm whether SP-NK1R signaling is crucial in mediating burn injury-induced ALI, we assessed MPO levels as an indicator of pulmonary neutrophil infiltration, lung microvascular permeability to test the severity of ALI after burn, and performed histopathological examination of lung tissue sections.

Our data confirm the presence of severe ALI following severe burn by an increase in lung MPO activity (Fig. 2.4A). However, burn-injured mice administered with L703606 showed markedly reduced MPO activity in lung as compared to the untreated mice at both 1h and 8h (Fig. 2.4A). These findings indicate that burn injury induced lung neutrophil accumulation partly depends on SP responses. Furthermore, studies have shown SP to be a potent chemoattractant as it enhances the migratory, cytotoxic functions and adherence of human leukocytes (O'Connor et al., 2004), therefore, this data suggests that neurogenic stimuli via SP results in excessive recruitment of neutrophils leading to an exaggerated inflammatory response in lung tissue following severe local burn injury.

Additional analysis of lung protection by L703606 was performed by assessing leakage of i.v. administered FITC-labeled albumin into the alveolar space as a measure of pulmonary microvascular permeability. Evidently, lung permeability was enhanced significantly following burn injury (Fig. 2.4B); however, pre-treatment with L703606 significantly abolished the exacerbation of lung permeability at 8h post burn (Fig. 2.4B). As such, the protective effect of L703606 is mediated by inhibition of SP induced plasma leakage which may otherwise result in increased lung edema and hypoxemia.

Histopathological examination of lung tissue sections showed significantly higher alveolar congestion, a significant increase in perivascular and interstitial inflammatory cellular infiltrates, interstitial edema and widening of alveolar septa in untreated mice at 1h and 8h post burn. Administration of L703606, significantly restored normal lung

histoarchitecture at both 1h and 8h following burn injury, as evident in normal and sham-injured mice (Fig. 2.4, C1 to 4 and D1 to 4).

2.3.4 The augmented SP response correlates well with serious lung injury after burn; on the other hand, *PPT-A* gene deletion in mice showed reduced neutrophil infiltration and ameliorated pulmonary microvascular permeability

Next, we further confirm our findings by using mice lacking the *PPT-A* gene, and *PPT-A*$^{-/-}$ mice challenged with exogenous SP. We assessed myeloperoxidase (MPO) activity; which is an enzyme primarily expressed in neutrophils and is used as a marker for quantification of tissue neutrophil sequestration. Burn-injured WT mice exhibited a more than 2 fold increase in recruitment of neutrophils compared to sham-injured WT mice; and this increment was significantly reduced in *PPT-A*$^{-/-}$ mice (Fig. 2.5A). Furthermore, bronchoalveolar lavage fluid (BALF) revealed polymorphonuclear leukocytes (PMNs) accumulation in the alveolar spaces, where a high influx of neutrophils was identified (Fig. 2.5B). Neutrophil numbers markedly increased in burn-injured WT mice BALF, and this number was greatly decreased in the alveolar spaces of *PPT-A*$^{-/-}$ mice, consistent with changes in MPO activity.

Endothelial cells are key players in sequestration of PMNs and regulation of the permeability barrier of the pulmonary vasculature (Ware and Matthay, 2000). Given the increased recruitment of cells into the airways, we expected changes in vascular

permeability. Evidence of lung protection in *PPT-A*$^{-/-}$ mice was determined by assessing leakage of i.v. administered FITC-labeled albumin into the alveolar space as a measure of testing the severity of pulmonary microvascular permeability and damage after burn. Clearly, endothelial leakage was elevated in burn-injured WT mice as compared to sham-injured WT mice and this was significantly alleviated in *PPT-A*$^{-/-}$ mice (Fig. 2.5C). To further evaluate lung injury, histopathological examination of lung tissue revealed significantly higher alveolar congestion, intense PMNs infiltration, alveolar septal wall thickness and interstitial edema in burn-injured WT mice (Fig. 2.5D). However, restoration of normal lung histoarchitecture 8h after burn injury was found in *PPT-A*$^{-/-}$ mice, as observed in sham-injured WT and *PPT-A*$^{-/-}$ mice (Fig. 2.5D). Together, these findings show that absence of *PPT-A* gene products are key in resolving acute lung injury after burn.

2.3.5 Protective effect of *PPT-A* gene deletion was reversed in *PPT-A*$^{-/-}$ mice challenged with exogenous SP following burn injury; whereas SP analogue peptide form did not aggravate lung damage

The *PPT-A* gene expresses four distinct mRNA forms through alternative splicing, all of which encode synthesis of SP. Only two of the four mRNA forms also encodes neurokinin-A (NKA) and its derivatives neuropeptide K and γ, therefore, most research continue to focus on studying SP (O'Connor et al., 2004).

To answer the question of whether burn-induced acute lung injury is specifically attributable to the pro-inflammatory effects of SP, we challenged *PPT-A*$^{-/-}$ mice with exogenous SP. Initially, dose dependent experiments on lung neutrophil accumulation were conducted. Sham or burn-injured *PPT-A*$^{-/-}$ mice received doses of SP starting from 0, 0.012, 0.12 and 1.2µg/kg, administered i.v. immediately after burn (Fig. 2.6A). Lung MPO activity showed a significant increase in burn-injured *PPT-A*$^{-/-}$ mice challenged with 0.12 and 1.2µg/kg of SP at 8h post-burn compared to 0µg/kg (Fig. 2.6A). No differences were seen at a dose of 0.012 µg/kg compared to 0µg/kg in burn-injured *PPT-A*$^{-/-}$ mice and comparing doses administered across the sham-injured *PPT-A*$^{-/-}$ mice groups only (Fig. 2.6A). These findings show that the protective effects of *PPT-A* gene deletion in the lungs were reversed upon administration of exogenous SP in a mouse burn injury model at doses of 0.12 and 1.2µg/kg. Notably, a significant rise in neutrophil infiltration in burned-injured *PPT-A*$^{-/-}$ mice, clearly starts at 0.12µg/kg implicating that the SP injection of 0.12µg/kg was sufficient to mobilize neutrophil accumulation in lungs; furthermore, this level also corresponds to the total physiological circulatory concentration of endogenous SP detected in plasma of normal mice of about 0.15ng/ml (Fig. 2.1). Therefore, a dose of 0.12µg/kg SP was chosen to be exogenously administered in *PPT-A*$^{-/-}$ mice for all future experiments.

Although SP, at a dose of 0.12µg/kg, was administered directly into the blood by i.v., we wanted to ascertain the actual presence of exogenous SP in the plasma and lungs. Time dependent SP levels were performed in sham and burn-injured *PPT-A*$^{-/-}$ mice (Fig. 2.7A and B). The results show that slightly less than half of 0.12µg/kg exogenous SP

administered, was actually present in the plasma of *PPT-A*$^{-/-}$ mice at 5mins after burn injury compared to 0mins, where no SP was administered (Fig. 2.7A). Within 30mins, the SP levels declined rapidly and then remained fairly constant to 8h. Likewise, in the lungs, SP levels rapidly infiltrated the lungs within 5mins as compared with 0mins (Fig. 2.7B). Although, SP levels detected at 5mins after burn in plasma was less then the actual dose administered, which is expected, overall, this data supports that our model of exogenous SP challenged to *PPT-A*$^{-/-}$ mice at a dose of 0.12µg/kg, i.v., was sufficient and effective enough to exert pro-inflammatory effects as demonstrated in Fig. 2.6A.

Next, we assessed lung neutrophil infiltration and injury in sham or burn-injured *PPT-A*$^{-/-}$ mice challenged with SP. Lung MPO activity, neutrophil count in BALF, microvascular permeability and histological examination experiments were performed. As expected, the introduction of SP to burned-injured *PPT-A*$^{-/-}$ mice significantly reversed the protective effects seen in burned-injured *PPT-A*$^{-/-}$ mice alone, without SP administration, similar to levels seen in burn-injured WT mice (Fig. 2.5A to D). No differences were seen across all three sham groups (Fig. 2.5A to D).

To further ascertain that the effects of burn-induced acute lung injury found is specifically attributable to the pro-inflammatory effects of SP, we used a known SP analogue peptide, (D-Arg^{1}, D-Trp$^{7, 9}$, Leu^{11})-Substance P. Amino acids positioned at 1, 7, 9 and 11 were substituted for H-D-Arg^{1}-Pro-Lys-Pro-Gln-Gln-D-Trp^{7}-Phe-D-Trp^{9}-Leu-Leu^{11}-NH_2 compared to the original SP sequence of H-Arg^{1}-Pro-Lys-Pro-Gln-Gln-Phe^{7}-Phe-Gly^{9}-Leu-Met^{11}-NH_2 (Folkers et al., 1986). $_{D}$-Tryptophan substitution at position 7

and 9 is favorable for antagonistic activity, while leucine in position 11 increases potency (Folkers et al., 1984; Folkers et al., 1983). The function of this analogue has been tested to occupy NK1R and antagonize it (Maggi et al., 1991). As expected, the same intravenous dose of 0.12µg/kg administered to WT mice 15 min before (pre-treatment) and 15 min after (post-treatment) sham or burn injury and to *PPT-A*$^{-/-}$ mice immediately after sham or burn injury failed to aggravate lung MPO activity (Fig. 2.8A and B) and alveolar congestion as revealed by histological examination (Fig. 2.8C to E).

2.3.6 Lung NK1R expression after burn injury

SP elicits its pro-inflammatory effects by activating NK1R. Hence, knowing NK1R expression levels in lungs after burn injury is of significant importance. Western blot and Immunohistochemical analysis (appearing as brown color) showed significantly increased NK1R levels at 8h in burn-injured WT mice compared to sham-injured WT mice and burn-injured *PPT-A*$^{-/-}$ mice (Fig. 2.9). No differences in lung NK1R levels were observed between burn-injured *PPT-A*$^{-/-}$ mice and in burn-injured *PPT-A*$^{-/-}$ mice injected with SP as well as compared to their respective sham groups (Fig. 2.9).

2.4 Discussion

The results of this study provide a major implication: given the large number of molecules thought to influence the pathophysiology of burn injury, we show that a single endogenous factor, SP, acts early with distinct roles, in instigating diverse pulmonary inflammatory events following severe local burn injury. Evidence derived from three

different and complementary approaches: pharmacological inhibition of SP-NK1R signaling, genetic deletion of *PPT-A* gene in mice and exogenous administration of SP to *PPT-A*$^{-/-}$ mice, supports these conclusions.

Results of the present study revealed that burn injury augments significant SP release in plasma and lungs. Furthermore, concomitant elevation of *PPT-A* gene levels were observed. Also, significantly higher levels of MPO activity, microvascular permeability and histological examination revealing higher alveolar congestion, interstitial inflammatory cellular infiltrates and edema was observed at 8h compared to 1h which correlated with elevated levels of SP and *PPT-A* mRNA at 8h than at 1h in lungs.

Activation of SP downstream pro-inflammatory effects requires its binding to NK1R. Interestingly, our results show that mRNA levels of NK1R were elevated at only 1h and later decreased to levels similar to sham-injured mice at 8h although SP, *PPT-A* and lung injury levels were significantly higher at 8h than at 1h. Therefore, these lead us to question whether NK1R expression levels have a role in regulating SP and its resultant effects of ALI. We verified this by using a specific antagonist to NK1R, L703606, which markedly suppressed lung SP expression at the transcriptional and protein level at both 1h and 8h after burn injury. Therefore, the data suggest that the modulation of SP by NK1R acts possibly by a mechanism where only blockade of NK1R down-regulates SP and *PPT-A* gene expression levels. Furthermore, this modulation of NK1R on SP occurs regardless of the number of NK1R expressed, because SP synthesis, *PPT-A* levels and ALI persistently increased despite a return of NK1R mRNA levels to basal levels at 8h in

burn-injured mice. Therefore, collectively, these results demonstrate that burn-induced ALI occurs via biological activity of SP-NK1R signaling, which consistently continues to rise, regardless of number of NK1R expressed as long as the NK1R complex is intact. However, once blockade of the NK1R complex occurs, the signaling is disrupted, and levels of SP along with the severity of ALI are diminished.

Next, we further supported our results by verifying the role of SP in burn-induced ALI by using mice deficient in the *PPT-A* gene and challenging the *PPT-A*$^{-/-}$ mice with exogenous SP. Consistent with our earlier findings, our results show that excessive neutrophil accumulation in the lungs and alveolar spaces of burn-injured WT mice, as evidenced by increased MPO activity and neutrophil count in BALF. The significantly higher migration of neutrophils, from blood to the alveolar airspace is one of the hallmarks of the development of ALI (Ware and Matthay, 2000). Thereby, as expected, ALI was observed by a concurrent significant increase in microvascular permeability and disruption of lung histoarchitecture. These effects were abolished in burn-injured *PPT-A*$^{-/-}$ mice and restored when *PPT-A*$^{-/-}$ mice were challenged with exogenous SP. These results imply that although SP and neurokinin-A are synthesized by the *PPT-A* gene (Nawa et al., 1984), the contribution of SP is critical in instigating pulmonary injury after burn. Furthermore, sham-injured *PPT-A*$^{-/-}$ mice did not show increased lung MPO activity even though these mice were administered the same dose of SP, implicating that SP alone at a concentration used in this study and in the absence of burn injury, does not induce lung injury. Additionally, administration of SP analogue peptide in burn-injured WT and *PPT-A*$^{-/-}$ mice failed to aggravate ALI, further verifying the pro-inflammatory

role of SP in burn-induced ALI. Taken together, our findings reveal that burn injury primes the body for the pro-inflammatory effects of SP. Thereby, confirming that SP is a critical factor for the development of lung inflammation and injury after burn.

Interestingly, our findings here indicate that the low dose of exogenous SP injected into *PPT-A*$^{-/-}$ mice is enough to instigate increased ALI, compared to higher endogenous SP levels needed in WT mice to cause similar severity of ALI status. We suggest that this may be due to *PPT-A*$^{-/-}$ mice being more responsive than WT mice to SP. SP instigates ALI by activating NK1R (O'Connor et al., 2004). This greater responsiveness in *PPT-A*$^{-/-}$ mice maybe because of more responsive NK1R in *PPT-A*$^{-/-}$ mice, and less responsive NK1R in WT mice. This effect could be explained by the absence of continuous endogenous SP release in *PPT-A*$^{-/-}$ mice. Several studies have shown evidences that extended exposure of SP invariably leads to NK1R desensitization which results in down-regulation of the response of a cell to SP (Bowden et al., 1994; Grady et al., 1995; Mantyh, 1991; Solway et al., 1993). Therefore, the production of continuous endogenous SP in WT mice may result in NK1R to be less responsive; hence, greater SP concentrations would be necessary to cause significant ALI. In addition, our data demonstrates that a minimum threshold of 0.12μg/kg SP administered to *PPT-A*$^{-/-}$ mice, which corresponds to about 0.075ng/ml of actual SP concentration levels present in plasma, is required to instigate severe ALI following burn injury. Also, when a lower dose of 0.012μg/kg SP was injected to *PPT-A*$^{-/-}$ mice, this dose failed to cause lung neutrophil accumulation after burn. In WT mice, a concentration level of about 0.3ng/ml SP found in plasma was enough to exhibit markedly increased ALI at 8h post-burn.

The ability of SP to affect various early events in the inflammatory cascade suggests its up-stream activity in the burn-injured host. By binding to NK1R, present throughout cells in the body, SP elicits a cascade of downstream pro-inflammatory events. As such, knowing how burn injury may affect the NK1R expression levels in lungs is of significant importance. Western blot results showed that lung NK1R levels in burn-injured WT mice were up-regulated at 8h compared to sham-injured WT mice. However, levels of NK1R in burn-injured *PPT-A*$^{-/-}$ mice did not show any changes in NK1R levels compared to sham-injured *PPT-A*$^{-/-}$ mice but exhibited significantly lower NK1R levels than burn-injured WT mice. This observation suggests that increased SP levels in conjunction with burn injury regulate NK1R expression. Conversely, a low dose of SP injection in burn-injured *PPT-A*$^{-/-}$ mice did not result in any change in NK1R levels. Nevertheless, despite having no up-regulation in NK1R levels, burn-injured *PPT-A*$^{-/-}$ mice injected with SP still exhibited similar lung injury status as in burn-injured WT mice at 8h. Therefore, it appears that basal levels of NK1R are sufficient for downstream SP-mediated signaling after burn and up-regulation of NK1R does not influence the extent of lung injury.

In summary, we show evidence that the pharmacological inhibition of SP-NK1R signaling and absence of a single endogenous factor, SP, significantly provides early protection against burn-induced ALI in mice. Thereby, blockade of SP maybe beneficial in preventing early inflammation and ALI in critically burn-injured patients.

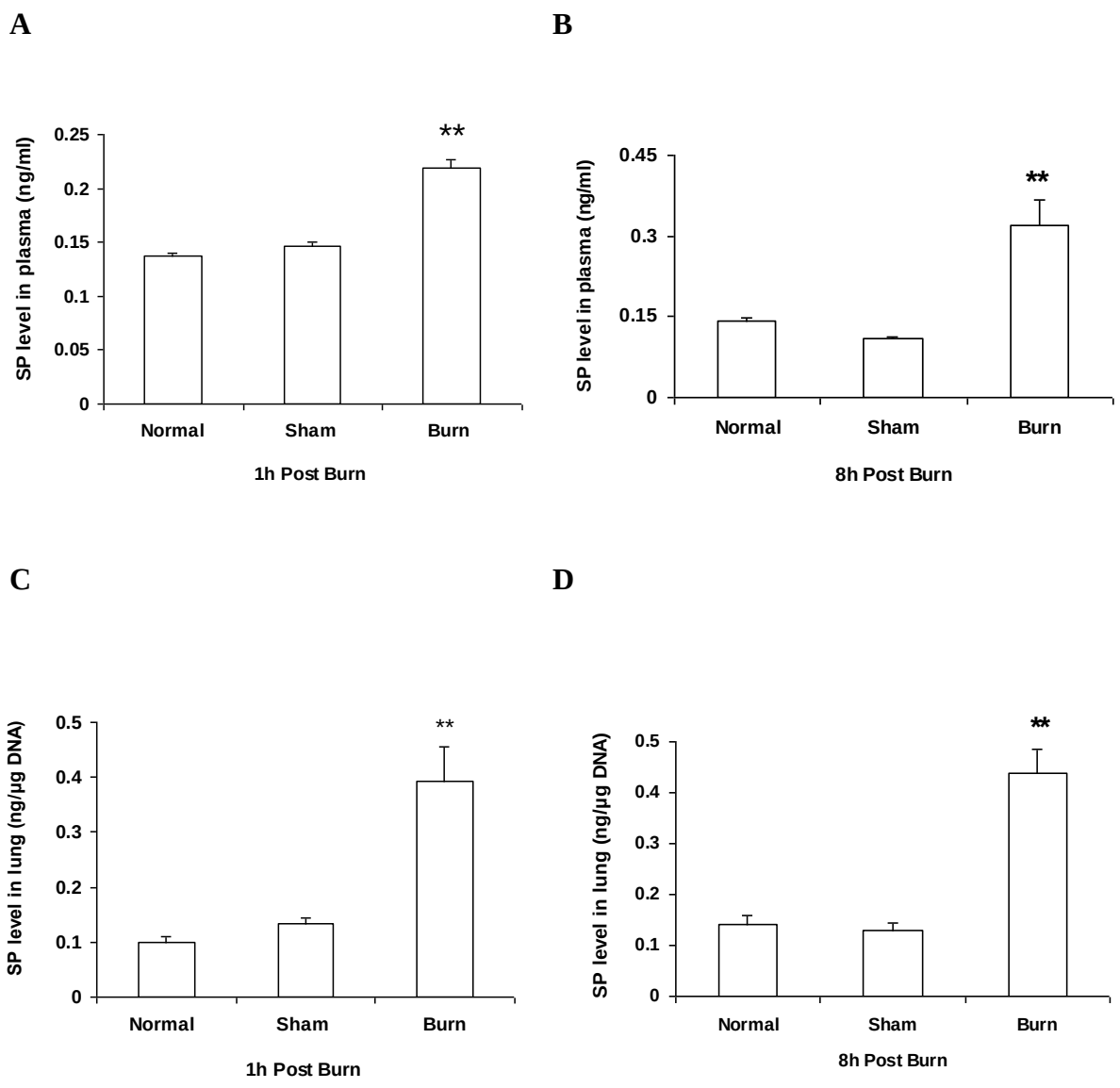


Figure 2.1 Significant increases in endogenous SP levels early after burn injury. SP levels in plasma (A and B) and lung (C and D) of WT mice 1h and 8h after sham or burn injury were measured by ELISA. Sham-burn mice served as controls. Results shown are the mean values ± SEM (n = 6-11 mice per group). **, $p<0.01$ vs sham.

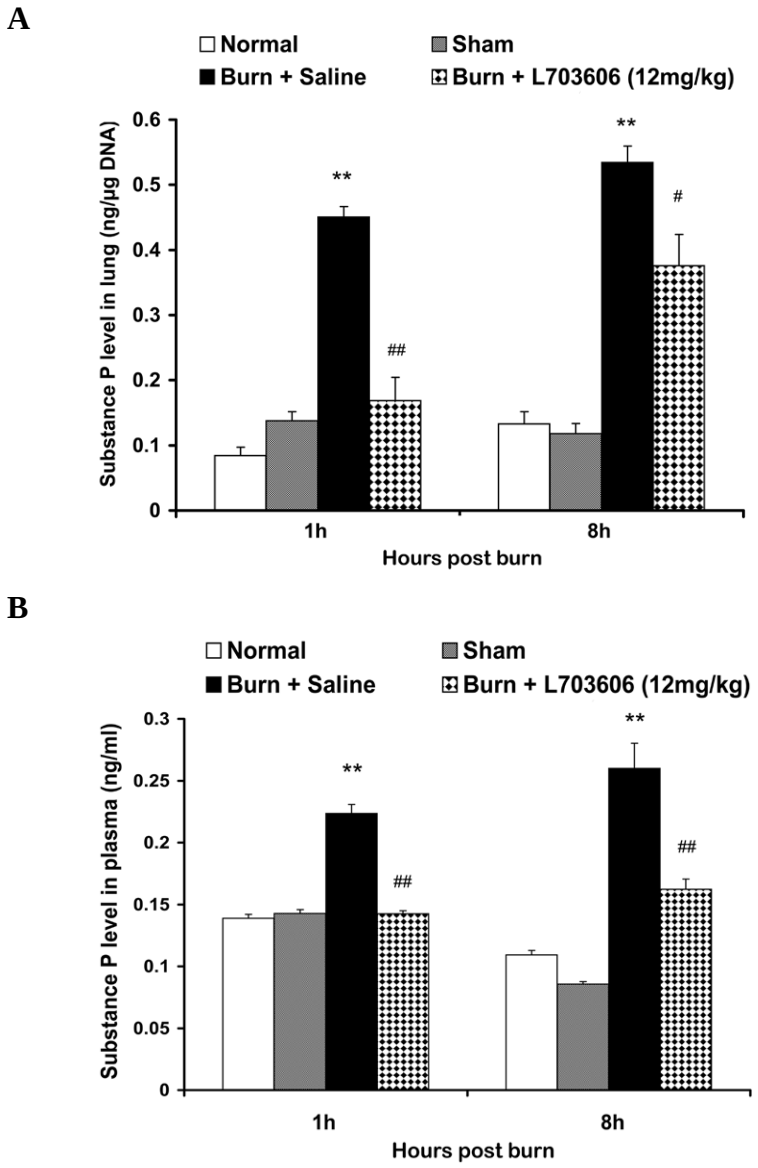
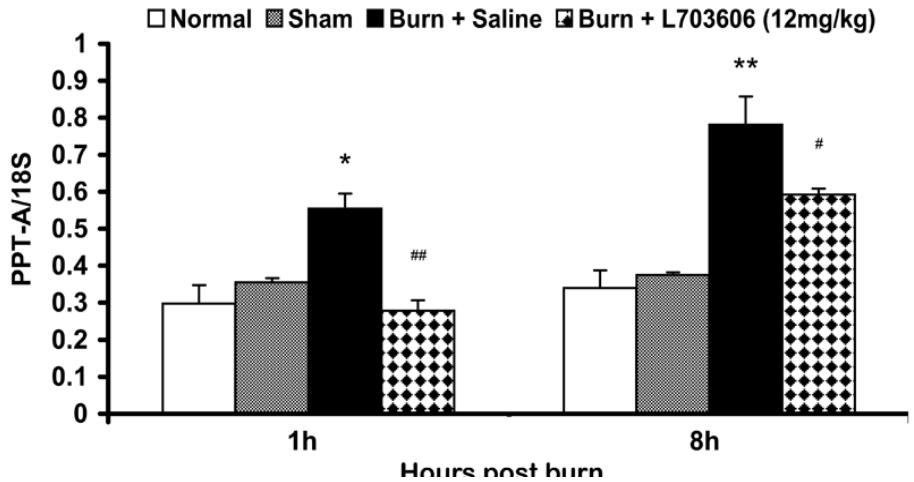


Figure 2.2 Blockade of NK1R significantly reduces SP levels after burn injury. Levels of SP, measured by ELISA, in lung (A) and plasma (B) were evaluated at 1h and 8h post burn. Mice were randomly given L703606 (12mg/kg, i.p.) or vehicle (saline) 1h before burn injury. Sham burn mice served as controls. Results shown are the mean values ± SEM. (n = 8 mice/group at each time point) **, $p<0.01$ vs sham; #, $p< 0.05$ vs burn + saline; ##, $p<0.01$ vs burn + saline.

A



B

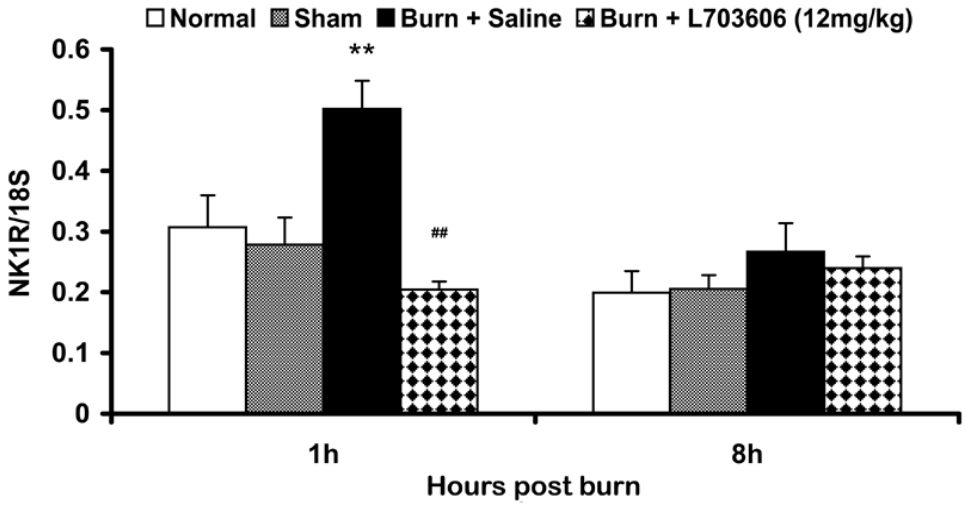
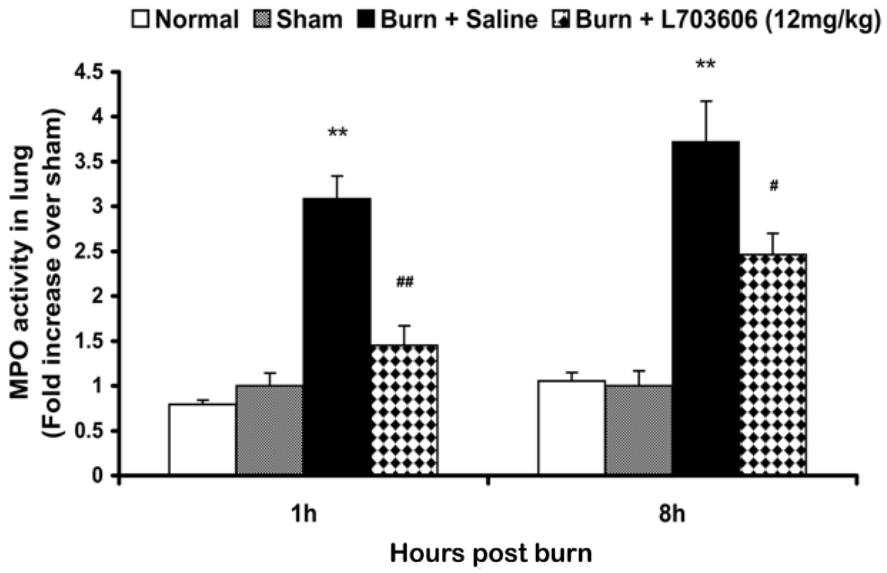
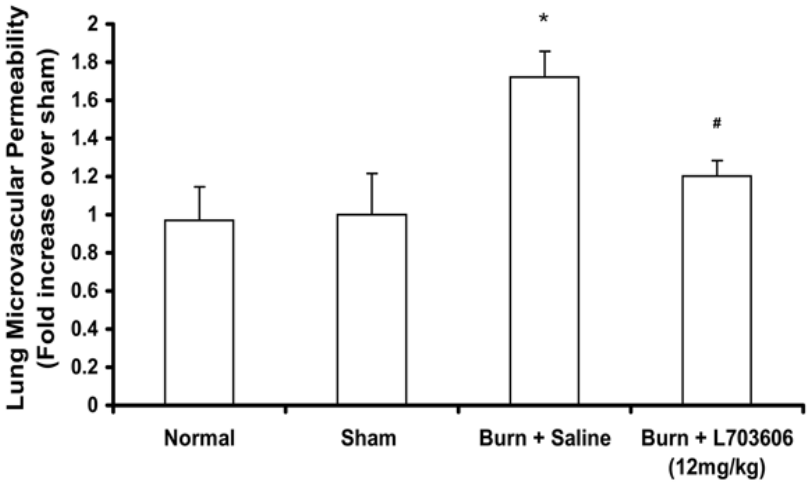


Figure 2.3 Expression levels of *PPT-A* and NK1R mRNA levels in lung after burn injury. The level of *PPT-A* (A) and NK1R (B) mRNA in lung was determined 1h and 8h post burn by semi-quantitative RT-PCR (determined as ratio of band densities of PPT-A or NK1R to 18S). Mouse 18S served as a control. Mice were randomly given L703606 (12mg/kg, i.p.) or vehicle (saline) 1h before burn injury. Sham burn mice served as controls. Results shown are the mean values ± SEM (n = 6 mice/group at each time point). *, $p<0.05$ vs sham; **, $p<0.01$ vs sham; #, $p< 0.05$ vs burn + saline; ##, $p<0.01$ vs burn + saline.

A


Normal
Sham
Burn + Saline
Burn + L703606 (12mg/kg)
MPO activity in lung
(Fold increase over sham)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
##
**
#
1h
8h
Hours post burn

B


Lung Microvascular Permeability
(Fold increase over sham)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
#
Normal
Sham
Burn + Saline
Burn + L703606
(12mg/kg)

C **D**

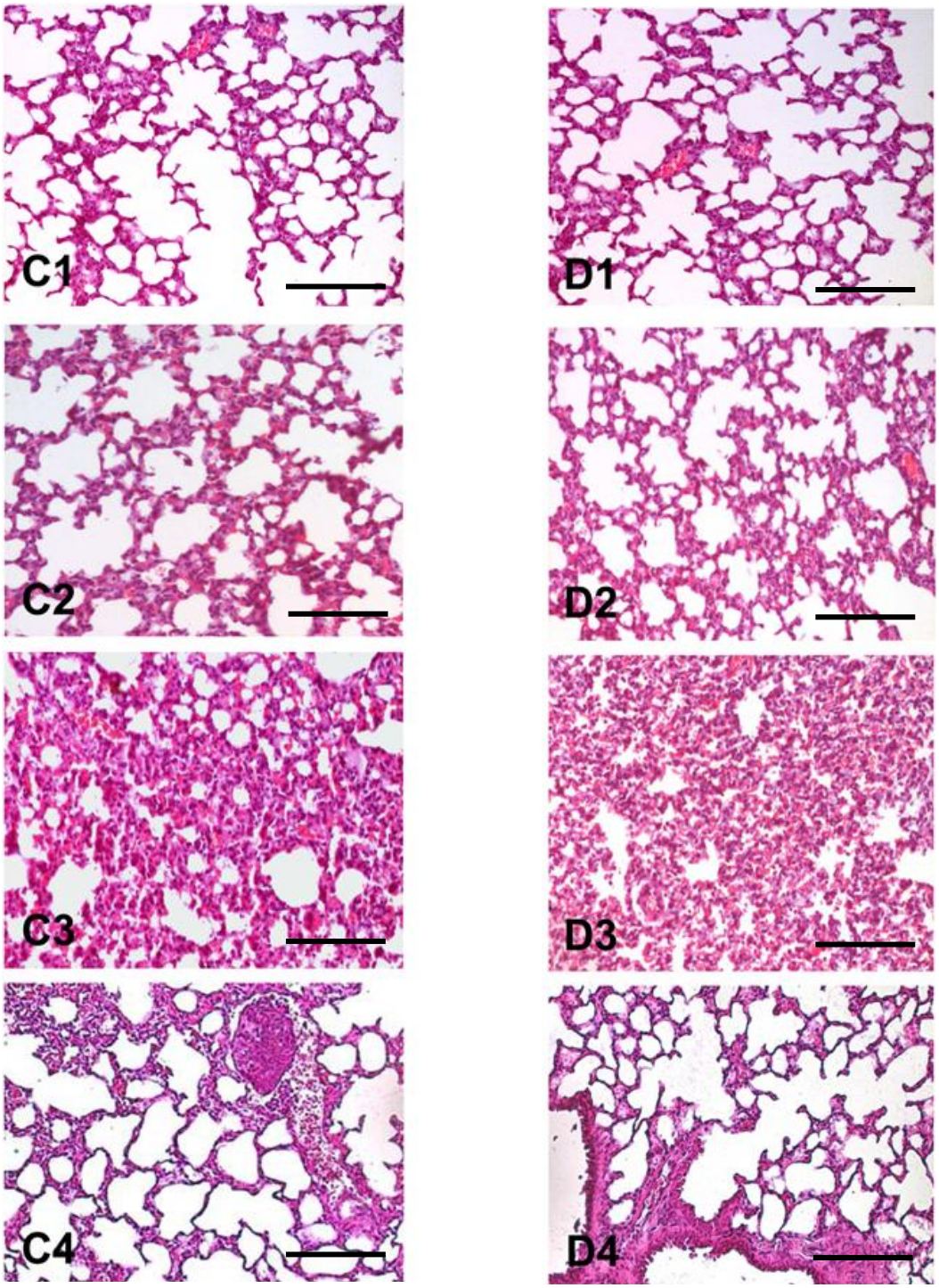
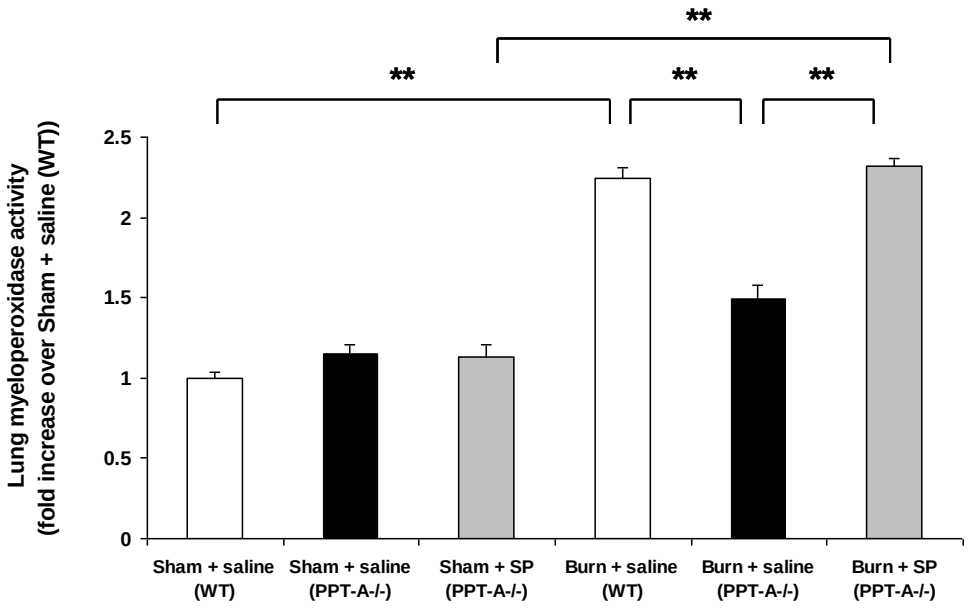
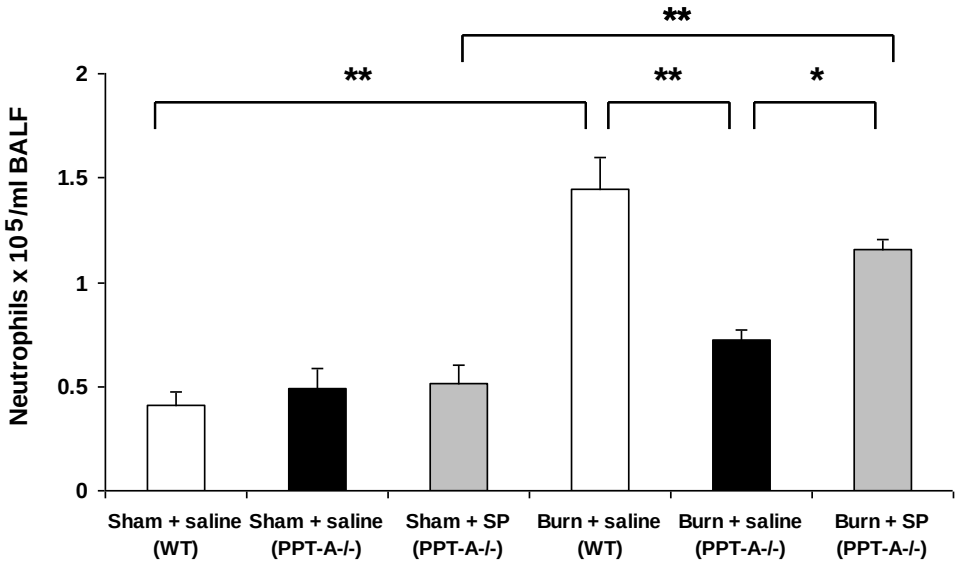


Figure 2.4 Treatment with L703606 significantly attenuated ALI in mice following burn injury. ALI was assessed by determining changes in MPO activity 1h and 8h post burn (A), the leakage of i.v. administered FITC-labeled albumin into the alveolar space 8h post burn as a measure of lung permeability (B), and H&E-stained representative lung sections of sham versus burn-injured mice at 1h and 8h post-burn (C and D). Mice were randomly given L703606 (12mg/kg, i.p.) or vehicle (saline) 1h before burn injury. Sham-burn mice served as controls. Lung sections shown include: normal mice (C1), sham mice (C2), burn + saline (C3), burn + L703606 (12mg/kg) (C4) 1h post burn; and normal mice (D1), sham mice (D2), burn + saline (D3), burn + L703606 (12mg/kg) (D4) 8h post burn. Results shown are the mean values ± SEM (n = 6-8 mice/group at each time point). *, $p<0.05$ vs sham; **, $p<0.01$ vs sham; #, $p< 0.05$ vs burn + saline; ##, $p<0.01$ vs burn + saline. Bars, 20μm.

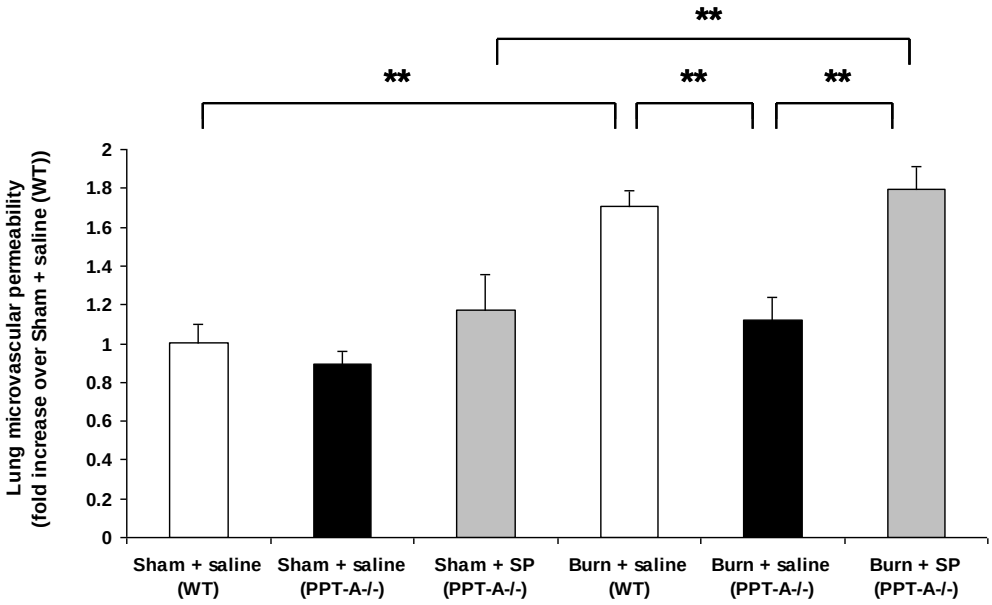
A


**
**
**
**
Lung myeloperoxidase activity
(fold increase over Sham + saline (WT))
2.5
2
1.5
1
0.5
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

B


**
**
**
*
Neutrophils x 10 5/ml BALF
2
1.5
1
0.5
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

C


**
**
**
**
Lung microvascular permeability
(fold increase over Sham + saline (WT))
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

D

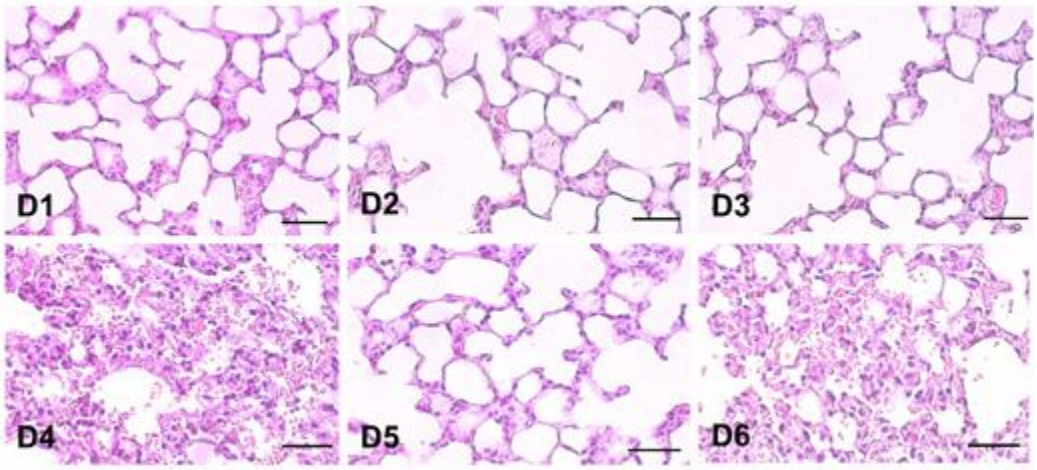

D1
D2
D3
D4
D5
D6

Figure 2.5 Reduced neutrophil infiltration and alleviated ALI in burn-injured *PPT-A*$^{-/-}$ mice. Lung MPO activity (A), neutrophil counts in BALF (B) and lung microvascular permeability assessment (C) at 8h in WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. Results shown are the mean values ± SEM (n = 4-7 mice per group). *, $p<0.05$; **, $p<0.01$. (D) Histopathological evaluation (H&E staining) at 8h of lung PMN infiltration and injury in WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. Sham burn mice served as controls. Lung sections include: sham-injured WT mice (D1), sham-injured *PPT-A*$^{-/-}$ mice (D2), sham-injured *PPT-A*$^{-/-}$ mice injected with SP (D3), burn-injured WT mice (D4), burn-injured *PPT-A*$^{-/-}$ mice (D5) and burn-injured *PPT-A*$^{-/-}$ mice injected with SP (D6). (n = 6 mice per group). Bars, 20µm.

A

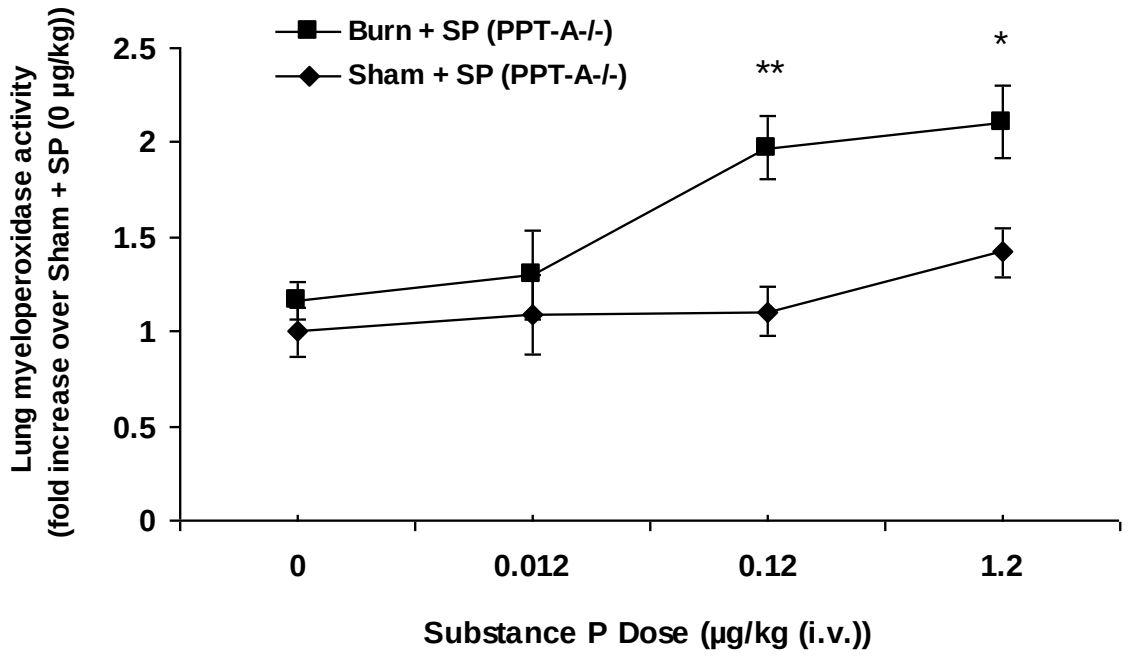
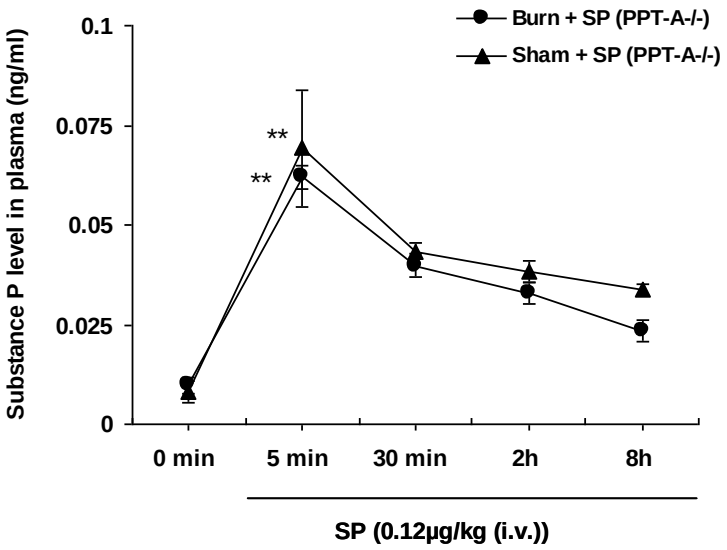


Figure 2.6 Administration of exogenous SP markedly increased lung neutrophil infiltration in a dose dependent manner following burn injury in *PPT-A*$^{-/-}$ mice. (A) Lung MPO activity at 8h in *PPT-A*$^{-/-}$ mice injected with various doses of SP immediately after sham or burn injury. Results shown are the mean values ± SEM (n = 6-8 mice for each dose group). **, $p<0.05$ compared to burn-injured *PPT-A*$^{-/-}$ mice + SP (0µg/kg) and sham-injured *PPT-A*$^{-/-}$ mice + SP (0.12µg/kg); *, $p<0.05$ compared to burn-injured *PPT-A*$^{-/-}$ mice + SP (0µg/kg) and sham-injured *PPT-A*$^{-/-}$ mice + SP (1.2µg/kg).

A



B

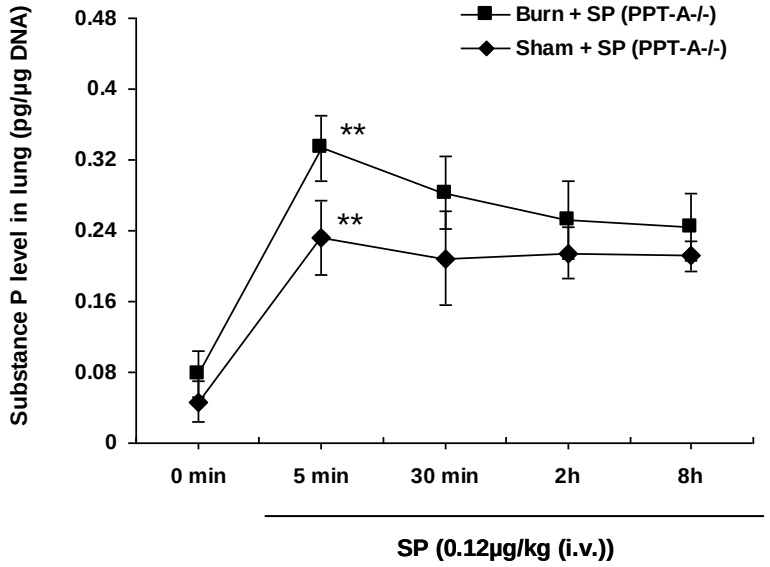
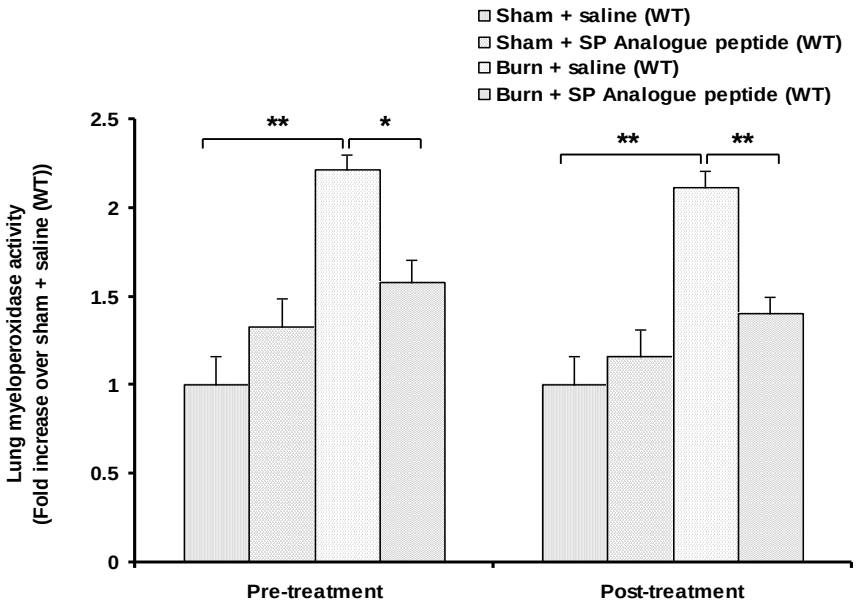
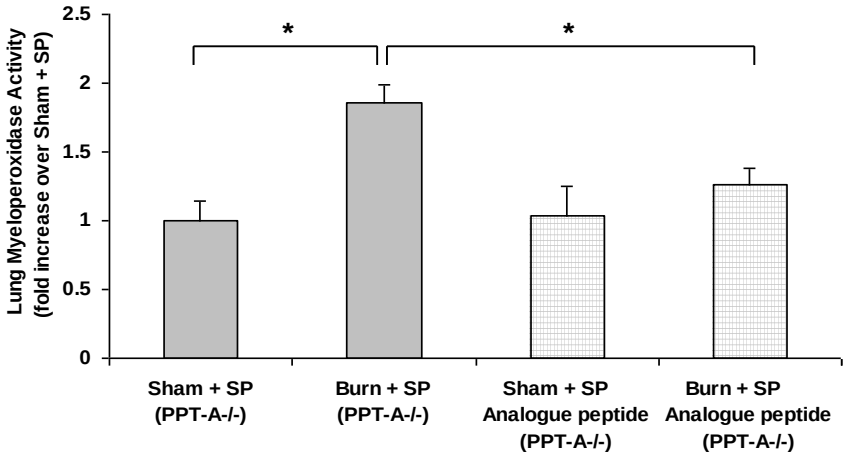


Figure 2.7 Confirmation of the actual levels of exogenous SP present in plasma and lung of *PPT-A*$^{-/-}$ mice. Actual levels of exogenous SP at various time points in plasma (A) and lungs (B) of *PPT-A*$^{-/-}$ mice administered immediately after sham or burn injury, were determined by ELISA. Results shown are the mean values ± SEM (n = 7-14 mice per group per time point). **, $p<0.01$ compared to SP levels at 0min (representing mice without SP injection).

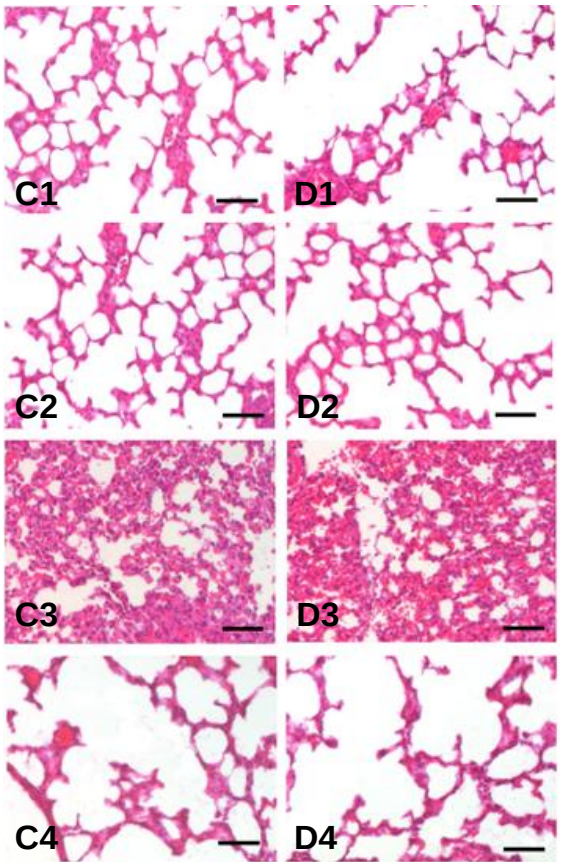
A


Sham + saline (WT)
Sham + SP Analogue peptide (WT)
Burn + saline (WT)
Burn + SP Analogue peptide (WT)
Lung myeloperoxidase activity
(Fold increase over sham + saline (WT))
2.5
2
1.5
1
0.5
0
**
*
**
**
Pre-treatment
Post-treatment

B


Lung Myeloperoxidase Activity
(fold increase over Sham + SP)
2.5
2
1.5
1
0.5
0
*
*
Sham + SP
(PPT-A-/-)
Burn + SP
(PPT-A-/-)
Sham + SP
Analogue peptide
(PPT-A-/-)
Burn + SP
Analogue peptide
(PPT-A-/-)

C **D** **E**



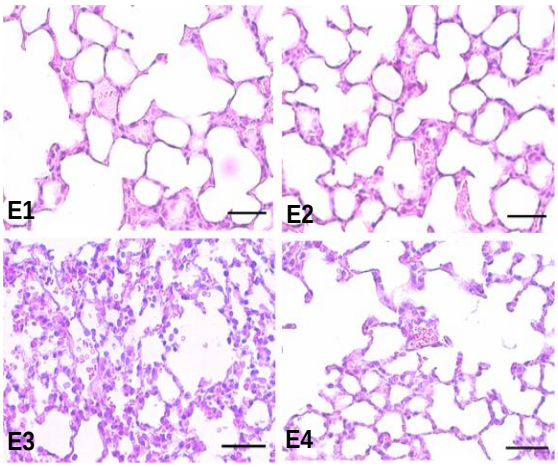
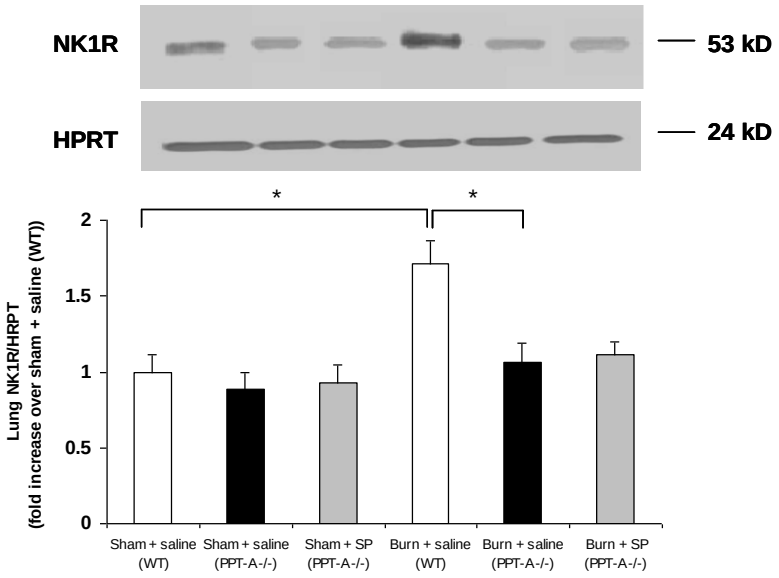


Figure 2.8 SP analogue peptide failed to induce ALI in WT and *PPT-A*$^{-/-}$ mice following burn injury. Lung MPO activity at 8h in WT mice injected with SP analogue peptide (0.12µg/kg, i.v.) or vehicle (saline) 15min before (pre-treatment) and after (post-treatment) sham or burn injury (A) and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or SP analogue peptide (0.12µg/kg, i.v.) immediately after sham or burn injury (B). Results shown are the mean values ± SEM (n = 5-7 mice per group). *, $p<0.05$. (C-E) Histopathological evaluation (H&E staining) of lung PMN infiltration and injury at 8h in WT mice administered with SP analogue peptide (0.12µg/kg, i.v.) or vehicle (saline) 15min before (pre-treatment) (C) and 15min after (post-treatment) (D) sham or burn injury and *PPT-A*$^{-/-}$ mice administered with SP analogue peptide (0.12µg/kg, i.v.) or SP (0.12µg/kg, i.v.) immediately after sham or burn injury (E). Sham burn mice served as controls. Lung sections include: sham-injured WT mice (pre- or post-treatment) (C1, D1), sham-injured WT mice injected with SP analogue peptide (pre- or post-treatment) (C2, D2), burn-injured WT mice (pre- or post-treatment) (C3, D3), burn-injured WT mice injected with SP analogue peptide (pre- or post-treatment) (C4, D4); sham-injured *PPT-A*$^{-/-}$ mice injected with SP (E1), sham-injured *PPT-A*$^{-/-}$ mice injected with SP analogue peptide (E2), burn-injured *PPT-A*$^{-/-}$ mice injected with SP (E3) and burn-injured *PPT-A*$^{-/-}$ mice injected with SP analogue peptide (E4). (n = 6 mice per group). Bars, 20µm.

A



B

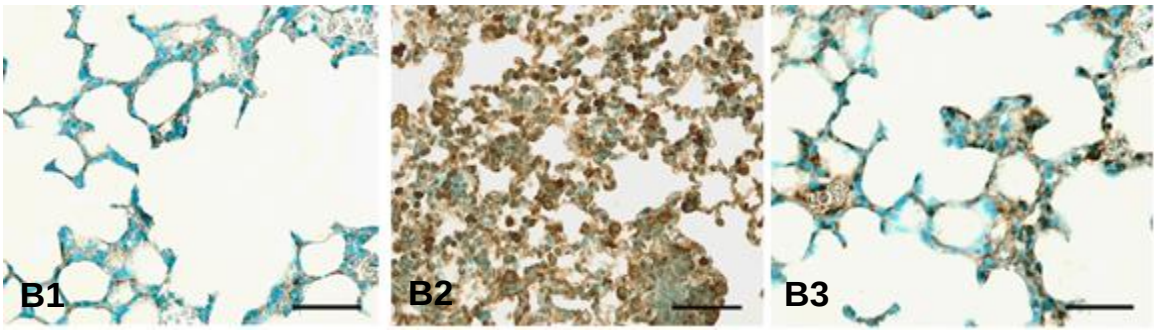


Figure 2.9 Expression levels of Lung NK1R following burn injury. (A) Western blot photograph of lung NK1R at 8h in WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury and corresponding densitometry quantification of bands. NK1R and its corresponding HPRT bands shown in each lane are representative results obtained from different blots. Results shown are the mean values ± SEM (n = 4 mice per group). *, $p<0.05$. (B) Immunohistochemical localization of NK1R at 8h in lungs of sham-injured WT mice (B1), burn-injured WT mice (B2) and burn-injured *PPT-A*$^{-/-}$ mice (B3). Sham burn mice served as controls. (n = 5 mice per group). Bars, 50µm.

Chapter III Effect of SP on pulmonary cytokines, chemokines and zinc metalloproteinases production after burn injury

3.1 Introduction

The classical bioactive neuropeptide, substance P (SP), encoded by the preprotachykinin-A gene, is a major mediator of pro-inflammatory responses in many pathological conditions. It binds preferentially to the neurokinin-1 receptor (NK1R), present in all major organs, including the lungs and on numerous immune cells, to elicit numerous systemic inflammatory effects such as increased microvascular permeability and plasma extravasation, immune cell influx, increased edema, vasodilation and glandular secretions thereby, contributing to heightened systemic inflammation.

The clinical consequences of the systemic inflammatory response syndrome (SIRS) are characterized by tachycardia, tachypnea, fever, leukocytosis, hypotension, and in very severe cases, shock and multiple organ dysfunction syndrome (MODS) (Sherwood and Traber, 2002). In severely burn-injured patients, SIRS is initiated immediately after the onset of the burn injury (Marko et al., 2003; Monafo, 1996). The presence of SIRS in burn patients is an important predictor of poor prognosis (Muckart and Bhagwanjee, 1997). This is supported further by burn patients reported to have severe SIRS and later died of sterile MODS, particularly ALI/ARDS leading to lung failure, who were clinically uninfected at the time of death (Sheridan et al., 1998). The results of multiple clinical and animal studies have established that the persistence of SIRS can lead to early

MODS with an attendant high mortality, occurring in 20 to 30% individuals (Biffl et al., 1996). Additionally, the severity of SIRS and its persistence in burn patients has been shown to be proportional to the magnitude of the burn injury, and is associated with increased morbidity and mortality (Sherwood and Traber, 2002). Therefore, the severity of the initial inflammatory events is an important predisposing factor in determining the initial and prolonged effects of SIRS on the host (Sherwood and Traber, 2002).

Upon severe burn injury, an acute release of a cascade of pro-inflammatory cytokines is induced leading to the activation of SIRS. Well known examples of the underlying molecules responsible are the classical TNF-α, IL-1β and IL-6. In addition, chemokines play a pivotal role to the pathophysiology of burn injury. Therefore, it is in our interest to investigate the role of pro-inflammatory mediator, SP, in instigating cytokine and chemokine production. Additionally, we were interested in determining the effect of burn injury on regulatory molecules of SP, such as neutral endopeptidase (NEP) and regulatory molecules of extra-cellular matrix proteins, matrix metalloproteinases (MMP). Therefore, in this chapter we focus on understanding the effect of SP and these inflammatory mediators in instigating remote ALI after severe local burn injury.

3.2 Materials and methods

3.2.1 Mouse burn injury model

For details, see section 2.2.1, page 48.

3.2.2. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

For details, see section 2.2.6, page 52. The primer sequences for detection of IL-1β, IL-6, TNF-α, MIP-1α, MIP-2 and 18S, optimal annealing temperature, optimal cycles, and product sizes are as shown in Table 3.1.

3.2.3 Cytokine, chemokine and matrix metalloproteinases analysis

Single-analyte ELISA assays were performed for the measurement of cytokines (IL-1β, IL-6 and TNF-α), chemokines (MIP-1α and MIP-2) and matrix metalloproteinases (MMP-9 and MMP-2) in homogenized lung tissue, ELISA kits, all from R&D Systems were used according to the manufacturer's instructions. The lower limits of detection of the levels of IL-1β, IL-6, TNF-α, MIP-1α, MIP-2, were 15.625, 15.625, 31.25, 3.91 and 15.625 pg/ml respectively; while MMP-9 and MMP-2 were 15.6 and 16.5 ng/ml respectively. The ELISA results were reproducible with interassay variability of <9.5% and intra-assay variability of <6.5%. Results were then corrected for the DNA content of the tissue samples (Labarca and Paigen, 1980) and were expressed as picograms per microgram of DNA.

3.2.4 Measurement of neutral endopeptidase activity

NEP enzymatic activity was measured spectrophotomerically as previously described with minor modifications (Yamaoka and Kawana, 2007). Lung tissue was homogenized

in T-PER tissue protein extraction reagent (Pierce) and centrifuged at 12,000g for 15 min at 4ºC. Each sample (30µg protein) was incubated with 1mM succinyl-Ala-Ala-Phe-p-nitroanilide (Suc-Ala-Ala-Phe-pNA; Bachem; Peninsula Laboratories) as a substrate in 0.1 M Tris·HCl (pH 7.6) in the presence of 1 µl (0.02 units/µl) porcine kidney aminopeptidase AP-N (Roche Diagnostics). In this coupled activity assay, NEP cleaves Suc-Ala-Ala-Phe-pNA between Ala and Phe, yielding Phe-pNA. AP-N subsequently cleaves Phe-pNA, generating pNA as the final product. The increase in specific absorbance at 405 nm (as a result of the accumulation of free *p*-nitroaniline) was determined each min after incubation at 37°C for 30 min with a micro-plate reader. Substrate alone and substrate with AP-N and Tris buffer blanks were run in parallel. Protein concentration was determined by the Bradford method.

3.2.5 Immunohistochemical analysis

For details, see section 2.2.9, page 54. Immuno-staining was performed using rat monoclonal anti-NEP (1/50 dilution; Santa Cruz Biotechnology).

3.2.6 Statistics

For details, see section 2.2.10, page 55.

Table 3.1 PCR primer sequences, optimal conditions, and product sizes

Gene	Primer Sequence	Optimal Conditions	Size (bp)
r18S	Sense: 5'-GTAACCCGTTGAACCCCATT-3‘ Antisense: 5'-CCATCCAATCGGTAGTAGCG-3'	28 cycles Annealing: 59ºC	150
IL-1β	Sense: 5'-AAGGAGAACCAAGCAACGAC-3‘ Antisense: 5'-GAGATTGAGCTGTCTGCTCA-3'	34 cycles Annealing: 63ºC	381
IL-6	Sense: 5'-TGCTGGTGACAACCACGGCC-3' Antisense: 5'-GTACTCCAGAAGACCAGAGG-3'	34 cycles Annealing: 63ºC	308
TNF-α	Sense: 5'-CCTGTAGCCCACGTCGTAGC-3' Antisense: 5'-TTGACCTCAGCGCTGAGTTG-3'	34 cycles Annealing: 65ºC	374
MIP-2	Sense: 5'-GCTGTCAATGCCTGAAGACC-3‘ Antisense: 5'-TAGTTCCCAACTCACCCTCTC-3'	36 cycles Annealing: 65ºC	189
MIP-1α	Sense 5'-ACTGCCCTTGCTGTTCTTCTCT-3‘ Antisense 5‘-AGGCATTCAGTTCCAGGTCAGTGA-3'	34 cycles Annealing: 61ºC	261

3.3 Results

3.3.1 SP-NK1R signaling significantly augmented pro-inflammatory cytokines and chemokines at the transcriptional and protein levels following severe burn injury.

Severe injury results in the onset of the production of a cascade of pro-inflammatory cytokines, termed as a "cytokine storm", which leads to the downstream phenotypic changes in the host inflammatory response (Bhatia and Moochhala, 2004; Ware and Matthay, 2000). Therefore, to investigate the link between SP-NK1R signaling in generating a cytokine mediated lung injury after burn, we determined the role of SP-NK1R signaling in affecting these mediators at the transcriptional and protein level.

Our results showed markedly increased mRNA expression levels of IL-1β, TNF-α and IL-6; and these were inhibited upon treatment with L703606 (Fig. 3.1A to C). At the protein expression level, a similar effect was found which correlated well with the mRNA levels showing significantly increased IL-1β, TNF-α and IL-6 at both 1h and 8h, all of which were markedly suppressed by pre-treatment of L703606 except for IL-6 which exhibited a decrease in levels only at 8h post burn but not at 1h (Fig. 3.2A to C). Notably, a marked reduction in IL-6 mRNA levels was found at 1h although there was no change at the protein level.

Subsequently, we addressed the role of SP-NK1R signaling on chemoattractants production in mediating recruitment of inflammatory cells after burn injury. Earlier, we assessed lung neutrophil infiltration as an early marker of ALI by determining MPO activity; therefore it was appropriate to study the expression of macrophage-inflammatory protein 2 (MIP-2), a potent neutrophil chemoattractant secreted mainly by alveolar macrophages (Puneet et al., 2005); and MIP-1α which is a product of activated monocytes and macrophages and also an important activator of T cells, monocytes, and macrophages (Puneet et al., 2005).

Significantly increased transcriptional levels of MIP-2 were found after burn injury at 1h and 8h (Fig. 3.1D). This correlated well with the protein levels at 1h and 8h (Fig. 3.2D), which also rose in parallel with elevated levels of MPO activity (Fig. 2.4A). Likewise, disruption of SP-NK1R signaling by L703606 suppressed MIP-2 protein levels (Fig. 3.2D) and as such a reduction in MPO activity was observed at both 1h and 8h as well (Fig. 2.4A). Gene expression levels of MIP-1α were increased at only 1h but were no longer elevated at 8h (Fig. 3.1E). L703606 significantly prevented MIP-1α gene expression at 1h (Fig. 3.1E). At the protein level, MIP-1α expression at both 1h and 8h was significantly suppressed by L703606 compared to untreated mice (Fig. 3.2E).

3.3.2 Absence of *PPT-A* gene impaired pro-inflammatory cytokine and chemokine production after burn but not in mice challenged with exogenous SP

Next, we further confirm our results by using mice lacking the *PPT-A* gene, and *PPT-A-/-* mice challenged with exogenous SP. Burn-injured *PPT-A-/-* mice exhibited alleviated lung inflammation evident by a significant reduction in pulmonary levels of pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and chemokines MIP and MIP-1α compared to burn-injured WT mice (Fig. 3.3A to E). Administration of exogenous SP to *PPT-A-/-* mice reversed this effect in all five cytokines and chemokines aggravating lung inflammation (Fig. 3.3A to E). Therefore, these data show that *PPT-A* gene deletion is important in preventing the release of SP-mediated cytokines and chemoattractant induction of inflammatory cells, which lead to the onset of ALI after burn.

3.3.3 Effect of *PPT-A* gene products on zinc metalloproteinases expression and activity in lungs after burn injury

Members of the zinc metalloproteinase superfamily are a major group of enzymes that regulate protein levels by using zinc for their proteolytic activities (Hooper, 1994). Neutral endopeptidase (NEP), matrix metalloproteinase (MMP)-9 and MMP-2 are such examples. NEP represents one of the most studied neuropeptide-degrading enzymes. It is a membrane bound enzyme which is in lungs located mainly at the surface of epithelial cells and is known to degrade and inactivate SP, thereby, modulating the number of intact SP molecules (Di Maria et al., 1998). NEP is present in high quantities in the healthy

lung, and is decreased in a number of airways diseases such as asthma and bronchitis (Di Maria et al., 1998; Nadel, 1990). Our results show that after burn injury in WT mice, NEP activity was significantly diminished compared to sham-injured WT mice (Fig. 3.4A). Its activity was markedly increased in burn-injured *PPT-A*$^{-/-}$ mice; while exogenous administration of SP to burn-injured *PPT-A*$^{-/-}$ mice again lowered NEP activity (Fig. 3.4A). Additionally, immuno-staining of lung sections for NEP (appearing as brown color) showed similar results (Fig. 3.4B), suggesting that burn-induced increase in SP levels may modulate NEP activity.

MMP-9 and MMP-2 are extra-cellular enzymes that regulate extracellular matrix (ECM) proteins, and are particularly important in the pathogenesis of many inflammatory diseases in many organs including the lungs (Atkinson and Senior, 2003). They are present in low quantities in the healthy lung, but much more abundant in several lung diseases (Atkinson and Senior, 2003). Our results reveal that lung MMP-9 levels are elevated after burn in WT mice and significantly reduced in burn-injured *PPT-A*$^{-/-}$ mice; this effect was reversed in burn-injured *PPT-A*$^{-/-}$ mice injected with exogenous SP (Fig. 3.5A). However, no changes in lung MMP-2 levels were detected (data not shown). Hence, these findings suggest that SP regulates lung MMP-9 levels more than MMP-2 in lungs after burn injury.

3.4 Discussion

SP has been shown to directly activate and induce the chemotaxis of numerous immuno-inflammatory cells, including, neutrophils, macrophages, lymphocytes, monocytes, eosinophils and mast cells to release a diverse spectrum of signaling messengers such as cytokines, chemokines, and other mediators (O'Connor et al., 2004). After burn injury, the heightened inflammation response and ALI characterized by significant neutrophil infiltration in the lungs was observed in parallel with higher pro-inflammatory IL-1β, TNF-α and IL-6 mRNA and protein production 1h and 8h post burn. Chemokines, MIP-2 and MIP-1 α were markedly elevated indicating the active role of SP induced chemoattractants production in mediating trafficking of inflammatory cells. Treatment with L703606 significantly suppressed levels of all these cytokines and chemokines. Furthermore, levels of all five molecules were significantly reduced in *PPT-A*$^{-/-}$ mice; and again elevated upon SP injection to *PPT-A*$^{-/-}$ mice. Thereby, the data demonstrate that SP plays an essential role in regulating neutrophil recruitment, as seen in MPO results, through chemokines such as MIP-2 and MIP-1 α and that the SP-induced cytokines release may further stimulate neutrophils recruited to play an active role in exacerbating lung inflammation and injury.

Regulation of SP levels is critical in controlling SP-induced inflammation in numerous inflammatory diseases, including those of the lung (Di Maria et al., 1998; Roques et al., 1993). The biological actions of SP are terminated by NEP, and studies show that it is an important regulator of SP levels (Lu et al., 1997; Roques et al., 1993). Burn injury

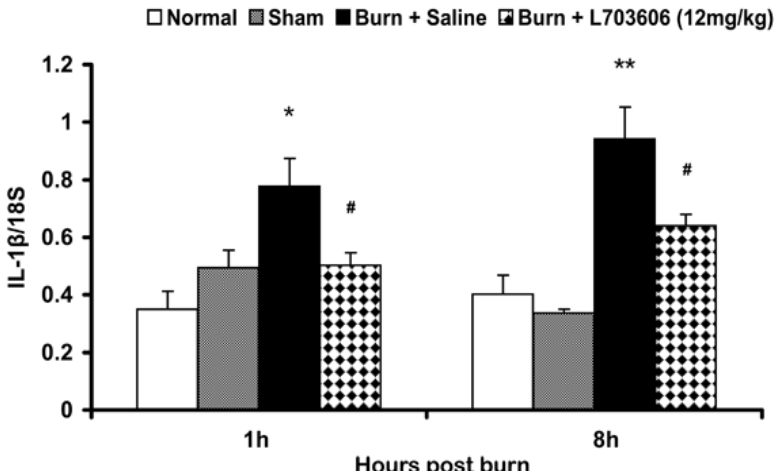
induced a significant reduction in NEP activity in lungs of burn-injured WT mice as compared to sham-injured WT mice, occurring in parallel with a significant rise in SP levels. However, this effect was not observed in *PPT-A*$^{-/-}$ mice, whereby, the absence of SP in sham and burn-injured *PPT-A*$^{-/-}$ mice, both showed elevated NEP activity. Hence, it appears that the presence of SP modulates NEP activity in lungs after burn. This was further confirmed when exogenous SP was introduced to sham and burn-injured *PPT-A*$^{-/-}$ mice, in which NEP activity was significantly lowered in burn-injured *PPT-A*$^{-/-}$ mice compared to sham-injured *PPT-A*$^{-/-}$ mice. The mechanism behind a loss in NEP activity has been reported to be due to its inhibition by oxidation from free radicals (Dusser et al., 1989). Interestingly, here, SP may serve as an indirect mediator to regulate NEP activity; where increased SP levels after burn, induces recruitment of hyper-active inflammatory cells, such as neutrophils, which have been shown to regulate NEP (Shipp et al., 1991), by releasing excessive oxidants, such as free radicals. Also, with increased tissue destruction, the contribution of oxidant release is further increased. Thus, with the inhibition of NEP activity early after burn, endogenous SP levels continue to persist at high levels, and lung injury is perpetuated.

Matrix metalloproteinases such as MMP-9 are primarily responsible for the turnover and degradation of ECM proteins (Atkinson and Senior, 2003). However, their role in inflammatory conditions remains unclear. Studies have implicated that excessive levels of MMP-9 in inflamed tissues results in excessive proteolysis of tissues, thus, paving the way for infiltrating inflammatory cells into tissues, and causing tissue destruction (Atkinson and Senior, 2003; Coussens et al., 2000). On the contrary, other studies regard MMP-9 as a reparative factor for the re-epithelialization of new proteins along with its

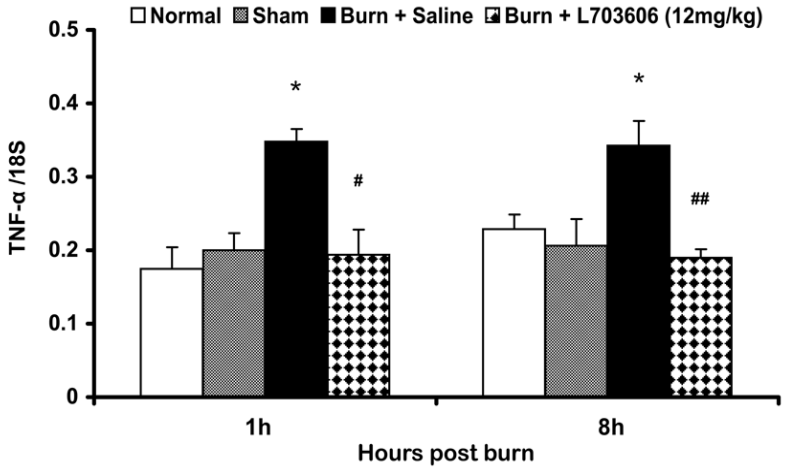
ability to degrade non-matrix proteins, including SP (Backstrom and Tokes, 1995), thus enhancing wound healing (Betsuyaku et al., 2000; Parks and Shapiro, 2001). Our results reveal that MMP-9 levels are markedly increased after burn compared to sham-injured WT mice. Despite an increase in MMP-9 levels in burn-injured WT mice, SP levels remains significantly high, while lung injury was concurrently intensified. These results suggest that SP may have a role in modulating MMP-9 levels after burn, influencing it to be more destructive than beneficial in the lungs. Further evidence of SP effect on MMP-9 levels was demonstrated by removal of SP in burn-injured *PPT-A*$^{-/-}$ mice which resulted in lowered MMP-9 levels; while exogenous administration of SP to burn-injured *PPT-A*$^{-/-}$ mice restored the MMP-9 lung levels. MMP-9 is known to be induced by pro-inflammatory cytokines including TNF-α, IL-1 and IL-8 (Ram et al., 2006). After burn, SP induces an increase in these cytokines; hence, this up-regulation of cytokines may in-turn elevate MMP-9 levels, resulting in tissue injury. Additionally, our results indicated that burn injury does not alter lung MMP-2 levels (data not shown). Therefore, burn-induced SP release may work via MMP-9 and not MMP-2 to cause lung tissue injury. A significant role of MMP-9 over MMP-2 after burn has also been reported to disrupt blood-brain barrier integrity in the rat (Swann et al., 2007). Nonetheless, establishment of the exact roles of MMP-9 after burn requires further investigation.

In summary, our results demonstrate that SP up-regulates the release and production of pro-inflammatory mediators which work to exacerbate systemic inflammation after severe local burn injury via NK1R signaling. Inhibition of SP by *PPT-A* gene deletion alleviates SP mediated ALI systemic inflammation and ALI after burn injury.

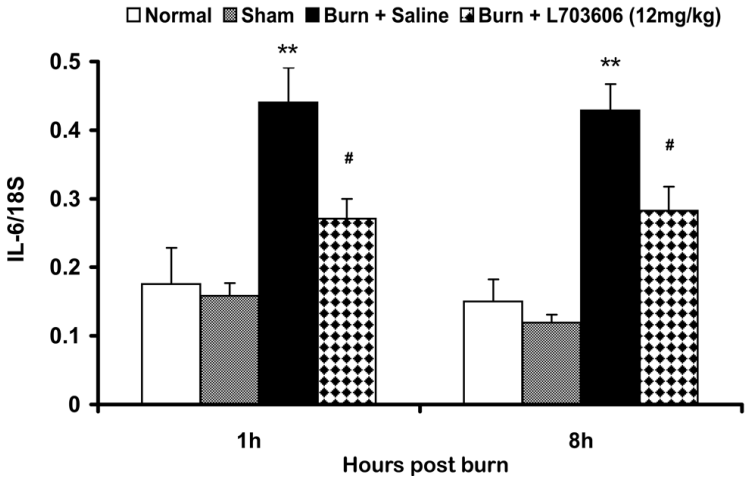
A


Normal
Sham
Burn + Saline
Burn + L703606 (12mg/kg)
IL-1β/18S
1.2
1
0.8
0.6
0.4
0.2
0
*
#
**
#
1h
8h
Hours post burn

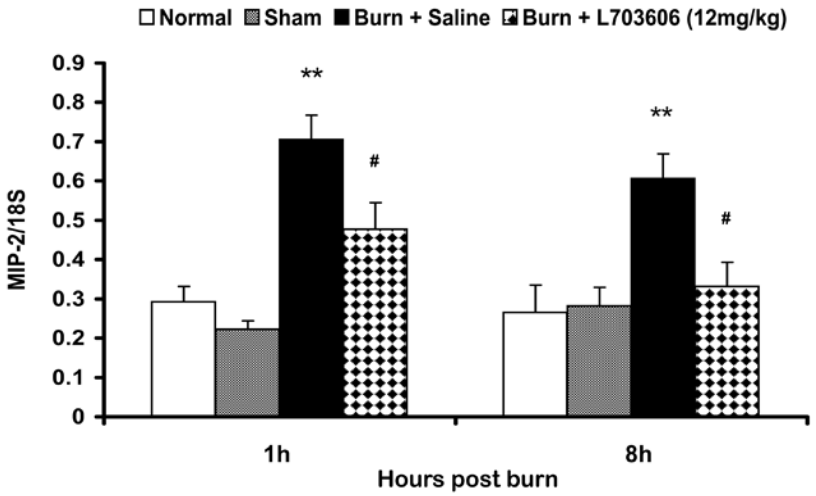
B


Normal
Sham
Burn + Saline
Burn + L703606 (12mg/kg)
TNF-α /18S
0.5
0.4
0.3
0.2
0.1
0
*
#
*
##
1h
8h
Hours post burn

C


Normal
Sham
Burn + Saline
Burn + L703606 (12mg/kg)
IL-6/18S
0.5
0.4
0.3
0.2
0.1
0
**
#
**
#
1h
8h
Hours post burn

D



E

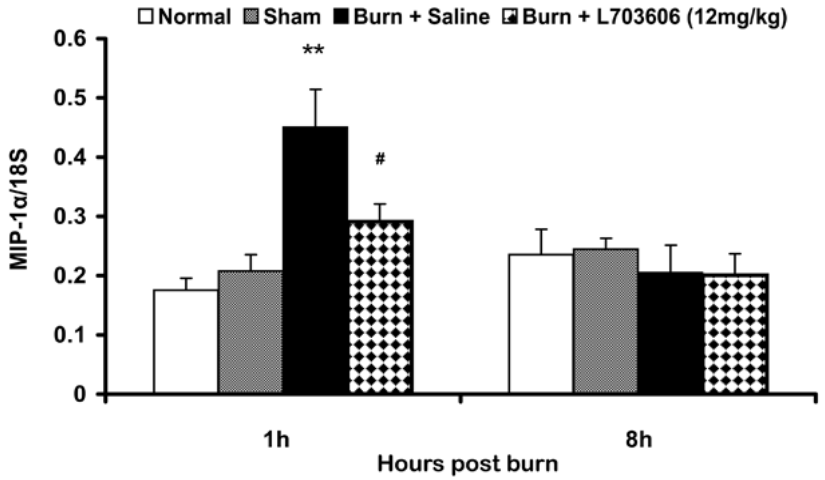
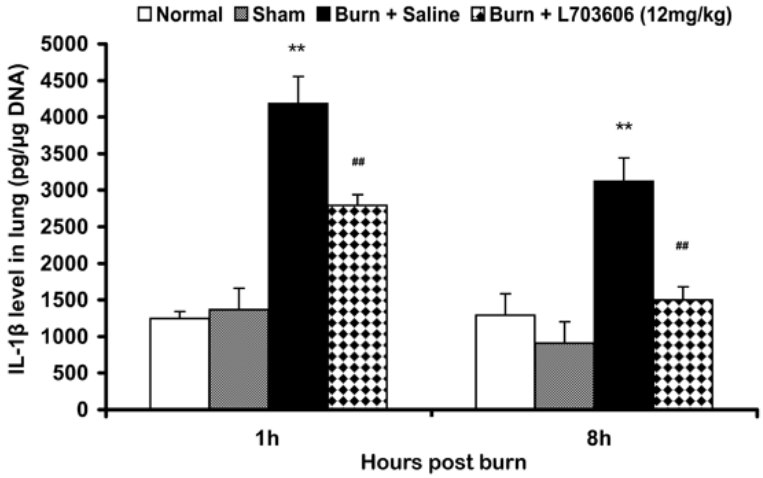
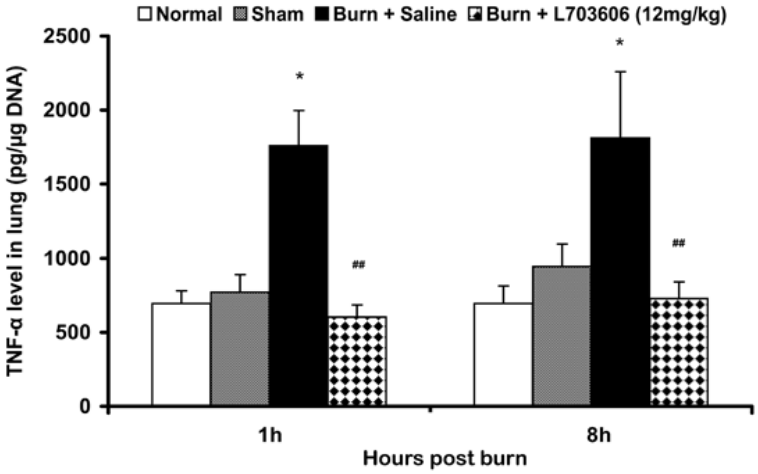


Figure 3.1 Lung mRNA levels of cytokine and chemokine in burn-injured mice treated with L703606. The mRNA levels of TNF-α, IL-1β, IL-6, MIP-2 and MIP-1α in lung was determined 1h and 8h post burn by semi-quantitative RT-PCR (determined as ratio of band densities of TNF-α, IL-1β, IL-6, MIP-2 or MIP-1α to 18S). Mouse 18S served as a control. Mice were randomly given L703606 (12mg/kg, i.p.) or vehicle (saline) 1h before burn injury. Sham burn mice served as controls. Results shown are the mean values ± SEM (n = 6 mice/group at each time point). *, $p<0.05$ vs sham; **, $p<0.01$ vs sham; #, $p< 0.05$ vs burn + saline; ##, $p<0.01$ vs burn + saline.

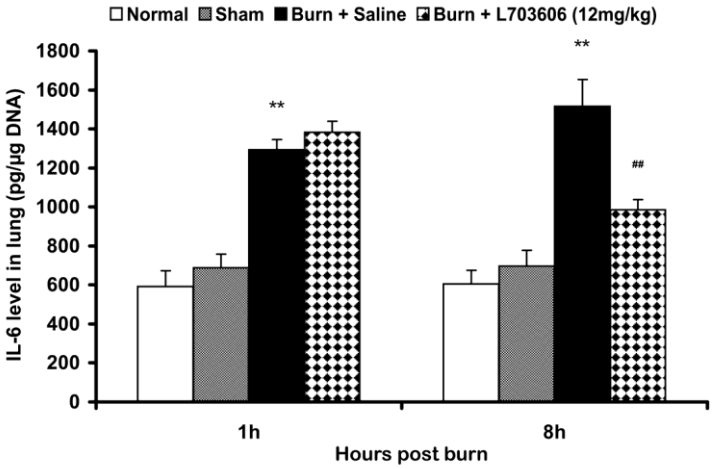
A


Normal
Sham
Burn + Saline
Burn + L703606 (12mg/kg)
IL-1β level in lung (pg/µg DNA)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
##
**
##
1h
8h
Hours post burn

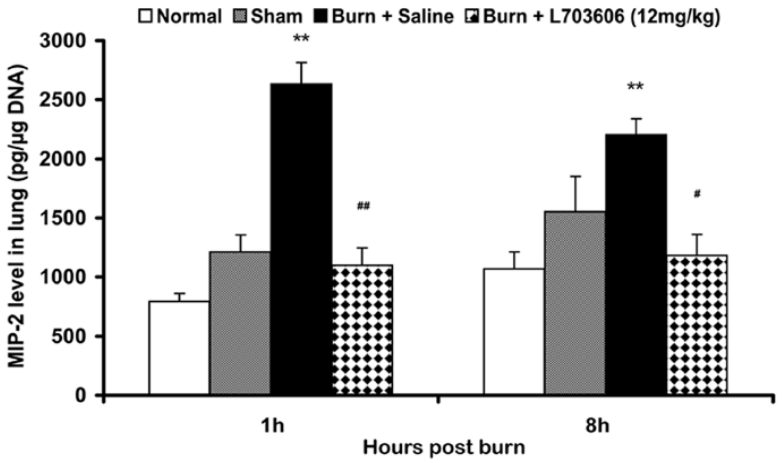
B


Normal
Sham
Burn + Saline
Burn + L703606 (12mg/kg)
TNF-α level in lung (pg/µg DNA)
2500
2000
1500
1000
500
0
*
##
*
##
1h
8h
Hours post burn

C


Normal
Sham
Burn + Saline
Burn + L703606 (12mg/kg)
IL-6 level in lung (pg/µg DNA)
1800
1600
1400
1200
1000
800
600
400
200
0
**
**
##
1h
8h
Hours post burn

D



E

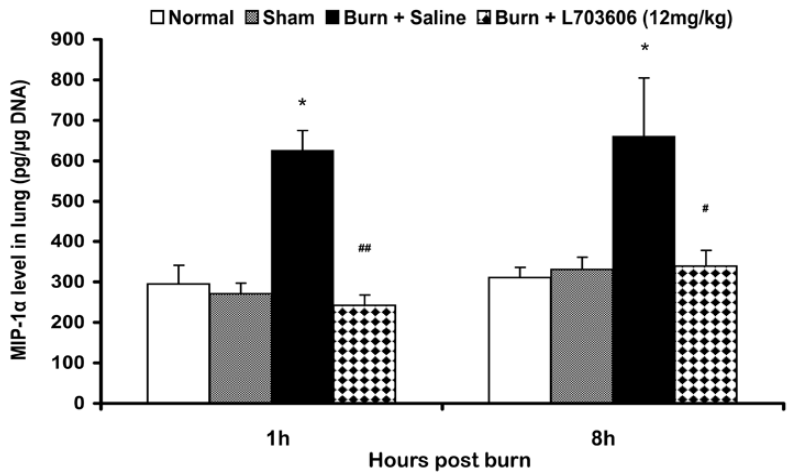
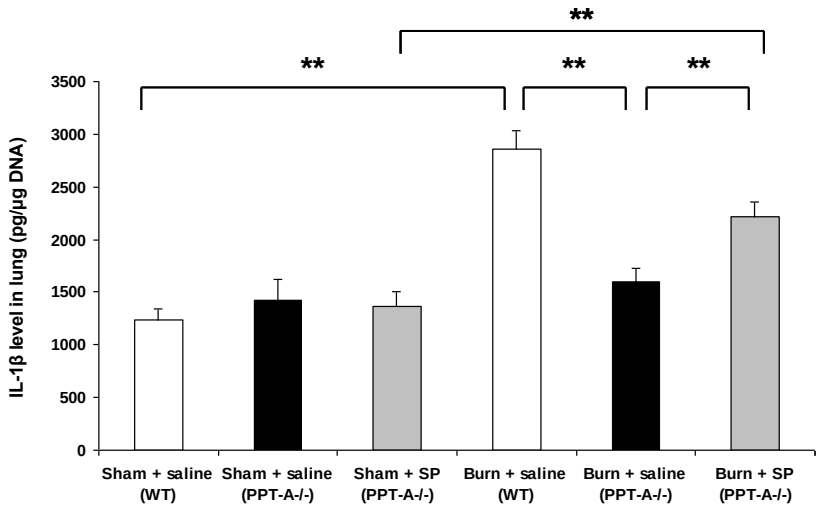
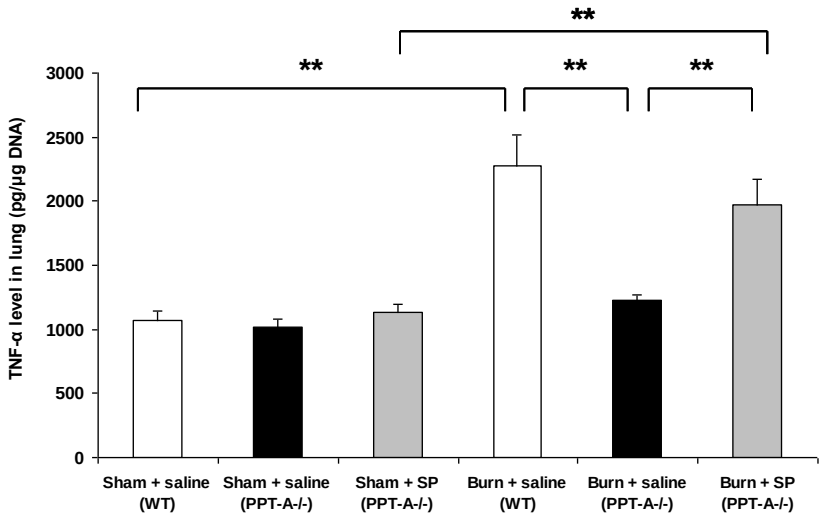


Figure 3.2 Effect of L703606 pretreatment on lung cytokine and chemokine levels in burn-injured mice. Levels of TNF-α (A), IL-1β (B), IL-6 (C), MIP-2 (D) and MIP-1α (E) in lung were measured by ELISA at 1h and 8h post burn. Mice were randomly given L703606 (12mg/kg, i.p.) or vehicle (saline) 1h before burn injury. Sham-burn mice served as controls. Results shown are the mean values ± SEM (n = 6-8 mice/group at each time point). *, $p<0.05$ vs sham; **, $p<0.01$ vs sham; #, $p< 0.05$ vs burn + saline; ##, $p<0.01$ vs burn + saline.

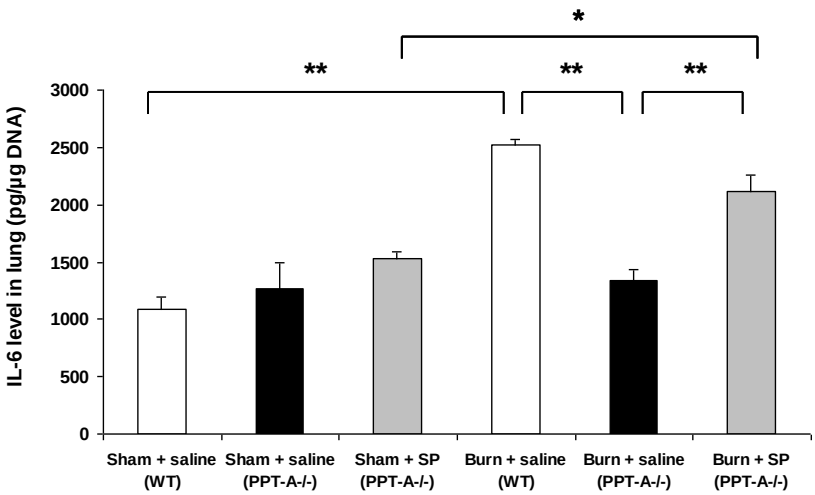
A


**
**
**
**
IL-1β level in lung (pg/µg DNA)
3500
3000
2500
2000
1500
1000
500
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

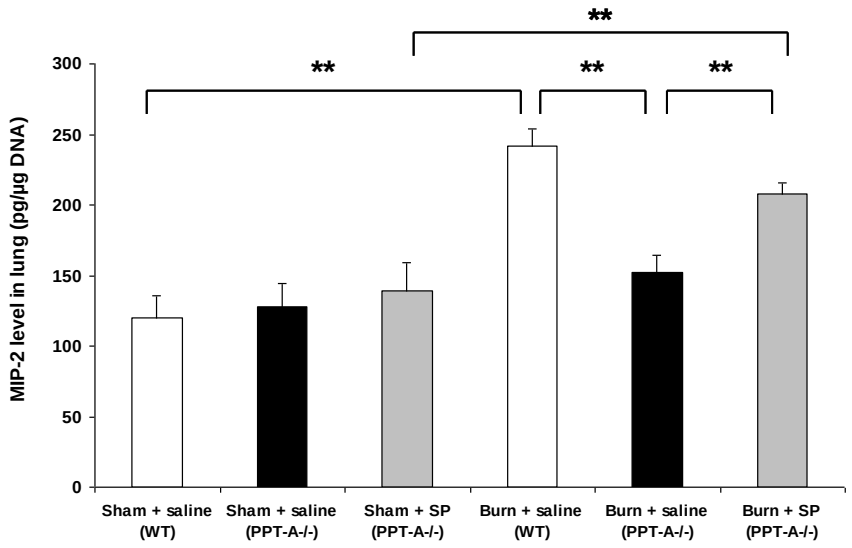
B


**
**
**
**
TNF-α level in lung (pg/µg DNA)
3000
2500
2000
1500
1000
500
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

C


*
**
**
**
IL-6 level in lung (pg/µg DNA)
3000
2500
2000
1500
1000
500
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

D



E

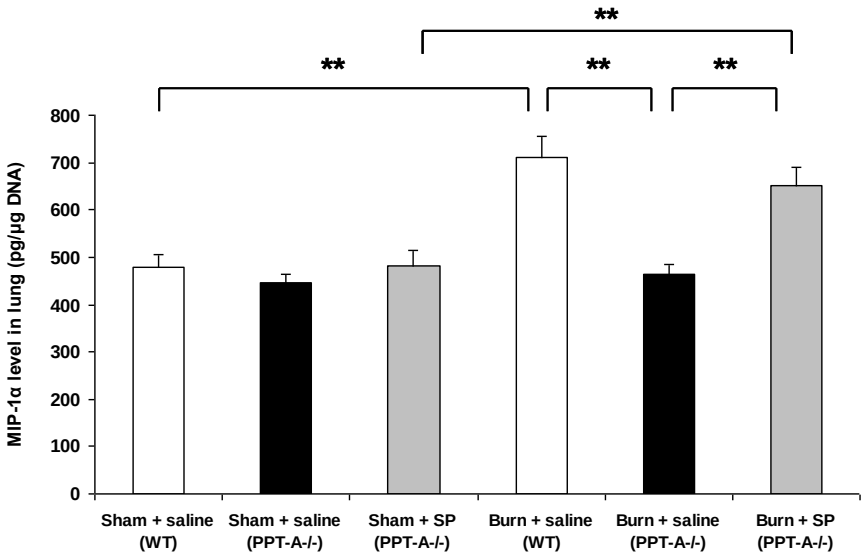
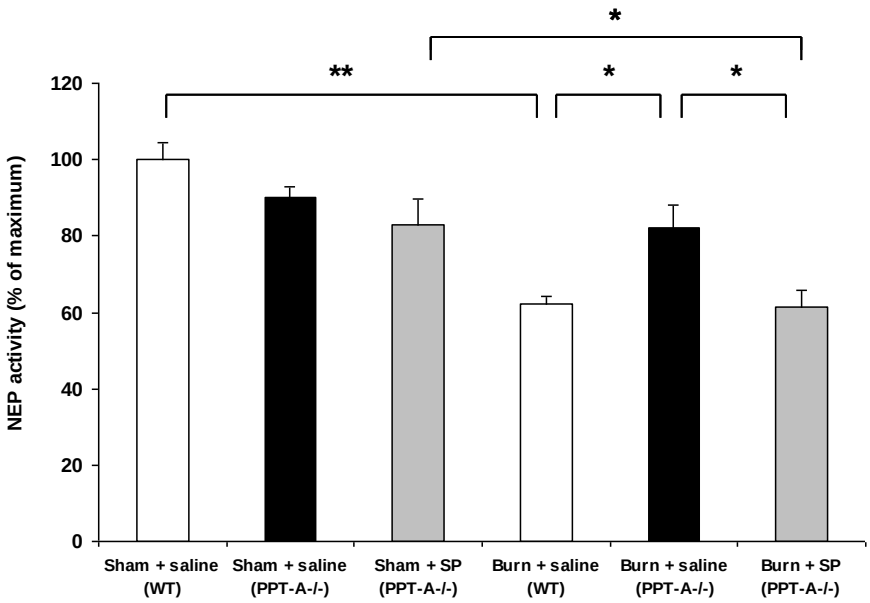


Figure 3.3 Reduced pro-inflammatory cytokines and chemokines in burn-injured *PPT-A*$^{-/-}$ mice. Levels of IL-1β (A), TNF-α (B), IL-6 (C), MIP-2 (D) and MIP-1α (E) in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) 8h after sham or burn injury, were determined by ELISA. Results shown are the mean values ± SEM (n = 6-8 mice per group). *, $p<0.05$; **, $p<0.01$.

A


*
**
*
*
120
100
80
60
40
20
0
NEP activity (% of maximum)
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

B

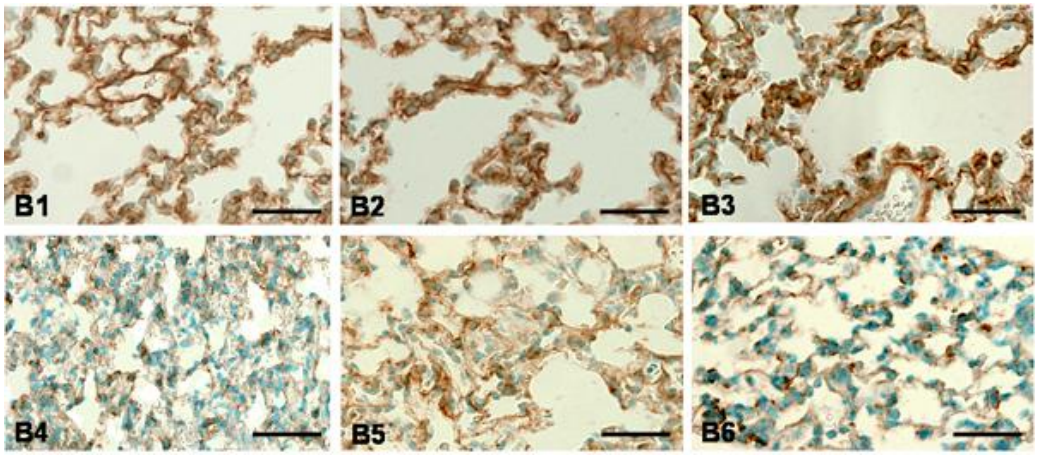

B1
B2
B3
B4
B5
B6

Figure 3.4 NEP activity and expression levels after burn injury. (A) NEP activity at 8h in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. Results shown are the mean values ± SEM (n = 4-7 mice per group). *, $p<0.05$; **, $p<0.01$. (B) Immunohistochemical expression of NEP (appearing as brown color) at 8h in WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. Lung sections shown include: sham-injured WT mice (B1), sham-injured *PPT-A*$^{-/-}$ mice (B2), sham-injured *PPT-A*$^{-/-}$ mice injected with SP (B3), burn-injured WT mice (B4), burn-injured *PPT-A*$^{-/-}$ mice (B5) and burn-injured *PPT-A*$^{-/-}$ mice injected with SP (B6). (n = 6 mice per group). Bars, 50µm.

A

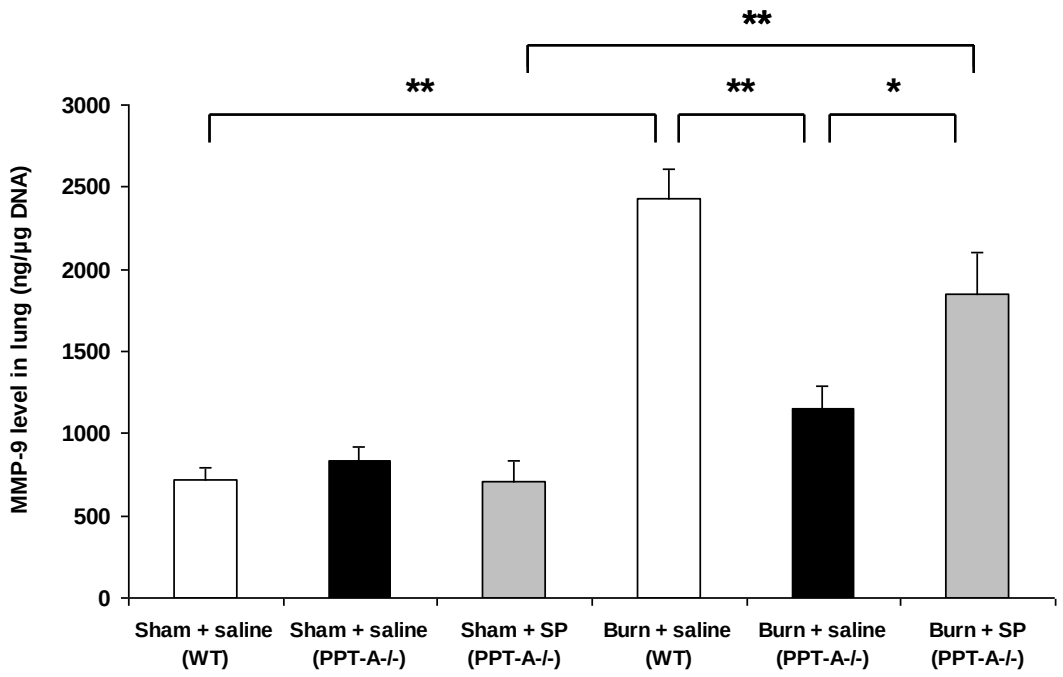


Figure 3.5 MMP-9 expression levels after burn injury. MMP-9 levels at 8h in lungs of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury, were measured by ELISA. Results shown are mean values ± SEM (n = 5-8 mice per group). *, $p<0.05$; **, $p<0.01$.

Chapter IV Effect of SP on inflammatory cells after burn injury

4.1 Introduction

Endothelial cell mediated recruitment and activation of leukocytes is a critical feature of the innate immune system in response to injury, which plays a critical role in host defense and tissue repair (Nathan, 2002; Witko-Sarsat et al., 2000). However, excessive or misdirected leukocyte accumulation in tissue components can lead to an exuberant host inflammatory response as seen in many inflammatory diseases (Luster, 1998). This focused pathology indicates an essential necessity for identifying the role of mediators that instigate effector leukocytes leading to the pathogenesis of inflammatory diseases (Luster, 1998). Thereby, blockade of the excessive accumulation of blood-borne effector cells in tissues should offer a possibility for effective and selective anti-inflammatory therapy (Luster, 1998; Ulbrich et al., 2003; Yonekawa and Harlan, 2005).

SP is known to exert its pro-inflammatory effects via immuno-regulation of numerous important inflammatory cells in the circulation (O'Connor et al., 2004). However, the role of SP and its receptor, NK1R, in regulating leukocyte numbers and recruitment after severe local burn injury remains unclear. As discussed in previous chapters, SP induces significant vascular endothelial permeability after burn injury and up-regulates chemokines, hence, it was imperative that we investigate the role of SP in regulating the circulatory population of leukocyte numbers and its effect on endothelium adhesion molecules expressed following severe local burn injury.

4.2 Materials and methods

4.2.1 Mouse burn injury model

For details, see section 2.2.1, page 48.

4.2.2 White blood corpuscles-differential count (WBC-DC)

Blood was collected by cardiac puncture into EDTA-containing tubes at each time point. Samples were analyzed by Celldyne-3700 (Abbott Diagnostics) for WBC-DCs.

4.2.3 Adhesion molecules analysis

Single-analyte ELISA assays were performed for the measurement of adhesion molecules (P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) in homogenized lung tissue, ELISA kits, all from R&D Systems were used according to the manufacturer's instructions. The lower limits of detection of the levels of P-selectin, E-selectin, ICAM-1, VCAM-1 were 31.25, 31.25, 62.5 and 62.5 pg/ml respectively. The ELISA results were reproducible with interassay variability of <9.5% and intra-assay variability of <6.5%. Results were then corrected for the DNA content of the tissue samples (Labarca and Paigen, 1980) and were expressed as picograms per microgram of DNA.

4.2.4 Statistics

For details, see section 2.2.10, page 55.

4.3 Results

4.3.1 Regulation of leukocyte and platelet cells in the circulatory population by SP-NK1R signaling following severe local burn injury

Previously, our results showed that endogenous levels of SP in plasma were elevated after burn injury and that this SP level was significantly lowered by L703606 (Fig. 2.2B). Therefore, we wanted to investigate the effect of SP-NK1R signaling on inflammatory cells including neutrophils, lymphocytes, monocytes, and as well as platelets in the circulation as this would further verify the role of SP in mediating systemic inflammation after burn injury.

White blood cell, neutrophil, monocyte and lymphocyte absolute numbers along with percentage of neutrophil and monocyte subsets all showed significantly higher numbers in the circulation after burn injury compared to sham-injured mice (Table 4.1 and 4.2). However, administration of L703606 which inhibited SP-NK1R signaling significantly lowered the levels of all these inflammatory cells. Additionally, absolute platelet count was markedly reduced following burn injury, however, treatment with L703606 resulted in significant improvement in blood platelet levels (Fig. 4.1A).

4.3.2 Burn injury significantly increased the expression levels of adhesion molecules in lungs of WT mice, but not in *PPT-A*$^{-/-}$ mice

Migration of leukocytes into tissues relies on a tightly regulated multi-step adhesion signaling cascade involving up-regulation of endothelial selectins and immunoglobulin 'super-family' members (Luster, 1998). A significant increase in P-selectin and E-selectin (Fig. 4.2A and B) (important for efficient tethering and reducing rolling velocities), intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 levels (Fig. 4.2C and D) (important for arrest and diapedesis), was observed in lungs of burn-injured WT mice compared to sham-injured WT mice. Deletion of *PPT-A* gene significantly reversed the up-regulation of these adhesion molecules after burn injury; while administration of exogenous SP restored back their levels (Fig. 4.2A to D).

4.4 Discussion

Our findings demonstrated the effect of SP-NK1R signaling on the circulatory population of immuno-inflammatory cells following severe local burn injury. Neutrophils, monocytes, lymphocytes and total white blood cell count were all significantly increased at 1h and 8h after burn injury while these effects was inhibited upon administration of L703606. Additionally, the trend of endogenous SP concentration in plasma at both 1h and 8h exhibited a parallel correlation (Fig. 2.1A and B); as SP was elevated in plasma following burn injury, there was a concurrent rise in neutrophils, monocytes,

lymphocytes and total white blood cell count. Similarly, upon inhibition of NK1R by L703606, SP levels in plasma were markedly lowered and inflammatory cell numbers were decreased in the circulation.

Studies have shown that SP modulates neuro-immune effects by numerous signaling molecules such as cytokines (Cuesta et al., 2002), chemokines (Yokoyama et al., 1993), reactive oxygen species (Serra et al., 1988) and other mediators (O'Connor et al., 2004). The mechanism behind the production of these mediators occurs through hyper-activation of immuno-inflammatory cells through excessive signaling of SP to the NK1 receptors which in times of normal physiological conditions are usually not involved in the cellular response to SP (O'Connor et al., 2004). Additionally, results from *in vitro* studies of hematopoiesis further substantiate that SP can hyper-stimulate inflammatory cells. For example, SP has been shown to induce bone marrow progenitors of both erythroid and myeloid lineages (Rameshwar et al., 1993) which particularly include immune cell population subsets such as neutrophils (Iwamoto et al., 1993), eosinophils (Iwamoto et al., 1993), lymphocytes (Guo et al., 2002), monocytes (Ho et al., 1997), macrophages (Ho et al., 1997) and mast cells (Ansel et al., 1993) which all express NK1R on the surface of their cells. Hence, in line with these studies, our results suggests that after burn injury, significant increases in SP-NK1R complex signaling on numerous immuno-inflammatory cells are rapidly stimulated to produce a cascade of pro-inflammatory mediators which proceed to intensify ALI.

Interestingly, the effect on platelet cells were the opposite, wherein a decline in circulating platelets following burn injury was observed but improved to a large extent after disruption of SP-NK1R signaling. Studies have demonstrated that platelets have the ability store SP and express NK1R (Graham et al., 2004). Furthermore, stimulation of platelets isolated from healthy mice by tachykinins resulted in platelet aggregation, while inhibition of the NK1R significantly lowered thrombus formation (Jones and Gibbins, 2008). In a study, platelet numbers were found to decrease following burn injury (Fujimi et al., 2006). In our model of burn injury, our results demonstrate a similar trend of a significant reduction in circulating platelets. Additionally, we observed that the platelet numbers improved profoundly upon blockade of NK1R by L703606 at 1h and 8h following burn injury. Published clinical outcomes report that patients with severe burns who experience episodes of thrombocytopenia have a higher association of increased mortality (George et al., 2001; Takashima, 1997). However, the molecular mechanisms behind the reduction in platelet numbers after severe trauma remains unknown (Fujimi et al., 2006). Nonetheless, our data suggests that SP may have a role in leading to a decrease of platelet numbers and in modulating different cell types after severe local burn injury.

Our experiments also revealed an up-regulation of lung endothelial adhesion molecules, P-selectin, E-selectin, ICAM-1 and VCAM-1, in burn-injured WT mice. These effects were directly reversed in burn-injured *PPT-A*$^{-/-}$ mice; and when SP was administered to burn-injured *PPT-A*$^{-/-}$ mice, expression of these adhesion molecules were heightened again. Interestingly, it is important to note that although selectins are critical for initiating the rolling of inflammatory cells in the systemic vasculature; however, their role for

transmigration of these cells in lung inflammation is less clear, and studies have shown that it depends on the inflammatory stimulus. For example, LPS-induced migration of leukocytes into the alveolar space was not prevented by using antagonists against E-, P-, and L-selectin (Burns et al., 2001). Furthermore, neutrophil translocation into the lung was not affected when E-selectin and P-selectin gene expressions were deleted in mice and when L-selectin function was inhibited in a lung inflammatory model induced by *Streptococcus pneumoniae* (Mizgerd et al., 1996). In contrast, all 3 selectins have been shown to contribution to lung injury induced by complement or intratracheal deposition of IgG complexes (Mulligan et al., 1996), or bacterial LPS (Hayashi et al., 1999). Hence, these studies suggest that the involvement of selectins in lung inflammation may be stimulus-dependent (Reutershan and Ley, 2004). Thus, in the case of burn-induced ALI, we show that burn injury increases P- and E selectin, which is reduced in *PPT-A*$^{-/-}$ mice, suggesting that SP may play a role in modulating their expression after burn.

Therefore, taken together, our findings suggests that excessive production of SP instigates not only concurrent increased levels of cellular pro-inflammatory cytokines, and chemokines, which shows the active role of SP-induced chemoattractant production in mediating trafficking of increased inflammatory cells in the circulation, but as well as disruption of endothelial adhesion molecules. By doing so, SP thereby ascertains the extravasation, migration and subsequent accumulation of neutrophils in lungs after burn injury. Therefore, it is evident that SP is a critical player involved in many key events of the systemic inflammatory cascade following burn injury.

Table 4.1 Hematologic analysis of whole blood samples from sham- and burn-injured mice [a]

Animal group, by time following injury	Absolute cell numbers, x 10^3/µl, mean ± SEM			
	White blood cells	Neutrophils	Monocytes	Lymphocytes
1h post-burn				
Normal	1.27 ± 0.10	0.61 ± 0.03	0.02 ± 0.01	0.67 ± 0.07
Sham	1.33 ± 0.09	0.63 ± 0.04	0.02 ± 0.01	0.68 ± 0.08
Burn + Saline	2.71 ± 0.41[b]	1.16 ± 0.10[b]	0.07 ± 0.02[b]	1.68 ± 0.26[b]
Burn + L703606 (12mg/kg)	1.56 ± 0.12[c]	0.71 ± 0.05[c]	0.03 ± 0.01[d]	0.83 ± 0.09[c]
8h post-burn				
Normal	1.29 ± 0.09	0.60 ± 0.04	0.02 ± 0.01	0.67 ± 0.07
Sham	1.35 ± 0.10	0.59 ± 0.04	0.02 ± 0.01	0.62 ± 0.06
Burn + Saline	4.20 ± 0.21[b]	2.57 ± 0.18[b]	0.05 ± 0.01[b]	1.72 ± 0.20[b]
Burn + L703606 (12mg/kg)	3.22 ± 0.13[c]	1.69 ± 0.16[c]	0.03 ± 0.02[d]	0.59 ± 0.09[c]

[a] Anti-coagulated whole blood at 1h and 8h post-burn was analyzed for absolute cell numbers. Mice were randomly given L703606 (12mg/kg, i.p.) or vehicle (saline) 1h before burn. n = 8-12 mice per group per time point. Results shown are the mean values ± SEM.

[b] $p<0.01$, Burn + saline vs sham

[c] $p<0.01$, Burn + saline vs Burn + L703606 (12mg/kg)

[d] $p<0.05$, Burn + saline vs Burn + L703606 (12mg/kg)

Table 4.2 Percentage of leukocyte subsets in circulating blood from sham- and burn-injured mice[a]

Animal group, by time following injury	Percent leukocyte subset, x 10^3/µl, mean ± SEM		
	Neutrophils	Monocytes	Lymphocytes
1h post-burn			
Normal	23.44 ± 1.05	0.96 ± 0.17	57.92 ± 3.26
Sham	28.51 ± 2.48	0.91 ± 0.25	62.00 ± 3.65
Burn + Saline	56.15 ± 1.32[b]	2.15 ± 0.33[c]	39.91 ± 3.17[b]
Burn + L703606 (12mg/kg)	41.15 ± 2.11[d]	1.28 ± 0.24[e]	51.45 ± 2.44[e]
8h post-burn			
Normal	22.97 ± 0.97	0.87 ± 0.21	63.57 ± 2.80
Sham	26.48 ± 2.02	0.92 ± 0.17	67.12 ± 2.79
Burn + Saline	77.56 ± 5.31[b]	2.13 ± 0.42[b]	23.75 ± 6.72[b]
Burn + L703606 (12mg/kg)	61.12 ± 4.27[e]	1.10 ± 0.43[d]	30.24 ± 4.00

[a] Anti-coagulated whole blood at 1h and 8h post-burn was analyzed for percent neutrophils, monocytes and lymphocytes. Mice were randomly given L703606 (12mg/kg, i.p.) or vehicle (saline) 1h before burn. n = 8-12 mice per group per time point. Results shown are the mean values ± SEM.

[b] $p<0.01$, Burn + saline vs sham

[c] $p<0.05$, Burn + saline vs sham

[d] $p<0.001$, Burn + saline vs Burn + L703606 (12mg/kg)

[e] $p<0.05$, Burn + saline vs Burn + L703606 (12mg/kg)

A

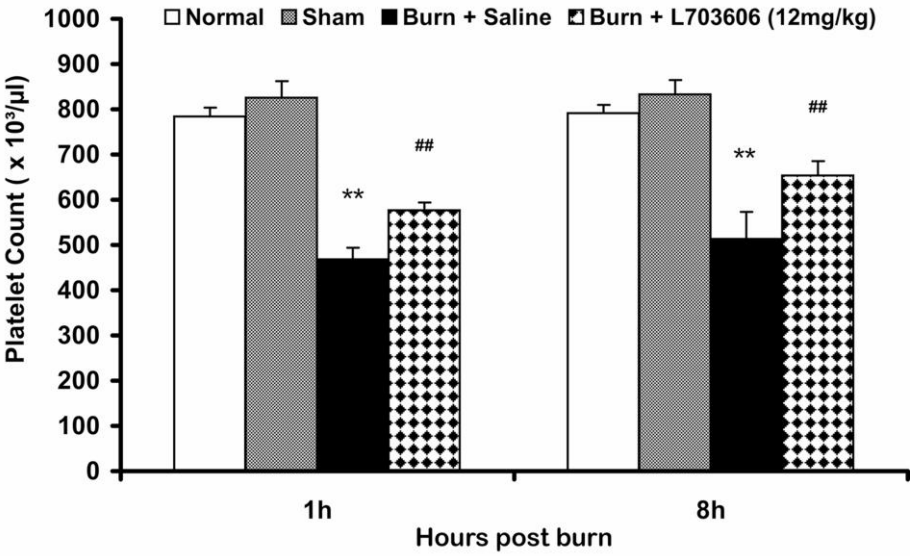
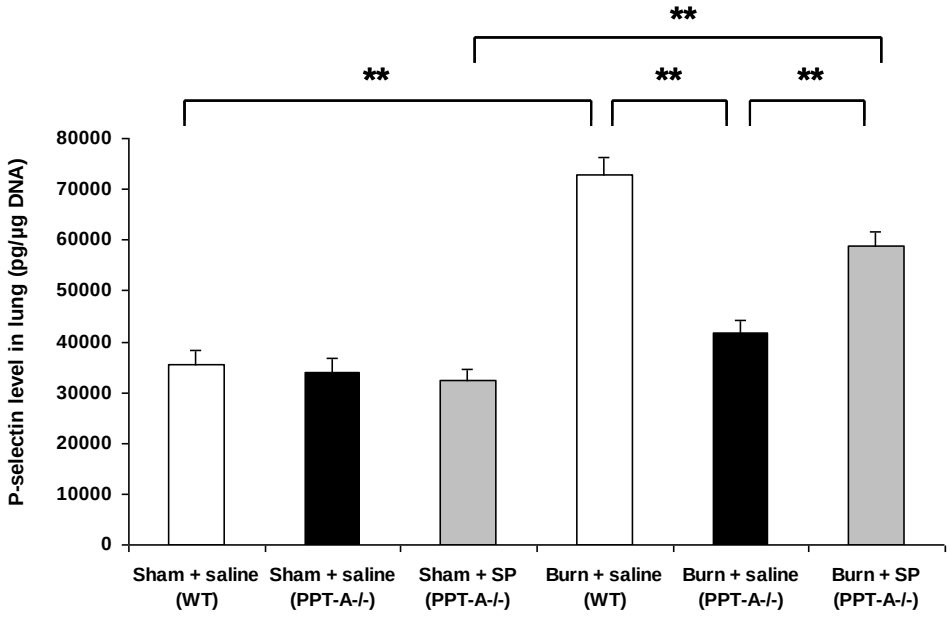
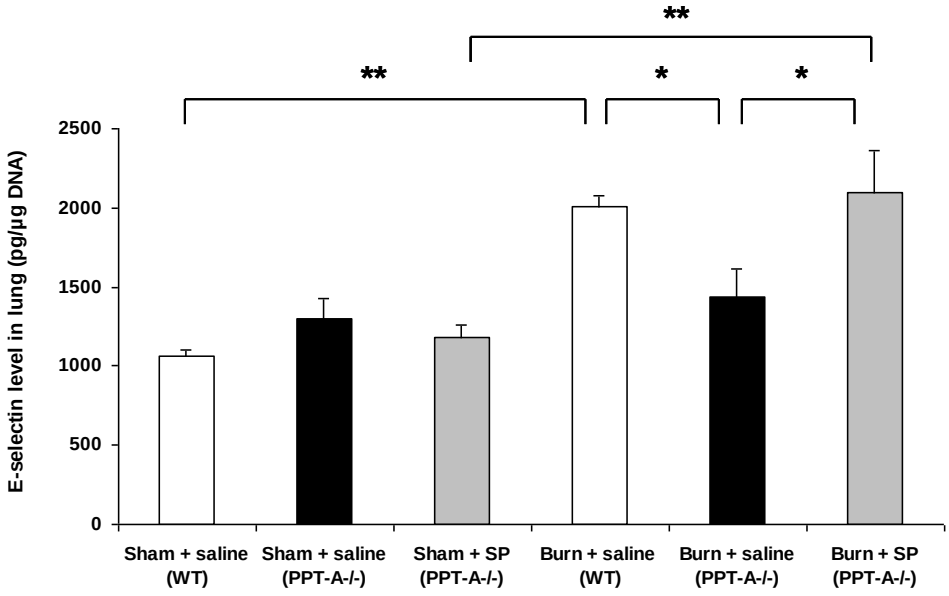


Figure 4.1. Role of SP-NK1R signaling on platelet count following burn injury. Platelet counts were determined at 1h and 8h post burn. Mice were randomly given L703606 (12mg/kg, i.p.) or vehicle (saline) 1h before burn injury. Sham burn mice served as controls. Results shown are the mean values ± SEM (n = 8-12 mice/group at each time point). **, $p<0.01$ vs sham; ##, $p<0.01$ vs burn + saline.

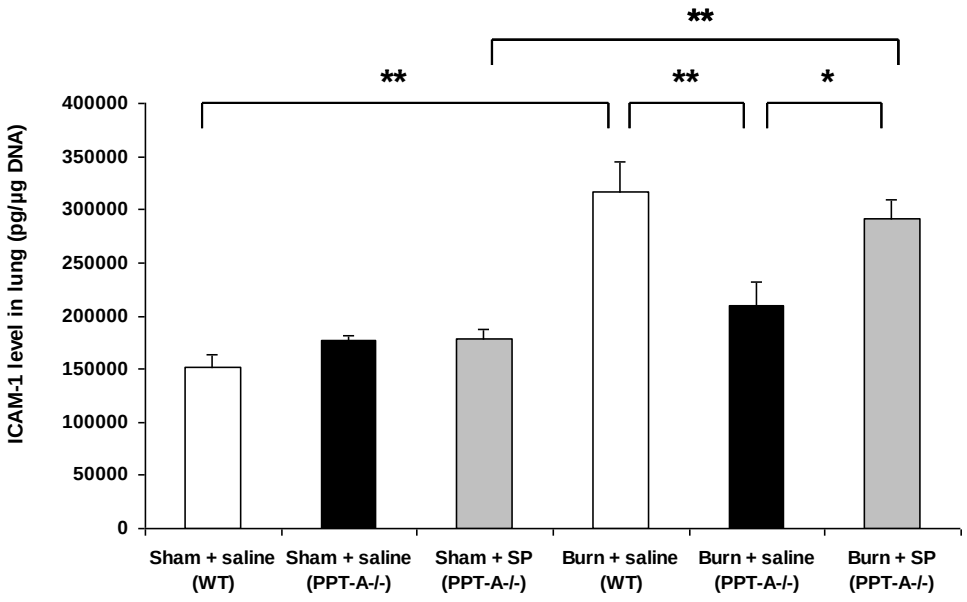
A


**
**
**
**
P-selectin level in lung (pg/µg DNA)
80000
70000
60000
50000
40000
30000
20000
10000
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

B


**
**
*
*
E-selectin level in lung (pg/µg DNA)
2500
2000
1500
1000
500
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

C


**
**
**
*
ICAM-1 level in lung (pg/µg DNA)
400000
350000
300000
250000
200000
150000
100000
50000
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

D

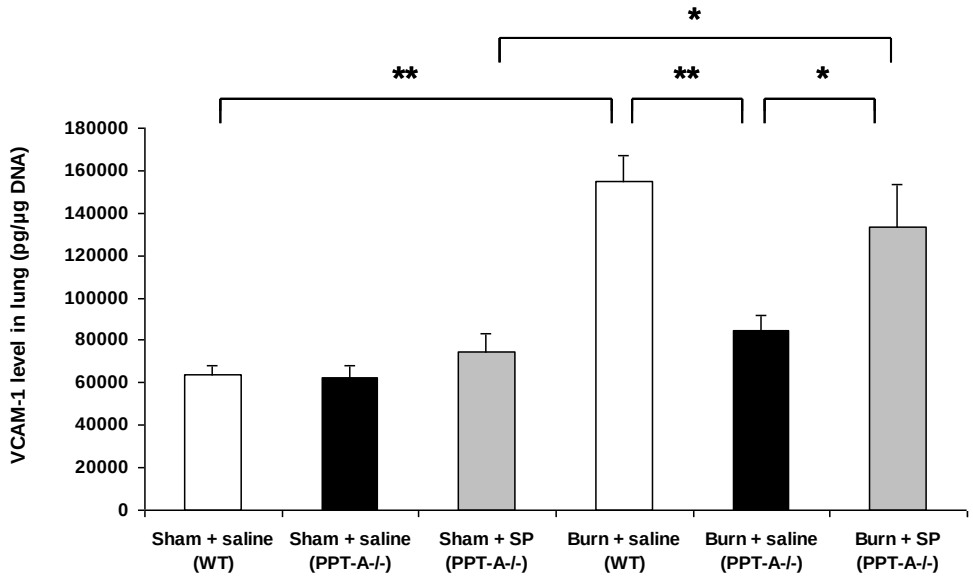

*
**
**
*
VCAM-1 level in lung (pg/µg DNA)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

Figure 4.2 Effect of *PPT-A* gene deletion on expression of adhesion molecules after burn injury. Levels of P-selectin (A), E-selectin (B), ICAM-1 (C) and VCAM-1 (D) in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12μg/kg, i.v.) or vehicle (saline) 8h after sham or burn injury, were measured by ELISA. Results shown are the mean values ± SEM (n = 6-8 mice per group). *, $p<0.05$; **, $p<0.01$.

Chapter V Effect of SP on respiratory function after burn injury

5.1 Introduction

Breathing is an extraordinarily steadfast and consistent behavior in vertebrates that regulates blood gas exchange, pH, temperature and diverse activities including sleep, physical exercise, pregnancy and ageing (Feldman and Del Negro, 2006). Impairment of the pulmonary and cardiovascular systems are the major causes of breathing disorders; however, it is important to note that abnormal functioning of the neutral control over breathing also has a major impact in respiratory diseases (Feldman and Del Negro, 2006). For example, mortality due to central respiratory arrest while sleeping is frequently encountered in patients with Parkinson's disease, multiple systems atrophy, amyotrophic lateral sclerosis and in the elderly (Feldman and Del Negro, 2006).

Studies have reported evidences on how the nervous system could influence respiratory function, of which, one connection is through neuropeptides. Several neuropeptides including SP (Chen et al., 1990; Chen et al., 1988; Gray et al., 2001), somatosatin (Kalia et al., 1984), thyrotropin-releasing hormone (McCown et al., 1986) and bombesin (Hedner et al., 1985) were shown to have significant effects on modulating breathing function when locally applied in the brain stem region. However, none have investigated the effect of endogenous SP on lung function following severe local burn injury. Furthermore, as discussed in previous chapters, SP was observed to significantly induce

remote ALI after burn, thus, for this reason, we were interested to investigate whether SP is concurrently involved in affecting respiratory function after burn injury.

5.2 Materials and methods

5.2.1 Mouse burn injury model

For details, see section 2.2.1, page 48.

5.2.2 Measurement of lung function

Eight and twenty-four hours after burn injury, in vivo respiratory patterns in unrestrained, conscious mice using a whole-body plethysmography system were measured repeatedly (Buxco Electronics, Troy, NY) (DeLorme and Moss, 2002). The whole-body plethysmograph chamber utilizes a barometric analysis technique that compares the pressure difference between the animal chamber and a reference chamber to measure airway physiological parameters. Resulting changes in pressure/volume readings in inspiration and expiration reflect the pulmonary function of the animal. After chamber calibration to ambient pressures and temperature, signals were recorded, and various respiratory variables were calculated using the manufacturer software. Before recording, mice were placed individually into the main chamber and allowed to acclimate for 5 mins. Mean respiratory variables were then calculated from 15 mins recordings of continuous

respiratory behavior (Gray et al., 2001). *In vivo* parameters obtained include: V_e (ml/min), RR (breaths/min), V_T (ml), PEF (ml/s), PIF (ml/s), T_i (s) and T_e (s).

5.2.3 Measurement of SP, MPO activity and histological examination

For details, see sections 2.22, 2.23 and 2.25 for SP measurement, MPO activity, and histological analysis respectively, page 50 to 52.

5.2.4 Statistics

For details, see section 2.2.10, page 55.

5.3 Results

5.3.1 Progressive improvement of lung function in burn-injured mice lacking *PPT-A* gene products over 8h and 24h

Mammalian respiratory rhythm generation is controlled critically by central neural modulation (Feldman and Del Negro, 2006). Throughout the central nervous system, SP is widely distributed, however, more importantly; the presence of SP has been shown to be intensified particularly in areas of the body involved in the control of autonomic function, such as respiration (Bongianni et al., 2008). Not surprisingly, the excitatory effects of SP on respiratory activity have been documented (Chen et al., 1990; Gray et al.,

2001; Marano et al., 1988). Therefore, we were interested to determine the effect of *PPT-A* gene deletion in unrestrained conscious mice and subsequent injection with exogenous SP on breathing rhythm after burn injury over an extended time at 8h to 24h, using whole body plethysmography.

Burn injury augmented significant reductions in minute ventilation (V_E) owing to concomitant decreases in respiratory rate (RR) and tidal volume (V_T) in WT mice compared to sham-injured mice at 8h and 24h (Fig. 5.1A to C). V_E is defined as the volume of gas that moves in and out of the lungs in one minute and is calculated by multiplying V_T and RR (Guyton and Hall, 2000). Deletion of *PPT-A* gene in burn-injured mice significantly elevated RR at both 8h and 24h; however, this did not increase V_T at 8h but only at 24h compared to burn-injured WT mice (Fig. 5.1B and C). Without the maintenance of V_T, although there was a rise in RR at 8h, burn-injured *PPT-A*$^{-/-}$ mice did not show improvement in V_E at 8h. However, by 24h time, V_T levels begin to increase significantly compared to sham-injured *PPT-A*$^{-/-}$ mice along with RR, and as such, V_E improved significantly back to levels similar to sham *PPT-A*$^{-/-}$ mice (Fig. 5.1A to C). Injection of exogenous SP to burn-injured *PPT-A*$^{-/-}$ mice, significantly blunted RR, V_T and V_E compared to sham-injured *PPT-A*$^{-/-}$ mice administered SP at 8h and 24h; however, when compared to burn-injured *PPT-A*$^{-/-}$ mice alone, burn-injured *PPT-A*$^{-/-}$ mice injected with SP showed only improved RR at 8h, and by 24h, all three respiratory responses, RR, V_T and V_E had significant improvements.

Measurements of peak expiratory flow (PEF) are of value in identifying air flow limitations (Quanjer et al., 1997). Diseases that narrow the airways will have a more profound effect on limiting the PEF and lengthening the time required for exhalation (Wyka, 2002). After burn injury, WT mice exhibited a significant drop in PEF and lengthening of expiratory time (T_e) compared to sham-injured WT mice (Fig. 5.1D and E). Concurrently, a similar decrease in peak inspiratory flow (PIF) and increase in inspiratory time (T_i) was seen (Fig. 5.1F and G). Deletion of *PPT-A* gene resulted in a reduced T_e and T_i at both 8h and 24h after burn (Fig. 5.1E and G). A reversal was seen at both time points, where burned-injured *PPT-A*$^{-/-}$mice injected with SP had lengthened T_e and T_i compared to burned-injured *PPT-A*$^{-/-}$ mice alone and sham-injured *PPT-A*$^{-/-}$ mice treated with SP. PEF and PIF levels in burn-injured *PPT-A*$^{-/-}$ mice were not increased at 8h but rose up significantly at 24h compared to burned-injured WT mice (Fig. 5.1D and F); injection of SP to burn-injured *PPT-A*$^{-/-}$ mice reversed the response at 24h, where PEF and PIF were significantly diminished, close to burn-injured WT mice levels.

5.3.2 Significant disruption of lung function correlated with exacerbated ALI and SP elevation at 24 hours

The changes in lung function at 24h after burn had instigated us to confirm if there was a direct and opposite concurrent lung damage in these same mice, similar to the effects seen at 8h. MPO activity and lung histology revealed increased neutrophil infiltration and damage in burn-injured WT mice; these effects were significantly diminished in burned *PPT-A*$^{-/-}$ mice; while burn-injured *PPT-A*$^{-/-}$ mice injected with SP again showed a shift

back towards lung damage (Fig. 5.2A and B). Levels of SP in lung and plasma were also elevated at 24h after burn in WT mice (Fig. 5.2C and D).

5.4 Discussion

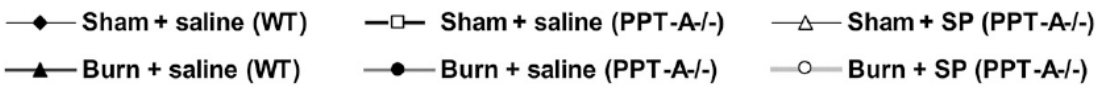
Pulmonary complications in burn patients are well reported, and frequently lead to the development of acute lung injury or lung failure, even when the lungs have not sustained direct burn injury (Kramer et al., 2002; Turnage et al., 2002). Therefore, it was imperative that we test the hypothesis of whether SP affects lung function in mice after burn injury using whole body plethysmography. Our data shows that concurrent with increased lung damage and elevated SP levels at 8h and 24h post-burn, lung function was significantly affected in all seven respiratory parameters; V_E, V_T, RR, PEF, PIF, T_e and T_i; after burn injury in WT mice. Studies have shown that intraventricular injections of SP caused marked increases in RR and V_T in cats and rabbits (Von Euler US and Gaddum JH, 1931). Intracerebroventricular injections of SP and microinjections of SP into the ventrolateral medulla also augmented minute ventilation owing to increases in V_T in rats (Chen et al., 1990; Hedner et al., 1984). In our burn injury model, ablation of SP by genetic deletion of *PPT-A* gene, showed a rescue in V_E, V_T and RR, closer to sham-injured mice levels by 24h time; while re-introduction of SP by i.v. administration in the tail vein of *PPT-A*$^{-/-}$ mice immediately after burn, reversed these effects.

Inspiration is an active procedure that requires work done by contraction of the diaphragm and respiratory muscles, which relies directly on the ability to expand the lungs against elastic recoil, air flow resistance and tissue resistance of lung and thorax

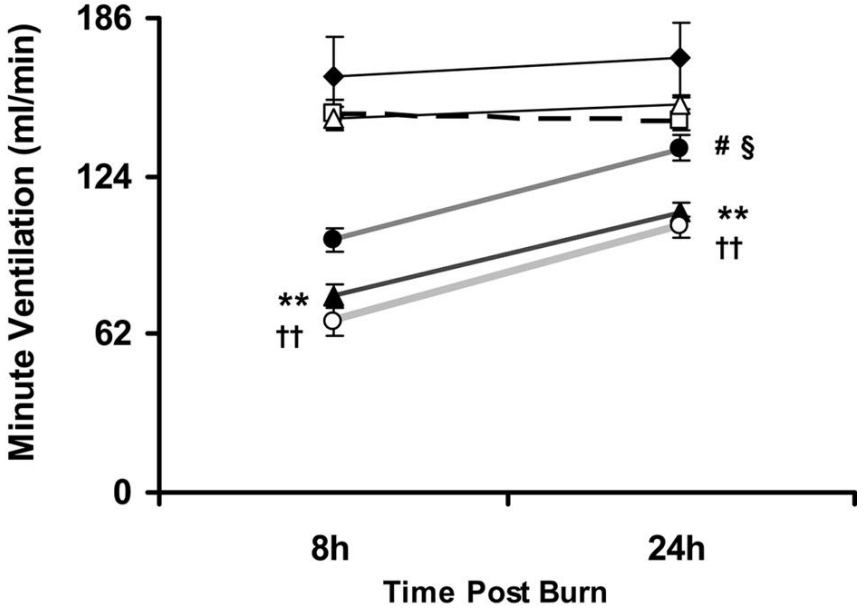
(Guyton and Hall, 2000). Expiration in healthy lungs is exclusively a passive procedure, and occurs in resting state, it is mediated by relaxation of the diaphragm and elastic recoil of lung and thorax, however, in the event of respiratory distress is actively maintained by respiratory muscles (Guyton and Hall, 2000). Exertion of respiratory muscles during exhalation becomes apparent when parameters such as PEF are decreased and T_e is lengthened (Guyton and Hall, 2000; Wyka, 2002). Burn-injured mice exhibited such an effect at 8 and 24h post-burn; *PPT-A*$^{-/-}$ mice displayed a rescue by 24h time; while levels were again disrupted upon SP challenge. PIF and T_i also demonstrated similar effects; thus, these data indicate that large inspiratory efforts had to be done by the lungs after burn injury to compensate for a higher need of oxygen followed by prolonged expiration.

The influence of SP in respiratory function extends beyond the lungs. It is established that the rhythm underlying breathing depends critically on neurons in the preBötzinger complex (preBötC), a region of the medullary oblongata proposed to contain the kernel of networks for rhythm generation (Smith et al., 1991). Interestingly, it was found that neurons in the preBötC express intense NK1R immuno-reactivity (Gray et al., 2001). Studies show that the stimulatory effect of microinjections of SP into preBötC, resulted in modulation of respiratory rhythm (Chen et al., 1990). While others showed that regional ablation of preBötC NK1R expressing neuron, severely disrupted breathing pattern in conscious rats, using whole body plethysmography (Gray et al., 2001). Similar results were also confirmed in larger animals such as goats (Wenninger et al., 2004). Clearly, these studies indicate that SP and its receptor are important for maintenance of normal breathing. Therefore, our results, just as these studies have shown, point to an obvious SP mediated neural-respiratory communications after burn injury. Our data demonstrate that

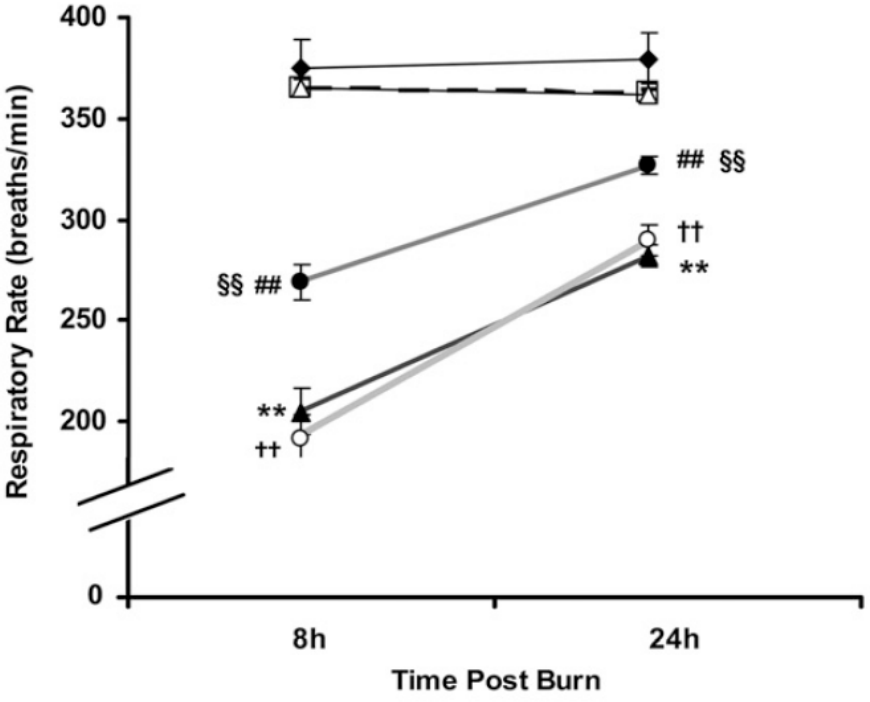
along with excessive production of SP in the lungs and plasma, lung function is significantly disrupted after burn injury in mice and therefore shows its importance in affecting multiple steps of SP-induced pulmonary inflammation.

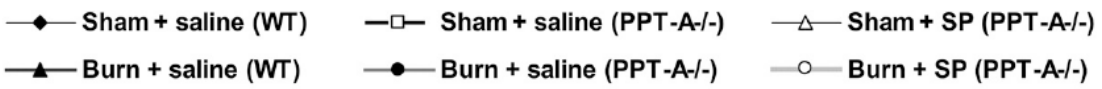

Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

A

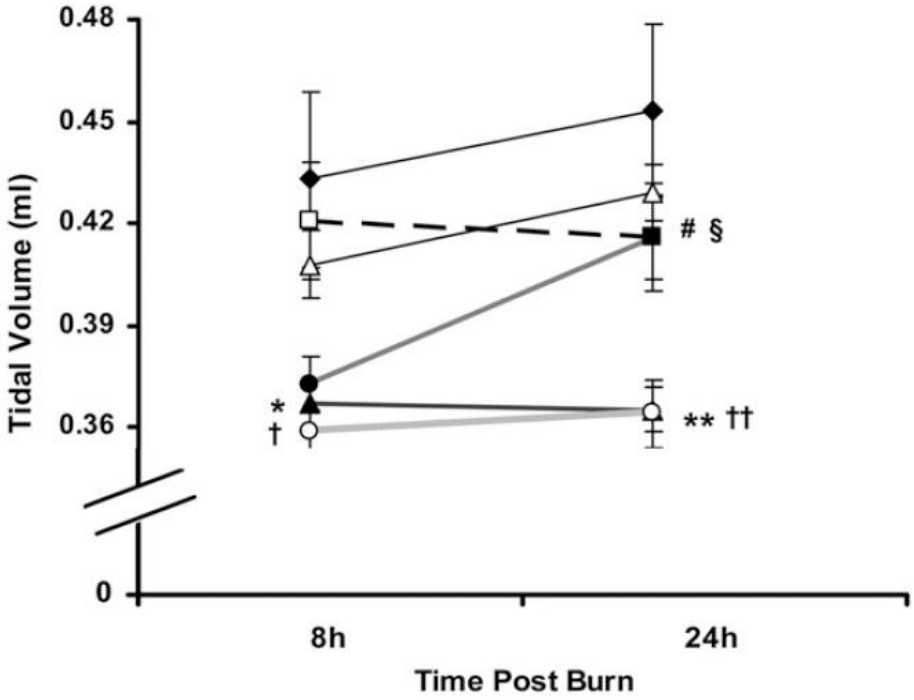

Minute Ventilation (ml/min)
186
124
62
0
§
**
††
**
††
8h
24h
Time Post Burn

B

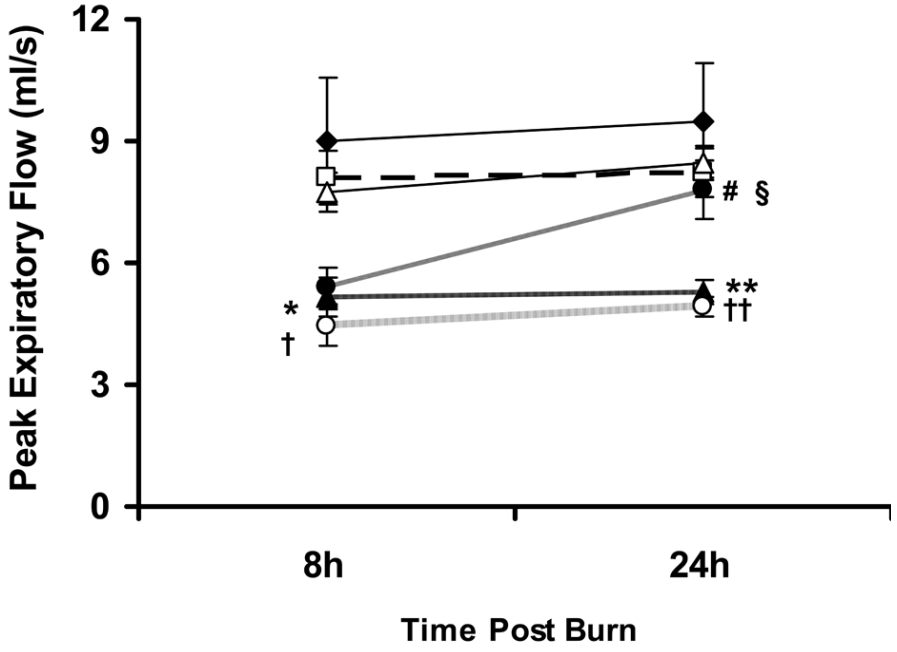

Respiratory Rate (breaths/min)
400
350
300
250
200
0
§§
††
**
§§ ##
**
††
8h
24h
Time Post Burn

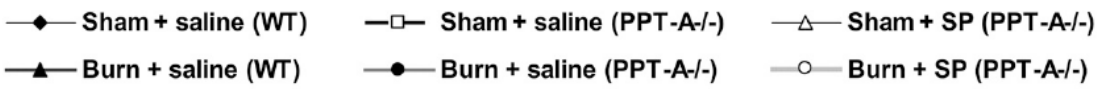

Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

C

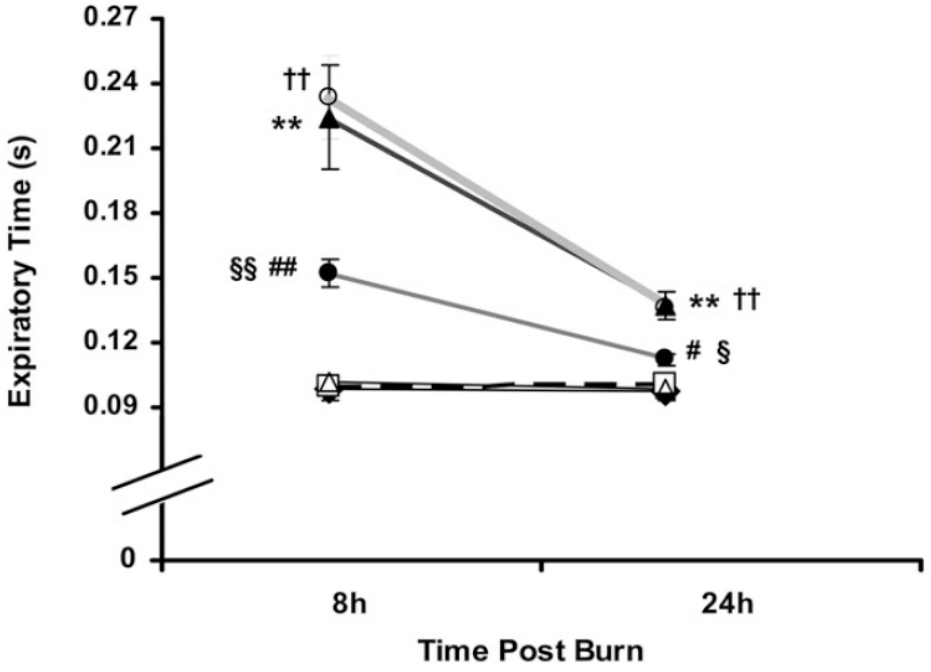

0.48
0.45
0.42
0.39
0.36
0
Tidal Volume (ml)
§
*
†
** ††
8h
24h
Time Post Burn

D

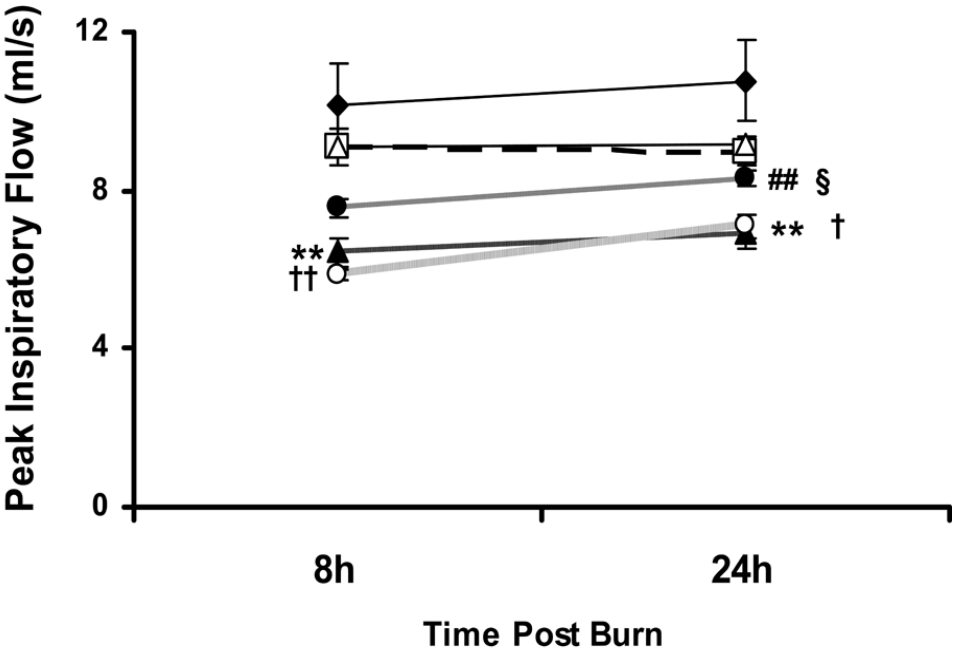

12
9
6
3
0
Peak Expiratory Flow (ml/s)
§
*
†
**
††
8h
24h
Time Post Burn

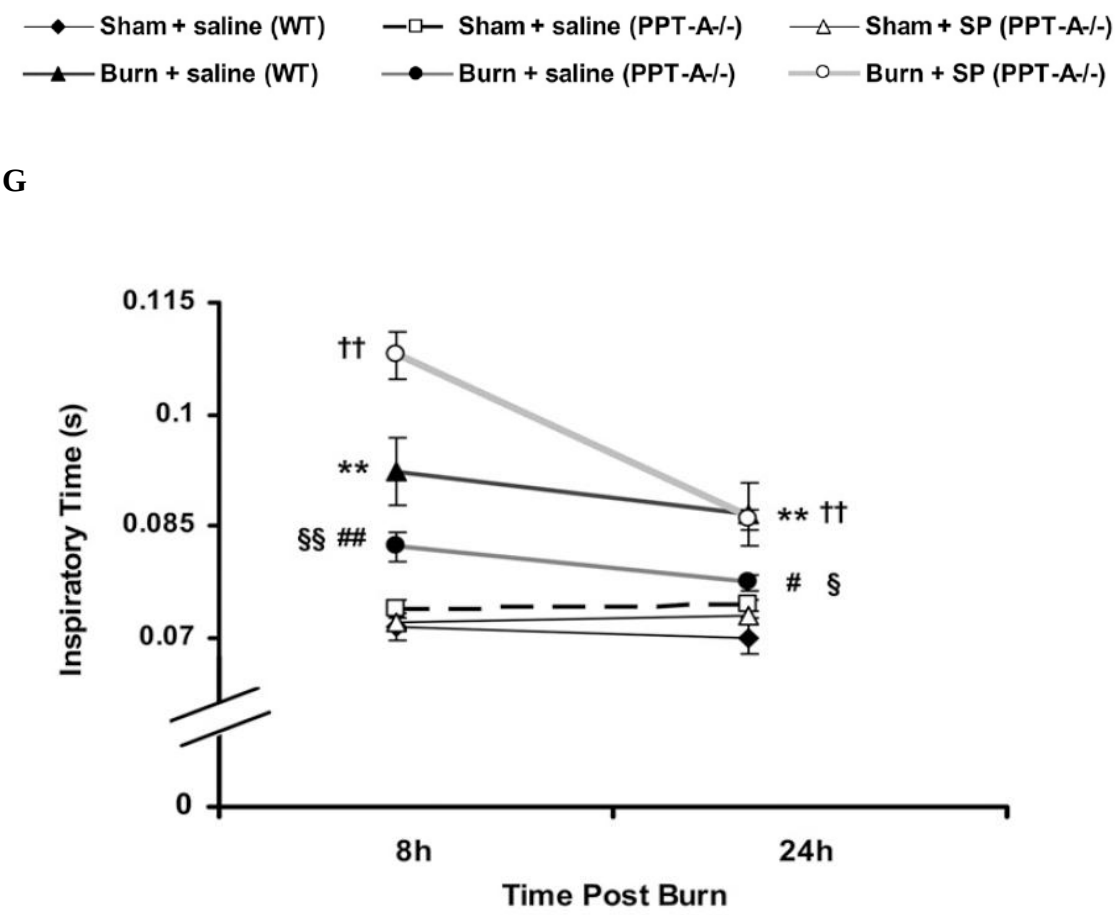

Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

E


Expiratory Time (s)
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0
††
**
§§ ##
** ††
# §
8h
24h
Time Post Burn

F

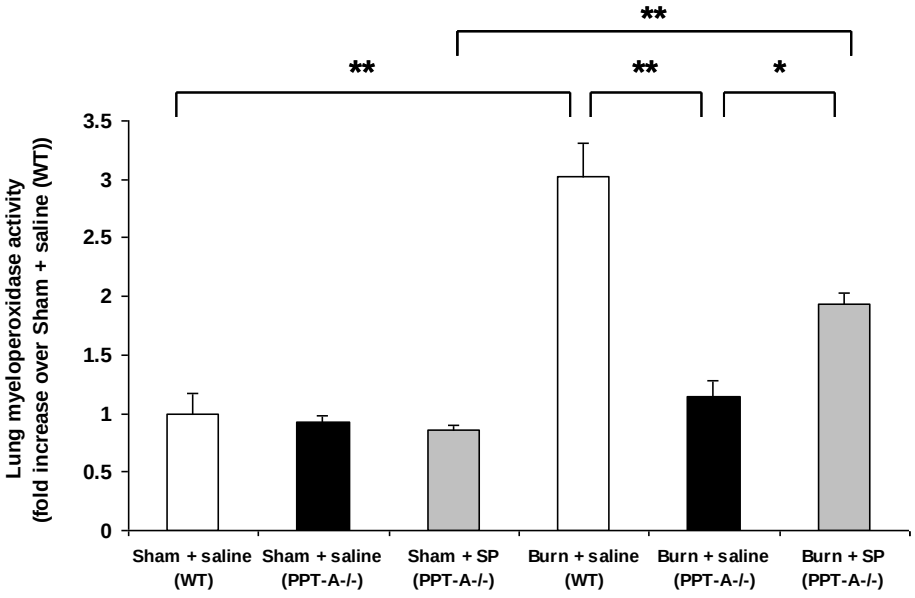

Peak Inspiratory Flow (ml/s)
12
8
4
0
**
††
§
** †
8h
24h
Time Post Burn



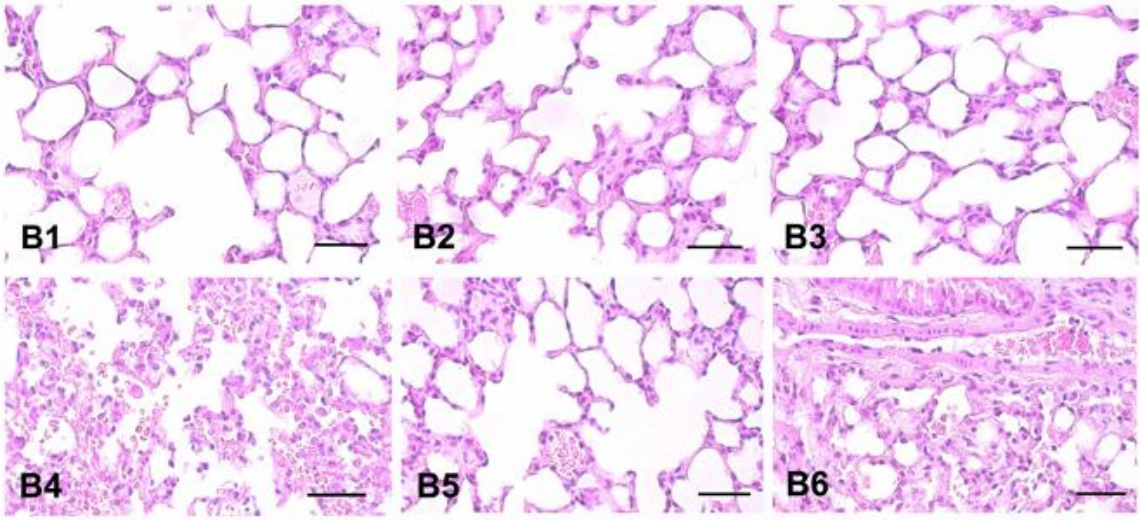
G

Figure 5.1 Progressive lung function responses after burn injury. Respiratory responses of V_e (A), RR (B), TV (C), PEF (D), T_e (E), PIF (F) and T_i (G) were recorded in unrestrained conscious WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) 8h after sham or burn injury, using whole body plethysmography. Results shown are the mean values ± SEM (n = 10-21 mice per group per time point). Mean values were calculated from 15mins of continuous respiratory behavior. *, $p<0.05$ comparing burn + saline (WT) vs sham + saline (WT); §, $p<0.05$ comparing burn + saline (*PPT-A*$^{-/-}$) vs burn + saline (WT); †, $p<0.05$ comparing burn + SP (*PPT-A*$^{-/-}$) vs sham + SP (*PPT-A*$^{-/-}$); #, $p<0.05$ comparing burn + saline (*PPT-A*$^{-/-}$) vs burn + SP (*PPT-A*$^{-/-}$).

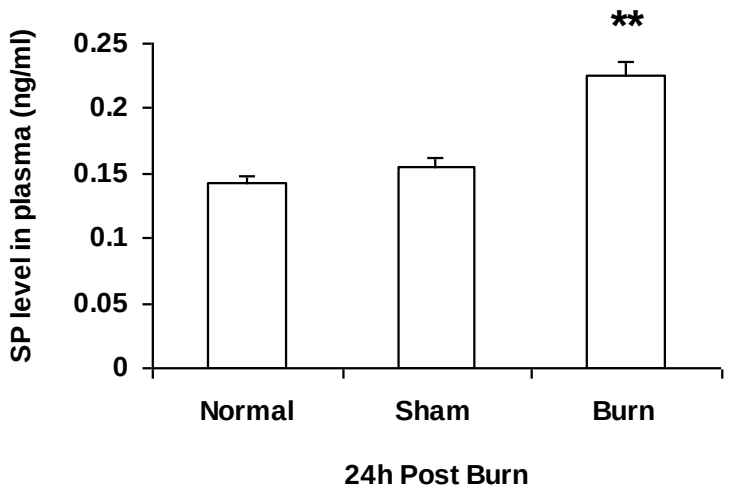
A


**
**
**
*
Lung myeloperoxidase activity
(fold increase over Sham + saline (WT))
3.5
3
2.5
2
1.5
1
0.5
0
Sham + saline (WT)
Sham + saline (PPT-A-/-)
Sham + SP (PPT-A-/-)
Burn + saline (WT)
Burn + saline (PPT-A-/-)
Burn + SP (PPT-A-/-)

B


B1
B2
B3
B4
B5
B6

C


**
0.25
0.2
0.15
0.1
0.05
0
SP level in plasma (ng/ml)
Normal
Sham
Burn
24h Post Burn

D

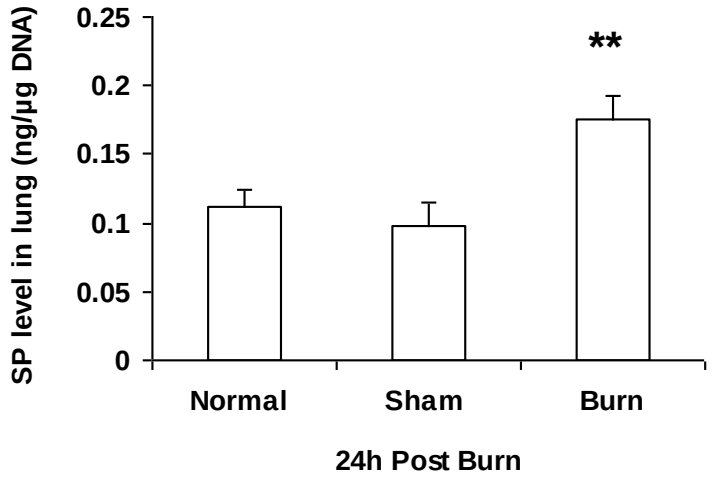

**
0.25
0.2
0.15
0.1
0.05
0
SP level in lung (ng/µg DNA)
Normal
Sham
Burn
24h Post Burn

Figure 5.2 ALI assessment correlated with augmented SP levels in plasma and lungs at 24h post burn. (A) MPO activity in the lungs of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) 24h after sham or burn injury. Results shown are the mean values ± SEM (n = 5-6 mice per group). *, $p<0.05$; **, $p<0.01$. (B) Histopathological evaluation (H&E staining) at 24h of lung PMN infiltration and injury in WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. Sham burn mice served as controls. Lung sections include: sham-injured WT mice (B1), sham-injured *PPT-A*$^{-/-}$ mice (B2), sham-injured *PPT-A*$^{-/-}$ mice injected with SP (B3), burn-injured WT mice (B4), burn-injured *PPT-A*$^{-/-}$ mice (B5) and burn-injured *PPT-A*$^{-/-}$ mice injected with SP (B6). (n = 6 mice per group). Bars, 20µm. (C-D) SP levels in plasma (C) and lung (D) of WT mice 24h after sham or burn injury. Results shown are the mean values ± SEM (n = 7-9 mice per group). **, $p<0.01$.

Chapter VI Effect of SP on extracellular signal-regulated kinase (ERK)-NFκB pathway and its association with pulmonary cyclooxygenase-2 and prostaglandin E metabolite expression levels after burn injury

6.1 Introduction

The molecular mechanisms by which SP elicits its pro-inflammatory effects have been shown to involve SP binding to its G protein-coupled receptor, NK1R, which then signals via the classical MAP kinase and NF-κB pathway (Chang et al., 2009; Williams et al., 2007). Activation of the key transcription factor, NF-κB, is well known to lead to the up-regulation of numerous mediators involved in inflammatory diseases, including cytokines, chemokines, cell adhesion molecules, acute phase proteins and important enzymes such as cyclooxygenase (COX)-1 and -2 (Barnes and Karin, 1997; Krakauer, 2004; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). COX-1 has the characteristics of a "housekeeping enzyme" as it is constitutively expressed in numerous cells. Its endogenous levels are found to play an important role in normal physiological functions, including regulation of vascular activity and maintenance of gastrointestinal function (Dubois et al., 1998). In contrast, COX-2 is an inducible enzyme stimulated by numerous molecules including mitogens, cytokines, oxidants and microbial products (Krakauer, 2004). COX-2 has been shown to be the primary isoform of the two enzymes responsible for the synthesis of prostanoid mediators of pain and inflammation, such as prostaglandin E_2 (PGE_2) (Krakauer, 2004). Studies have demonstrated PGE_2 to be a potent mediator of

pulmonary edema in acute lung injury (ALI) (Park and Christman, 2006). Increased COX-2 gene expression was also found in-conjunction with elevated ALI in mice while inhibition of COX-2 at the transcriptional and protein levels attenuated ALI in an acid model of ALI and carrageenan induced pleurisy model (Cuzzocrea et al., 2002; Ohara et al., 1998). Additionally, PGE_2 has been shown to exert significant vasodilation, pain and immune system dysfunction after burn injury (Hahn and Gamelli, 2000; Ogle et al., 1993; Schwacha et al., 1999). Significant levels of systemic prostaglandin levels were found in the plasma of severely burn-injured patients (Liu et al., 1996); while macrophages isolated from burn-injured experimental animals produced increased PGE_2 levels (Schwacha et al., 2002). More importantly, selective inhibition of COX-2 attenuated acute inflammation and improved immune function after burn injury (Grbic et al., 1991; Hahn and Gamelli, 2000).

Earlier in this study, we have shown that pre-treatment in burn-injured WT mice with L-703606, a potent and specific antagonist to NK1R, resulted in blockage of SP-NK1R signaling, which significantly lowered the release of lung neutrophils, pro-inflammatory cytokines and chemokines along with reduced cells counts of neutrophils, monocytes, and lymphocytes in the circulation (described in chapter II, III and IV). Hence, after burn injury, it is clear that SP works by activating NK1R, known to be present on lung cells and immune cells, to elicit its downstream pro-inflammatory effects. However, the downstream signaling pathway by which SP-NK1R signaling leads to in the lungs following severe local burn injury remains unknown. Furthermore, the association of COX-2 and PGE_2 with SP-related lung inflammatory responses after burn injury is not

known. Therefore, in the present study we aimed to investigate whether SP-NK1R signaling regulates ALI after burns via the ERK-NF-κB pathway, which is a major component of the MAP kinase pathways involved in inflammation. Additionally, we were interested to elucidate the effects of SP-NK1R signaling on COX-2 and PGE_2 expression levels in the lung, and the contribution of COX-2 and PGE_2 to ALI after burn. Therefore, to assess the role of COX-2 in ALI after burn injury, we used a potent and selective inhibitor of COX-2, parecoxib (Padi et al., 2004; Talley et al., 2000). It belongs to a family of medicines called Coxibs which work to relieve pain and inflammation. Parecoxib is a water-soluble prodrug of valdecoxib and is the first COX-2 specific inhibitor used for parenteral administration. At therapeutic doses, parecoxib does not appear to affect COX-1, which is crucial for maintaining normal function in the stomach and blood (Teagarden and Sandeep, 2007).

6.2 Materials and methods

6.2.1 Mouse burn injury model

For details, see section 2.2.1, page 48.

6.2.2 Time course study of lung tissue SP levels, COX-2 expression and activity levels, ERK1/2 activation and IκBα phosphorylation and degradation after burn injury

In separate groups, some WT mice were sacrificed at 0min, 30min, 1h, 2h, 4h and 8h after sham or burn injury. Lung samples were collected and stored at –80 °C for

subsequent measurements of SP levels, COX-2 expression and activity levels, ERK1/2 activation and IκBα phosphorylation and degradation. A final time point of 2h after induction of sham or burn injury was chosen for all future experiments.

6.2.3 Effect of parecoxib, a selective COX-2 inhibitor, in burn-induced ALI

Parecoxib (Dynastat), a potent and selective COX-2 inhibitor specifically developed for parenteral administration (Padi et al., 2004; Talley et al., 2000; Teagarden and Sandeep, 2007), was supplied in glass vials (40mg/vial, Pfizer) and dissolved in 0.9% sterile normal saline immediately before use. In a separate group, WT mice were treated with dose dependent concentrations of parecoxib (0, 5, 10, 20 and 30mg/kg, administered i.v) or vehicle (saline), 20min after sham or burn injury. A final dose of 30mg/kg of parecoxib, administered i.v., was chosen for all future experiments. In a another group, *PPT-A*$^{-/-}$ mice injected with SP (0.12μg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury, were subsequently administered parecoxib (30mg/kg, i.v.) 20min later.

6.2.4 Effect of PD98059, a selective inhibitor of MEK-1, in burn-induced ALI

PD98059, a potent and selective antagonist of MEK-1, which is the upstream kinase of ERK1/2 (10mg/kg, i.p.; Calbiochem, USA) was dissolved in vehicle (dimethyl sulfoxide/0.9%NaCl [1:50]; 5ml/kg, i.p.) and administered to WT or *PPT-A*$^{-/-}$ mice 1h before sham or burn injury as previously described (Minutoli et al., 2005; Tamaoki et al., 2004; Zhang et al., 2008; Zhang and Dong, 2005). Notably, we have shown previously

that the concentration of DMSO used in the vehicle to dissolve PD98059 has no effect on ERK kinase activation in the lungs (Zhang et al., 2008).

6.2.5 Effect of Bay 11-7082, a selective inhibitor of NF-κB, in burn-induced ALI

Bay 11-7082 (20mg/kg, i.p., Calbiochem), a specific inhibitor of NF-κB (Pierce et al., 1997), or vehicle (0.5% v/v DMSO in 0.9% sterile saline) was administered 30min before sham or burn injury in WT or *PPT-A*$^{-/-}$ mice. The dose of Bay 11-7082 used was chosen based on previous studies (Zhang et al., 2007). The final DMSO concentration used did not have any effect in our experiments and in other studies (Malaver et al., 2009; Strohm et al., 2000; Tamaoki et al., 2004).

6.2.6 Measurement of SP, MPO activity, histological examination, cytokine and chemokine analysis

For details, see sections 2.22, 2.23, 2.25 for SP measurement, MPO activity and histological examination respectively, page 50 to 52. For cytokines (IL-1β, IL-6 and TNF-α) and chemokines (MIP-2 and MIP-1α) analysis, see section 3.2.3, page 86.

6.2.7 Measurement of COX-2 activity

COX-2 activity was measured according to the manufacturer's instructions (Cayman Chemicals). Briefly, lung samples were homogenized in ice-cold lysis buffer (0.1M Tris-HCl, pH 7.5, supplemented with protease inhibitor mixture (Roche)) for 20s. The

homogenates were centrifuged (10,000 x *g*, 15min, 4°C), the supernatants collected and stored at -80ºC for subsequent analysis with commercially available COX activity assay kit (Cayman Chemicals). The results were corrected for the DNA content of the tissue sample and expressed as fold increase over control (Labarca and Paigen, 1980)

6.2.8 Measurement of PGE metabolite (PGEM) levels

Lung PGEM levels were determined by ELISA kits (Cayman Chemicals), according to the manufacturer's instructions. The results were corrected for the DNA content of the tissue sample and expressed as fold increase over control (Labarca and Paigen, 1980)

6.2.9 Preparation of nuclear extract and measurement of NF-κB activation

Nuclear extracts from lung (50mg) were prepared by using Nuclear Extraction Kit as described by the manufacturer (Active Motif, Tokyo, Japan). Protein concentrations in nuclear extracts were determined using Bradford assay (Bio-Rad, CA, USA). To monitor NF-κB activation in lung tissues, we used a TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif, Tokyo, Japan). The kit consists of a 96-well plate, into which oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') is bound. The active form of NF-κB in the nuclear extract specifically binds to this consensus site and is recognized by a primary antibody specific for the activated form of p65 of NF-κB. A horseradish peroxidase-conjugated secondary antibody provides the basis for the colorimetric quantification. The absorbance of the resulting solution was measured 2 min

later (450nm with a reference wavelength of 655 nm), using a 96-well microplate reader (Tecan Systems Inc., San Jose, CA, USA). The wild-type consensus oligonucleotide is provided as a competitor for NF-κB binding in order to monitor the specificity of the assay. Results were expressed as fold increase over the control group.

6.2.10 Western immunoblot

For details, see section 2.2.8, page 53. The primary antibodies used for detection for proteins of interest include: rabbit polyclonal anti-COX-2 (Cayman Chemicals; 1: 1000 dilution), rabbit anti-IκBα, phospho-IκBα, ERK1/2, phospho-ERK1/2 (Cell Signaling Technology, USA; 1:1000 dilution for all).

6.2.11 Statistics

For details, see section 2.2.10, page 55.

6.3 Results

6.3.1 Time course study of lung SP and COX-2 levels following burn injury

Endogenous lung SP levels were significantly increased as early as 30min after burn injury compared to WT mice at 0min post burn, which were not induced with burn injury (Fig. 6.1A). Moreover, the SP levels continued to remain significantly elevated at 1h, 2h, 4h, and up to 8h after burn injury. In an attempt to correlate if there were also

simultaneous changes in lung COX-2 levels as there were in SP levels after burn injury, we performed western blot analysis for the same time points to evaluate the lung expression levels of COX-2 in WT mice. Our results revealed a concurrent increase in COX-2 levels in a time dependent manner which peaked at 0.5h to 2h post burn (Fig. 6.2A). Additionally, a similar time dependent profile was observed in lung COX-2 activity levels over the same time points, with the highest level of COX-2 activity peaking significantly at 2h post burn compared to WT mice at 0min post burn (Fig. 6.2B). Therefore, these results show that burn injury induces a concomitant and significant overproduction of both SP and COX-2 in the lungs. Notably, the levels of both SP and COX-2 are prominently expressed at 2h post burn (Fig. 6.1 and 6.2), therefore, this time point was chosen for all future experiments.

6.3.2 Dose dependent effect of parecoxib on lung neutrophil infiltration following burn injury

To assess the severity of COX-2 mediated ALI after burn injury, we examined the effect of parecoxib on lung myeloperoxidase activity, which is an important index of lung neutrophil accumulation. Parecoxib was administered therapeutically at 20mins post burn to burn-injured WT mice in a dose dependent manner of 0, 5, 10, 20 and 30mg/kg intravenously, and MPO activity was subsequently measured at 2h post burn. The results reveal that only the dose of 30mg/kg of parecoxib markedly lowered lung MPO activity, while no significant inhibition was observed with all the other lower doses (Fig. 6.3). Therefore, the dose of 30mg/kg parecoxib was used for all future experiments.

6.3.3 Blockade of SP-NK1R signaling and COX-2 expression significantly protects against burn-induced ALI

The severity of ALI is clearly characterized by the invasion of neutrophils into the lungs (Wheeler and Bernard, 2007). Following burn injury, lung MPO levels were markedly increased which correlated with augmented SP and COX-2 expression levels in WT mice (Fig. 6.4A). Notably, the MPO activity levels were significantly attenuated in burn-injured WT mice treated with L703606, a specific NK1R antagonist at a dose of 12mg/kg, i.p., 1h before burn injury, and 30mg/kg of parecoxib, i.v., 20mins post burn, suggesting that SP-NK1R signaling and COX-2 play a pivotal role in modulating ALI after burn (Fig. 6.4A). Additionally, to further confirm our findings, we used mice deficient in the preprotachykinin-A (*PPT-A*) gene, which encodes for SP, to investigate the link between SP, COX-2 and ALI after burn injury. Our results showed that the heightened MPO activity levels observed in burn-injured WT mice was significantly abrogated in burn-injured *PPT-A*$^{-/-}$ mice, indicating that overproduction of SP can lead to a significant increment in MPO activity levels after burn. Consistent with these findings, the drop in MPO activity levels in burn-injured *PPT-A*$^{-/-}$ mice was significantly reversed in burn-injured *PPT-A*$^{-/-}$ mice injected with exogenous SP (Fig. 6.4A). Moreover, lower levels of MPO activity were again restored upon treatment with parecoxib in burn-injured *PPT-A*$^{-/-}$ mice injected with exogenous SP, thereby, confirming the important role of SP and COX-2 in mediating lung neutrophil infiltration after burn injury (Fig. 6.4A). Subsequently, we further extended our investigation by performing histopathological examination at 2h post burn. Consistently, alleviated alveolar congestion, lowered leukocyte infiltration,

alveolar septal wall thickness and interstitial edema were observed in WT mice treated with L703606 (Fig. 6.4B5) and parecoxib (Fig. 6.4B6) after burn and in burn-injured *PPT-A*$^{-/-}$ mice (Fig. 6.4B10) compared to burn-injured WT mice (Fig. 6.4B4). Burn-injured *PPT-A*$^{-/-}$ mice injected with SP (Fig. 6.4B11) again demonstrated severe ALI similar to burn-injured WT mice. In contrast, burn-injured *PPT-A*$^{-/-}$ mice injected with SP showed significant protection from ALI and restoration of normal lung histoarchitecture after treatment with parecoxib at 2h post burn (Fig. 6.4B12). Notably, in both MPO activity and histology, all sham-treated mice showed basal levels, particularly in sham-injured WT mice treated with L703606 and parecoxib, and in sham-injured *PPT-A*$^{-/-}$ mice injected with SP which were subsequently treated with parecoxib at 20mins post burn. As such, after confirming that the doses of 12mg/kg of L703606 and 30mg/kg of parecoxib had no adverse effect on lung neutrophil infiltration and histopathology of sham-injured WT or *PPT-A*$^{-/-}$ mice, these sham-injured mice were not included in other experiments. Therefore, collectively, these results show that the absence of SP and inhibition of SP-NK1R signaling and COX-2 are critical in resolving ALI after burn.

6.3.4 Inhibition of SP-NK1R signaling and COX-2 up-regulation greatly impairs cytokines and chemokines production following burn injury

Evaluation of pro-inflammatory cytokines, IL-1β, TNF-α and IL-6 and chemokines, MIP-2 and MIP-1α showed a marked rise in all of their levels after burn in WT mice (Fig. 6.5). Consistent with MPO and histology results, burn-injured WT mice treated with L703606 and parecoxib, along with burn-injured *PPT-A*$^{-/-}$ mice showed an evident rescue in

production of IL-1β, TNF-α, IL-6, MIP-2 and MIP-1α, known to exert profound effects in numerous inflammatory diseases. Importantly, levels of all five cytokines and chemokines were observed to be amplified in burn-injured *PPT-A*$^{-/-}$ mice injected with SP; while blockage of COX-2 with parecoxib in burn-injured *PPT-A*$^{-/-}$ mice injected with SP significantly reverted back their levels to become lower again (Fig. 6.5).

6.3.5 Activation of SP-NK1R signaling and COX-2 expression levels leads to the production of PGE_2 metabolite (PGEM) following burn injury

The enhanced lung COX-2 expression levels observed in our results, together with the evident reduction in MPO activity after administration of parecoxib in burn-injured WT mice, instinctively lead us to examine lung PGE_2 levels after burn. PGE_2 is known to be rapidly metabolized in the lungs (Piper, 1974; Piper et al., 1970; Wyllie et al., 1969), as such, measurement of PGEM is necessary to provide a reliable estimate of actual PGE_2 production (Bothwell et al., 1982; Granstrom et al., 1980; Maclouf et al., 1987). As expected, PGEM levels were heightened after burn, which correlated with augmented COX-2 expression levels in WT mice (Fig. 6.6A). Notably, treatment with L703606 and parecoxib significantly inhibited PGEM levels at 2h post burn. Furthermore, when burn-injured WT mice was compared to burn-injured *PPT-A*$^{-/-}$ mice, an obvious reduction in PGEM levels was observed in burn-injured *PPT-A*$^{-/-}$ mice. However, upon administration of exogenous SP to burn-injured *PPT-A*$^{-/-}$ mice, this effect was significantly reversed. More importantly, treatment with parecoxib abolished the elevated PGEM levels in burn-injured *PPT-A*$^{-/-}$ mice injected with SP (Fig. 6.6A). Taken together, our results show

compelling evidence that SP-NK1R signaling and COX-2 are important in influencing the extent of PGEM production after burn injury. This also indicates that SP-NK1R signaling and COX-2 up-regulation occurs upstream of PGEM production after burn injury, thereby showing that SP has the ability to initiate the up-regulation of key inflammatory mediators such as PGE_2 that can contribute extensively to the severity of ALI after burn.

6.3.6 Time course study of lung ERK1/2 activation, phosphorylation and degradation of IκBα levels following burn injury

To investigate the signaling mechanisms leading to a rise in COX-2 expression levels in the lungs and elevated ALI following burn injury, we evaluated the ERK-NF-κB pathway, an important signal transduction pathway involved in regulating numerous inflammatory responses (Kyriakis and Avruch, 2001). Since the levels of SP and COX-2 were significantly increased over 30min, 1h, 2h, 4h and 8h as seen earlier in this study, we were also interested to determine whether the kinetics of ERK1/2 phosphorylation as well as IκBα phosphorylation and degradation in the lungs were affected over the same time points following burn injury. In agreement with changes over time of increased pulmonary SP and COX-2 expression levels, the phosphorylation of ERK1/2 showed marked increases from 30min to 8h post burn compared to 0min (Fig. 6.7A). Furthermore, evaluation of IκBα phosphorylation showed a progressive increase over time with a significant rise at 2h post burn (Fig. 6.7B). Likewise the content of IκBα was reduced in a time dependent manner with a significant occurrence of degradation obtained at 2h post

burn (Fig. 6.7C). Taken together, these data show that burn injury induces activation of the ERK-NF-κB pathway which was observed to take place with concomitant increases in SP and COX-2 pulmonary levels over time. As such, this verified that further investigation on the ERK-NF-κB pathway following burn injury was imperative; therefore, we focused on the cellular changes of these molecules at 2h post burn for the rest of this study.

6.3.7 Induction of SP-NK1R signaling and ERK1/2 pathway markedly augmented COX-2 expression levels after burn injury

To determine the critical link between SP-NK1R signaling and ERK1/2 activation leading to changes in COX-2 expression levels, we performed western blot analysis using L703606 and PD98059, a potent and selective antagonist of MEK-1, administered at a dose of 10mg/kg, 1h before burn injury. A significant increment in COX-2 expression levels were observed following burn injury in WT mice at 2h post burn (Fig. 6.8A). Of particular importance was that this effect was abolished in burn-injured WT mice treated with both L703606 and PD98059 (Fig. 6.8A). Thus, these results show evidently that SP-NK1R and ERK1/2 signaling significantly influences the expression levels of COX-2 in the lungs after burn injury, and further imply that SP-NK1R and ERK1/2 signaling occurs upstream of COX-2 expression. Additionally, usage of burn-injured *PPT-A*$^{-/-}$ mice further substantiated the role of SP in modulating COX-2 expression, whereby burn-injured *PPT-A*$^{-/-}$ mice revealed significantly lowered lung COX-2 levels, however, upon administration of exogenous SP, the COX-2 levels were again elevated as anticipated

(Fig. 6.8A). More significantly, treatment with PD98059 in burn-injured *PPT-A*$^{-/-}$ mice injected with SP markedly suppressed COX-2 expression levels.

6.3.8 Increased SP-NK1R signaling enhanced ERK1/2 activation after burn injury

Next, we verified the effect of SP-NK1R signaling on ERK1/2 phosphorylation. Our results show that ERK1/2 had undergone significant phosphorylation after burn in WT mice (Fig. 6.9A). This was abrogated in burn-injured WT mice administered with L703606 and PD98059 and in burn-injured *PPT-A*$^{-/-}$ mice. The phosphorylation levels of ERK1/2 was restored in burn-injured *PPT-A*$^{-/-}$ mice injected with SP and again reduced when burn-injured *PPT-A*$^{-/-}$ mice injected with SP were treated with PD98059 (Fig. 6.9A). Therefore, these data clearly show that SP-NK1R signaling occurs upstream of ERK1/2 and its inhibition significantly suppresses the activation of ERK1/2 which leads further to activation of downstream COX-2 expression.

6.3.9 Effect of SP-NK1R signaling and ERK1/2 pathway on IκBα phosphorylation and degradation levels and activity of NF-κB after burn injury

The phosphorylation, ubiquitination and subsequent proteolytic degradation of the inhibitor IκBα protein results in the release of NF-κB, the activated form consisting mainly of a heterodimer of p65 and p50, which consequently translocates to the nucleus, where it initiates the transcription of numerous inflammatory proteins (Barnes and Karin, 1997). Therefore, we analyzed the activation levels of IκBα and NF-κB after burn injury.

Pre-treatment with L703606 in burn-injured WT mice drastically reduced the pulmonary levels of phospho-IκBα compared to the heightened levels detected in untreated burn-injured WT mice (Fig. 6.10A). Furthermore, this reduction was consistent in burn-injured WT mice pre-treated with PD98059 and in burn-injured *PPT-A*$^{-/-}$ mice. In contrast, the phospho-IκBα levels burn-injured *PPT-A*$^{-/-}$ mice injected with SP were again amplified; while these levels were suppressed upon pre-treatment with PD98059 (Fig. 6.10A). Assessment of the degradation of IκBα displayed a significant reduction in IκBα levels after burn in WT mice and in burn-injured *PPT-A*$^{-/-}$ mice injected with SP, whereas the content of IκBα levels observed in all other groups where significantly higher (Fig. 6.10B).

The DNA-binding activity of nuclear NF-κB in the lungs of burn-injured WT mice were markedly enhanced compared to sham-injured WT mice, burn-injured WT mice treated with L703606 and PD98059, and in burn-injured *PPT-A*$^{-/-}$ mice (Fig. 6.11A). Exogenous administration of SP to burn-injured *PPT-A*$^{-/-}$ mice amplified the activation of NF-κB while pretreatment with PD98059, significantly disrupted the activity level of NF-κB in the lungs after burn (Fig. 6.11A). Next, to ascertain if COX-2 is up-regulated by NF-κB after burn, we administered Bay 11-7082 to mice, a specific inhibitor of NF-κB (Pierce et al., 1997), at 20mg/kg, i.p., 30min before burn injury, followed by measurement of COX-2 activity levels at 2h post burn. Both burn-injured WT mice and burn-injured *PPT-A*$^{-/-}$ mice injected with SP showed increased levels of lung COX-2 activity at 2h post burn (Fig. 6.11B). However, pre-treatment with Bay 11-7082 showed significantly reduced lung COX-2 activity levels in burn-injured WT mice and in burn-injured *PPT-A*$^{-/-}$ mice

injected with SP (Fig. 6.11B). Taken together, our findings demonstrate that SP-NK1R signaling contributes significantly in regulating the ERK-NF-κB dependent signal transduction pathway in a mouse model of burn-induced ALI.

6.4 Discussion

Defining the molecular mechanisms by which endogenous pulmonary inflammatory mediators initiate signal transduction pathways is of critical importance in elucidating the pathogenesis of ALI and ARDS. Numerous studies provide evidence which indicate that SP and NK1R are major pro-inflammatory mediators of lung diseases (Groneberg et al., 2006; O'Connor et al., 2004). At the same time, a large body of evidence has accumulated after decades of research to show that COX-2 and PGE_2 play pivotal roles in respiratory diseases such as ALI, chronic obstructive pulmonary disease, pulmonary fibrosis and lung cancer (Carpagnano et al., 2009; Chen et al., 2008; Fukunaga et al., 2005; Hinz and Brune, 2002; Lappi-Blanco et al., 2006; Park and Christman, 2006). Some studies have evidences which indicate that COX-2 and PGE_2 might be associated with SP related inflammatory responses. For example, studies have shown SP to incite COX-2 and PGE_2 expression via JAK-STAT pathway in human colonic epithelial cells (Koon et al., 2006), and via MAP kinase pathway in human umbilical vein endothelial cells (Gallicchio et al., 2006). SP was also shown to induce COX-2 expression and NF-κB activation in human polymorphonuclear leukocytes (Gallicchio et al., 2009). Furthermore, SP stimulated PGE_2 via NK1R in isolated rat intrapulmonary bronchi and trachea preparations (Devillier et al., 1992; Szarek et al., 1998), and via PGE_2 receptor subtype 2 in murine

tracheal preparations (Fortner et al., 2001). However, the interaction(s) between SP, COX-2 and PGE_2 in burn-induced remote ALI is not known. Here, we report for the first time, the signal transduction pathway following SP-NK1R coupling which activates the ERK-NF-κB pathway, leading to subsequent COX-2 up-regulation and PGEM production, which then contributes to significant elevations in pulmonary pro-inflammatory cytokines, chemokines, neutrophils and exacerbated ALI following severe local burn injury in mice. Results from pharmacological inhibition of NK1R, *PPT-A*$^{-/-}$ mice and *PPT-A*$^{-/-}$ mice injected with exogenous SP supports these conclusions.

Results of our time course studies showed that biosynthesis of SP and COX-2 expression levels were both concomitantly increased early after burn injury. As early as 30min post burn, the levels of SP and COX-2 expression were significantly elevated in WT mice compared to 0min post burn. Similarly the COX-2 activity levels were augmented, while ERK1/2 and IκBα were highly phosphorylated, along with concurrent observations of IκBα degradation. Interestingly, although, COX-2 activity was found to increase at 30min post-burn, while IκB phosphorylation occurred at 2h post-burn, it is possible that COX-2 up-regulation can occur earlier as there are other pathways independent of IκB phosphorylation and NF-κB activation that can induce COX-2 levels. For example, the inducible expression of COX-2 is known to be up-regulated by numerous cytokines, growth factors and transcription factors (Krakauer, 2004; Wu, 1996). Hence, these proteins may contribute to the increased COX-2 expression levels at the earlier time point. Nevertheless, together, the occurrence of SP, COX-2, ERK1/2 and IκBα levels correlated well and remained highly up-regulated or activated in lungs for up to 8h post burn.

Thereby, these data suggest that the overproduction of SP and COX-2 levels after severe local burn injury is not a temporal early rise but are constantly maintained in lung cells for at least up to 8h post burn.

The usage of L703606, a specific NK1R antagonist, in our study determined that SP signaled via NK1R, a G-protein coupled receptor (GPCR), to lead to the activation of COX-2, which then resulted in increased ALI, cytokines and chemokines production. Furthermore, usage of PD98059, a selective inhibitor of MEK-1, which is the upstream kinase of ERK1/2, directly ascertained the downstream involvement of ERK1/2 in SP-NK1R mediated signaling in the lungs following burn injury. Pretreatment with PD98059 in burn-injured WT mice and burn-injured *PPT-A*$^{-/-}$ mice injected with exogenous SP consistently resulted in inhibition of ERK1/2, IκBα phosphorylation and degradation and attenuated the over-activation of NF-κB. Additionally, lung COX-2 activity levels were significantly reduced with the administration of Bay 11-7082, a specific inhibitor of NF-κB, in burn-injured WT mice and burn-injured *PPT-A*$^{-/-}$ mice injected with SP. Notably, although the exogenous dose of 0.12μg/kg SP administered to burn-injured *PPT-A*$^{-/-}$ mice corresponded to physiological endogenous SP plasma concentration in normal mice as explained in chapter II, here, our results indicate that this dose of 0.12μg/kg SP was sufficient for the activation of ERK1/2 for a period of 2h after burn injury. Hence, once the exogenous SP administered to *PPT-A*$^{-/-}$ mice is able to activate SP-NK1R signaling after burn injury, this may be sufficient to initiate a cascade of downstream ERK1/2 signaling which then leads to the exacerbation of ALI. Together, these findings collectively show that SP-NK1R coupling initiates a straightforward involvement of

ERK-NF-κB signaling pathway to induce up-regulation of COX-2 expression and ALI following burn injury.

To verify that SP can work in conjunction with other major mediators such as COX-2 to induce ALI, we used a selective inhibitor of COX-2, parecoxib, to assess the role of SP-induced COX-2 expression in burn-injured mice. Data derived from burn-injured *PPT-A*$^{-/-}$ mice injected with exogenous SP showed exacerbated ALI compared to alleviated ALI in burn-injured *PPT-A*$^{-/-}$ mice alone. Thereby, indicating that burn-induced ALI was specifically attributable to the pro-inflammatory effects of SP. However, upon treatment with parecoxib in burn-injured *PPT-A*$^{-/-}$ mice injected with exogenous SP, the observed heightened ALI severity was attenuated. Thus, confirming that SP has the ability to signal via COX-2 to instigate ALI after burn. Additionally, pulmonary cytokines and chemokines data from burn-injured *PPT-A*$^{-/-}$ mice injected with exogenous SP and subsequently treated with or without parecoxib revealed that SP can regulate IL-1β, TNF-α, IL-6, MIP-2 and MIP-1α levels via COX-2.

It is known that more than 90% of circulating PGE_2 is rapidly catabolized to inactive forms on the first pass in the lungs (Ivanov et al., 2003; Piper et al., 1970; Wyllie et al., 1969). Therefore, studies have used the measurement of PGEM as a common alternative to indicate the levels of PGE_2 (Bothwell et al., 1982; Duffield-Lillico et al., 2009; Granstrom et al., 1980; Gross et al., 2005; Maclouf et al., 1987). Significantly high levels of PGEM in the lungs were detected in burn-injured WT mice and in burn-injured *PPT-A*$^{-/-}$ mice injected with SP. The high levels of PGEM in these two groups of mice also

corresponded with concurrent increases in COX-2 expression and ALI. Treatment with parecoxib and L703606 showed significantly reduced levels of PGEM in burn-injured WT mice, while PGEM levels were also lowered in burn-injured *PPT-A*$^{-/-}$ mice injected with SP and subsequently treated with parecoxib. Consistently, the decreased levels of PGEM in these two groups of mice following parecoxib treatment showed suppressed COX-2 expression and alleviated ALI after burn. Therefore, these results may indicate that SP-NK1R signaling induces COX-2 expression which leads to PGE_2 secretion and exacerbation of ALI. Also, it is interesting to note that the alterations in PGEM levels after burn may not only be due to the increase in production of PGE_2 via the enzymatic actions of COX-2 after burn but also possibly due to a drop in PGE_2 clearance and degradation by the lungs. Given that after burn injury, the lungs become severely injured, hence, this may not only affect respiratory function but also lead to the disruption of crucial metabolic activities such as the metabolism of PGE_2. As such, excessive PGE_2 may accumulate to elicit pain, vasodilation, edema and immune dysfunction.

In conclusion, our results show for the first time that SP regulates pulmonary inflammatory responses following severe local burn injury via the signaling transduction pathway involving: NK1R-ERK1/2-IκBα-NF-κB-COX-2 pathway which induces a marked rise in pulmonary PGEM levels that are indicative of increased PGE_2 levels. Together, the overproduction of SP and COX-2 subsequently resulted in significantly exacerbated ALI after burn. Therefore, this study may contribute to a better understanding of the precise mechanisms initiated by SP in the pathophysiology of remote ALI after burn.

A

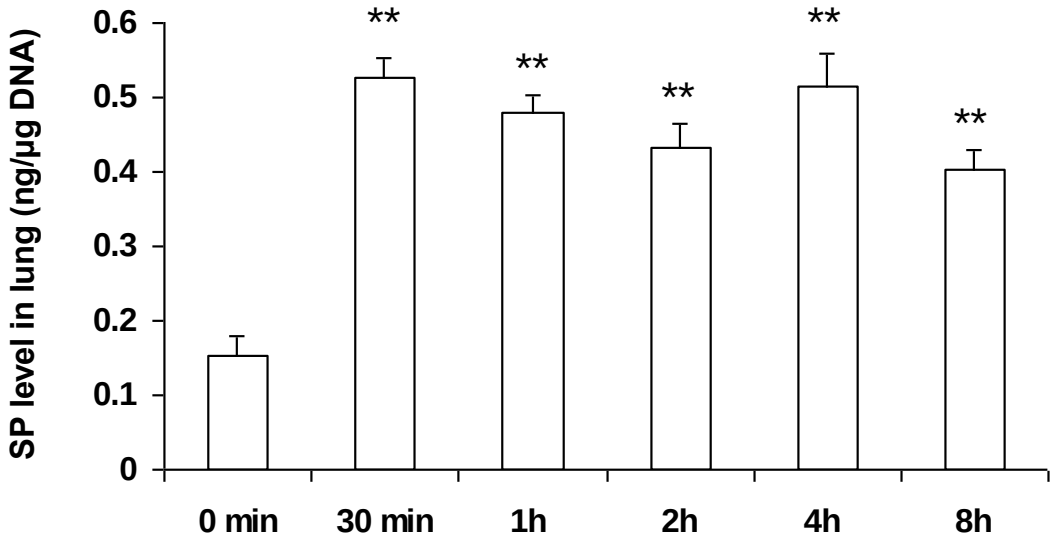
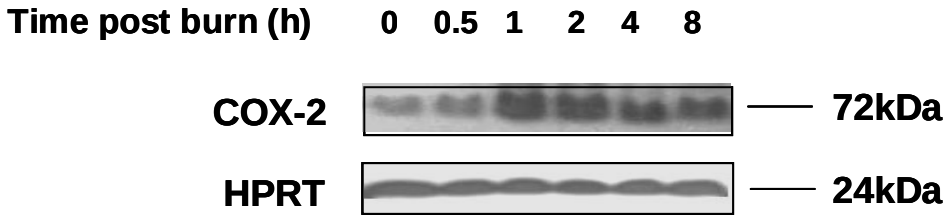
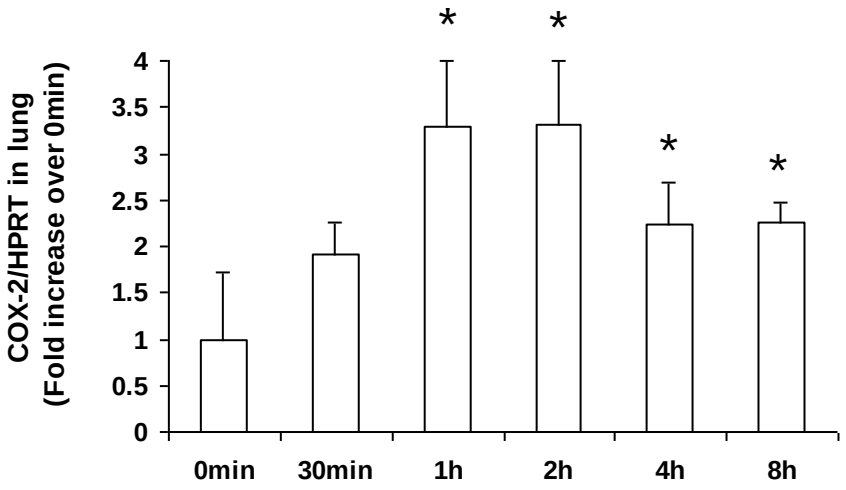


Figure 6.1 Time course study of SP levels in lung after burn injury. (A) SP levels in lung of WT mice were measured by ELISA at 0min, 30min, 1h, 2h, 4h and 8h after burn injury. Results shown are the mean values ± SEM (n = 4-6 mice per group). **, $p<0.01$.

A


Time post burn (h)
0
0.5
1
2
4
8
COX-2
72kDa
HPRT
24kDa


COX-2/HPRT in lung
(Fold increase over 0min)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
*
0min
30min
1h
2h
4h
8h

B

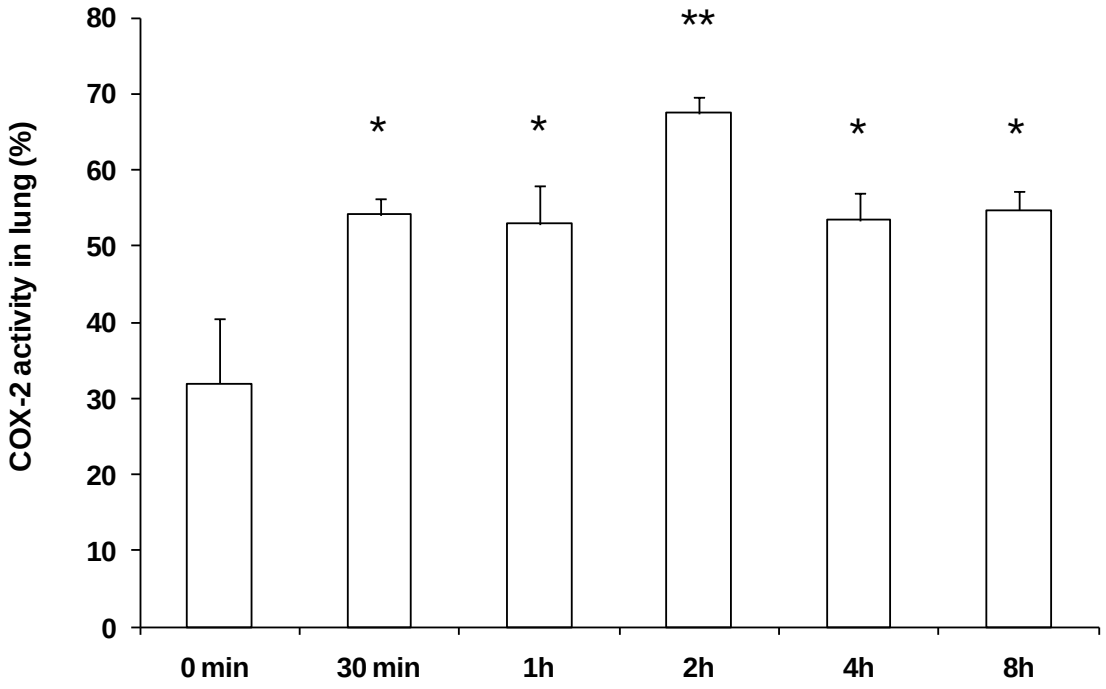


Figure 6.2 Time course study of COX-2 expression and activity in lungs after burn injury. (A) Western blot results showing COX-2 expression and (B) COX-2 activity levels in lung of WT mice at 0min, 30min, 1h, 2h, 4h and 8h after burn injury. Results shown are the mean values ± SEM (n = 4 mice per group for COX-2 expression levels, and 5-7 mice per group for COX-2 activity levels). *, $p<0.05$; **, $p<0.01$.

A

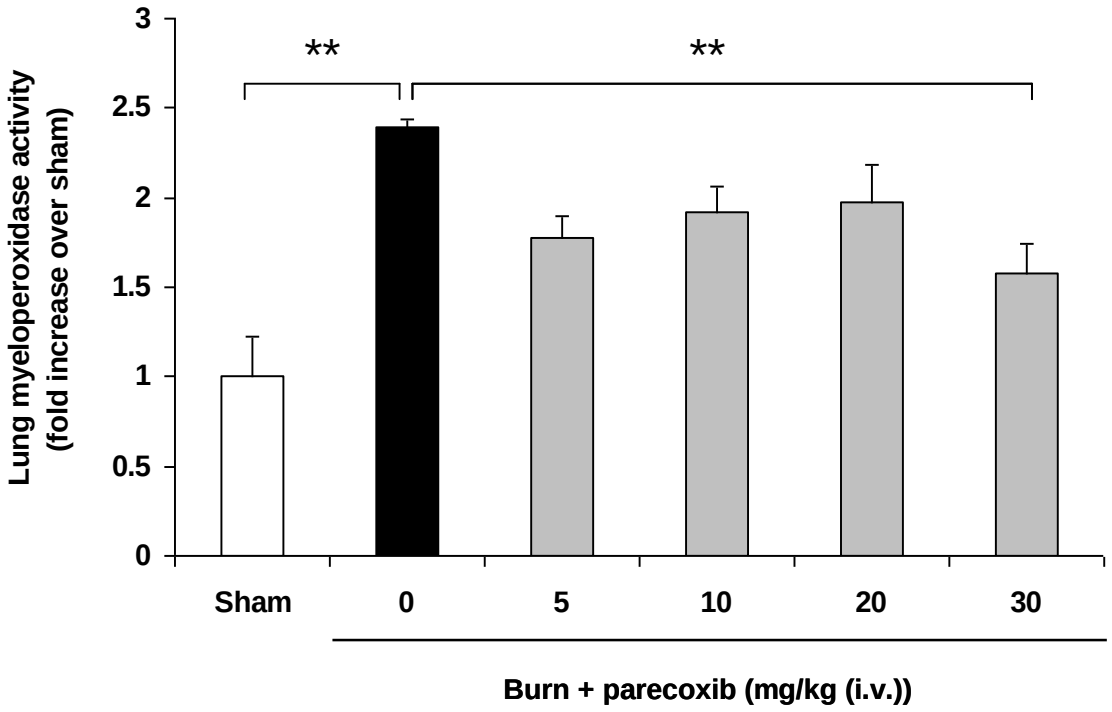
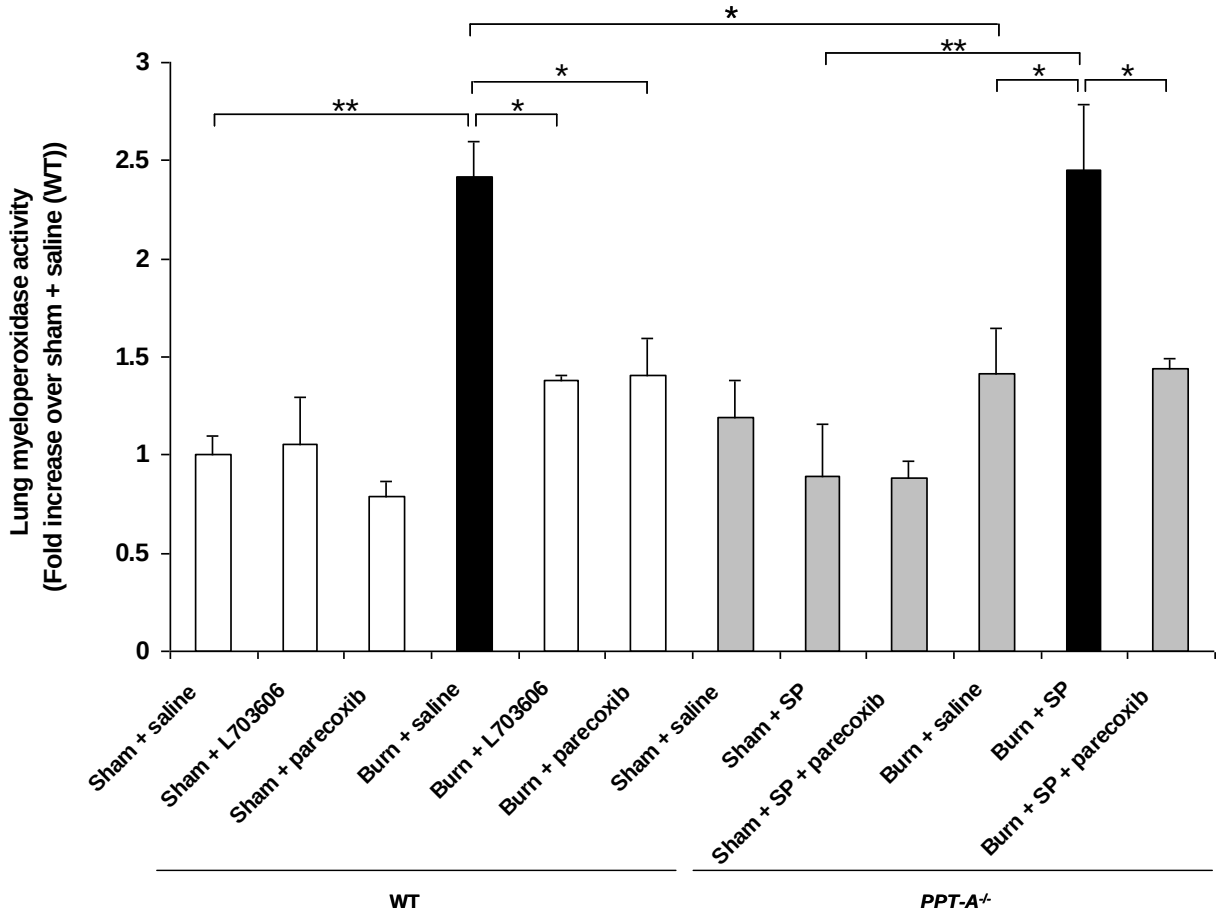


Figure 6.3 Dose dependent effect of parecoxib on lung neutrophil infiltration at 2h post burn in WT mice. (A) MPO activity at 2h after sham or burn injury in the lungs of WT mice treated with 0, 5, 10, 20 and 30mg/kg parecoxib, i.v., administered 20min post burn. Results shown are the mean values ± SEM (n = 5 mice per group). **, $p<0.01$.

A


Lung myeloperoxidase activity
(Fold increase over sham + saline (WT))
3
2.5
2
1.5
1
0.5
0
*
**
*
*
**
*
*
Sham + saline
Sham + L703606
Sham + parecoxib
Burn + saline
Burn + L703606
Burn + parecoxib
Sham + saline
Sham + SP
Sham + SP + parecoxib
Burn + saline
Burn + SP
Burn + SP + parecoxib
WT
PPT-A-/-

B

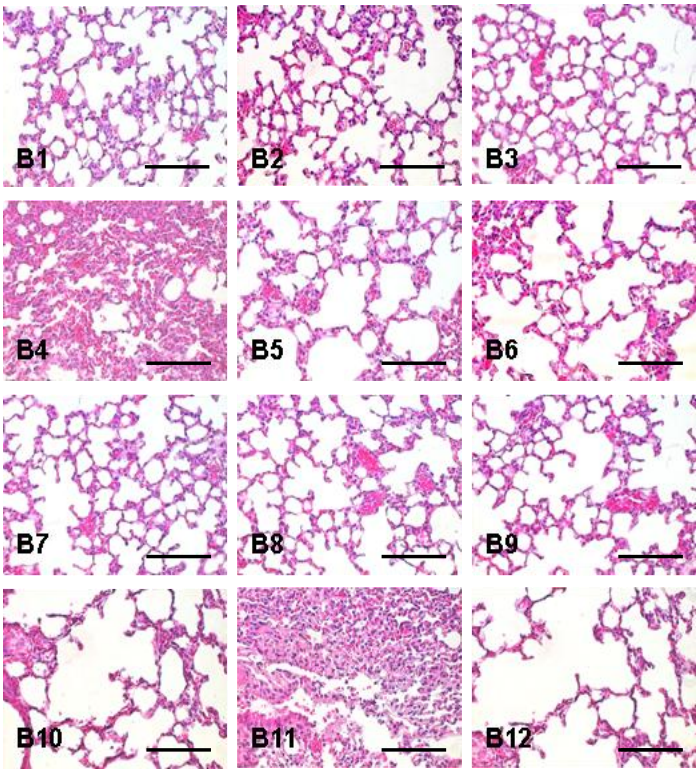
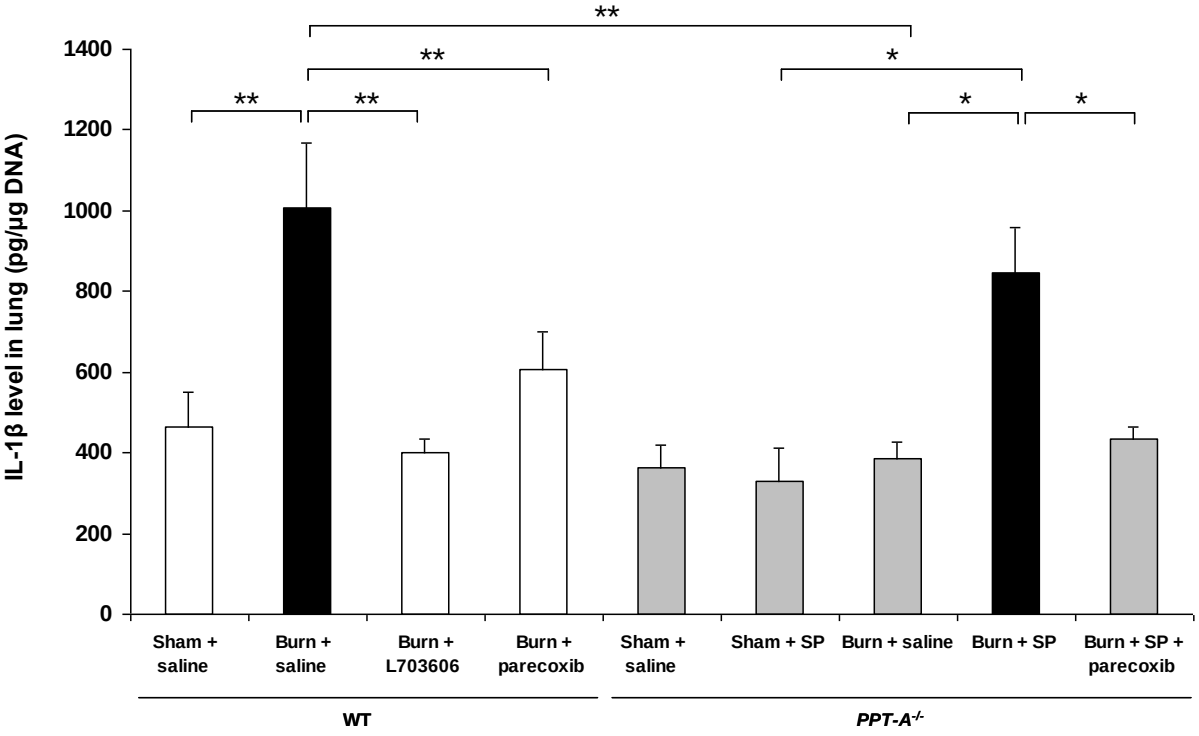
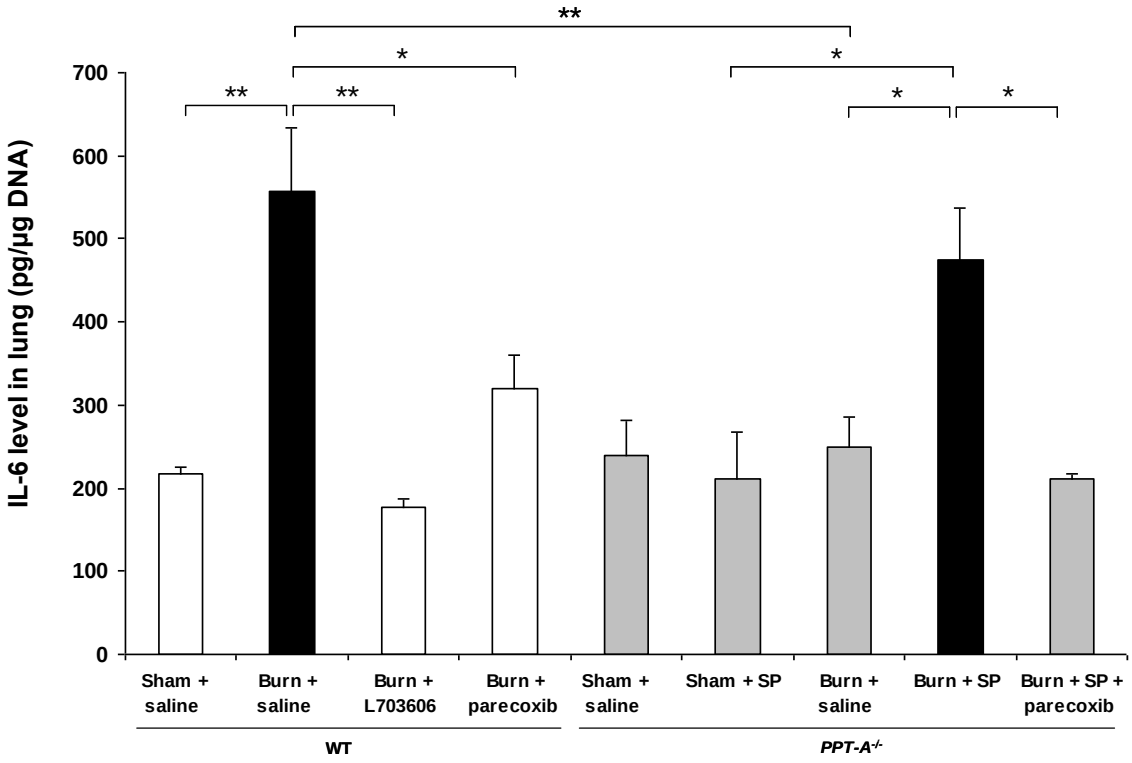


Figure 6.4 Inhibition of SP-NK1R signaling and COX-2 expression markedly reduced lung neutrophil infiltration and alleviated ALI after burn injury. (A) MPO activity and (B) histopathological evaluation (H&E staining) of PMN infiltration and injury at 2h in the lungs of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12μg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT mice, L703606 (12mg/kg, i.p., 1h before sham or burn injury) or parecoxib (30mg/kg, i.v., 20min post burn) was administered. *PPT-A*$^{-/-}$ mice injected with SP were also subsequently administered parecoxib (30mg/kg, i.v., 20min post burn). Results shown are the mean values ± SEM (n = 4-6 mice per group for MPO activity). *, $p<0.05$; **, $p<0.01$. Histology lung sections include: sham + saline (WT) (B1), sham + L703606 (WT) (B2), sham + parecoxib (WT) (B3), burn + saline (WT) (B4), burn + L703606 (WT) (B5), burn + parecoxib (WT) (B6), sham + saline (*PPT-A*$^{-/-}$) (B7), sham + SP (*PPT-A*$^{-/-}$) (B8), sham + SP + parecoxib (*PPT-A*$^{-/-}$) (B9), burn + saline (*PPT-A*$^{-/-}$) (B10), burn + SP (*PPT-A*$^{-/-}$) (B11), burn + SP + parecoxib (*PPT-A*$^{-/-}$) (B12). (n = 6-8 mice per group for histological analysis). Bars, 20μm.

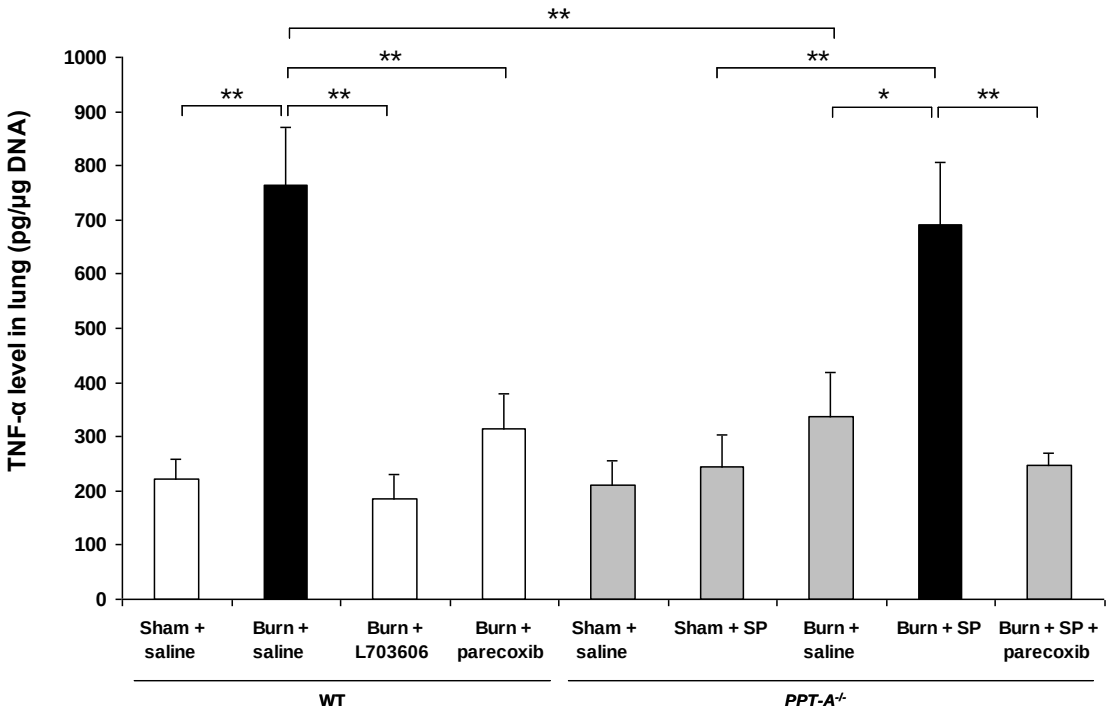
A


IL-1β level in lung (pg/µg DNA)
1400
1200
1000
800
600
400
200
0
**
**
**
**
*
*
*
Sham + saline
Burn + saline
Burn + L703606
Burn + parecoxib
Sham + saline
Sham + SP
Burn + saline
Burn + SP
Burn + SP + parecoxib
WT
PPT-A-/-

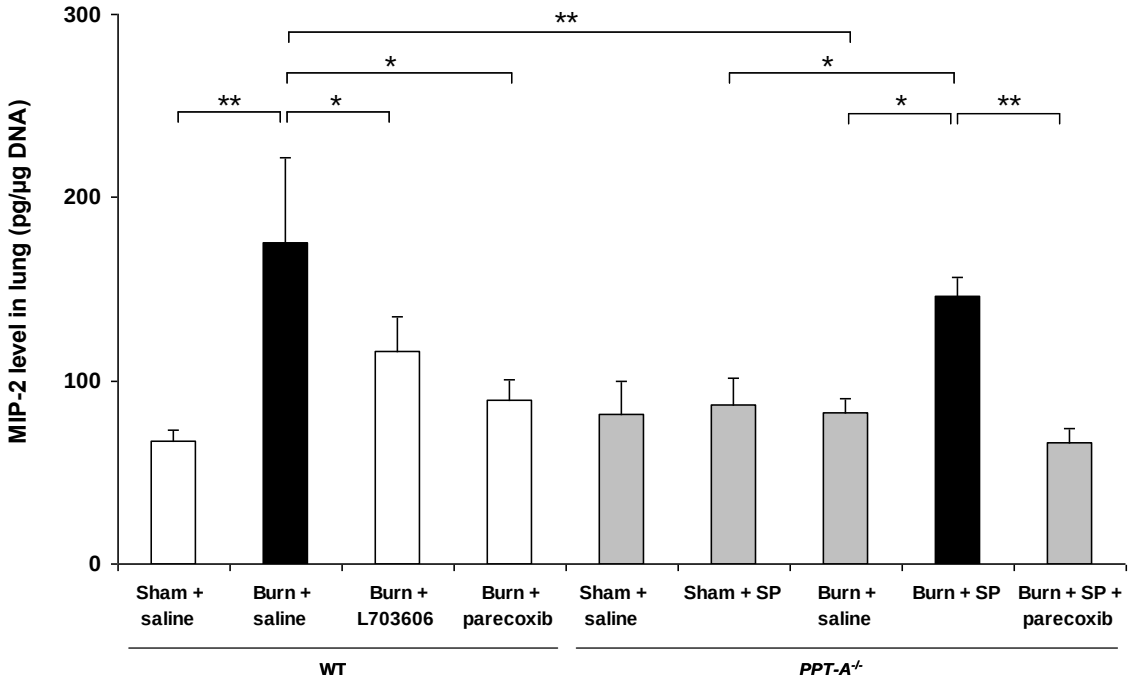
B


IL-6 level in lung (pg/µg DNA)
700
600
500
400
300
200
100
0
**
*
*
**
**
*
*
Sham + saline
Burn + saline
Burn + L703606
Burn + parecoxib
Sham + saline
Sham + SP
Burn + saline
Burn + SP
Burn + SP + parecoxib
WT
PPT-A-/-

C


TNF-α level in lung (pg/µg DNA)
1000
900
800
700
600
500
400
300
200
100
0
**
**
**
**
*
**
Sham + saline
Burn + saline
Burn + L703606
Burn + parecoxib
Sham + saline
Sham + SP
Burn + saline
Burn + SP
Burn + SP + parecoxib
WT
PPT-A-/-

D


MIP-2 level in lung (pg/µg DNA)
300
200
100
0
**
*
*
**
*
*
**
Sham + saline
Burn + saline
Burn + L703606
Burn + parecoxib
Sham + saline
Sham + SP
Burn + saline
Burn + SP
Burn + SP + parecoxib
WT
PPT-A-/-

E

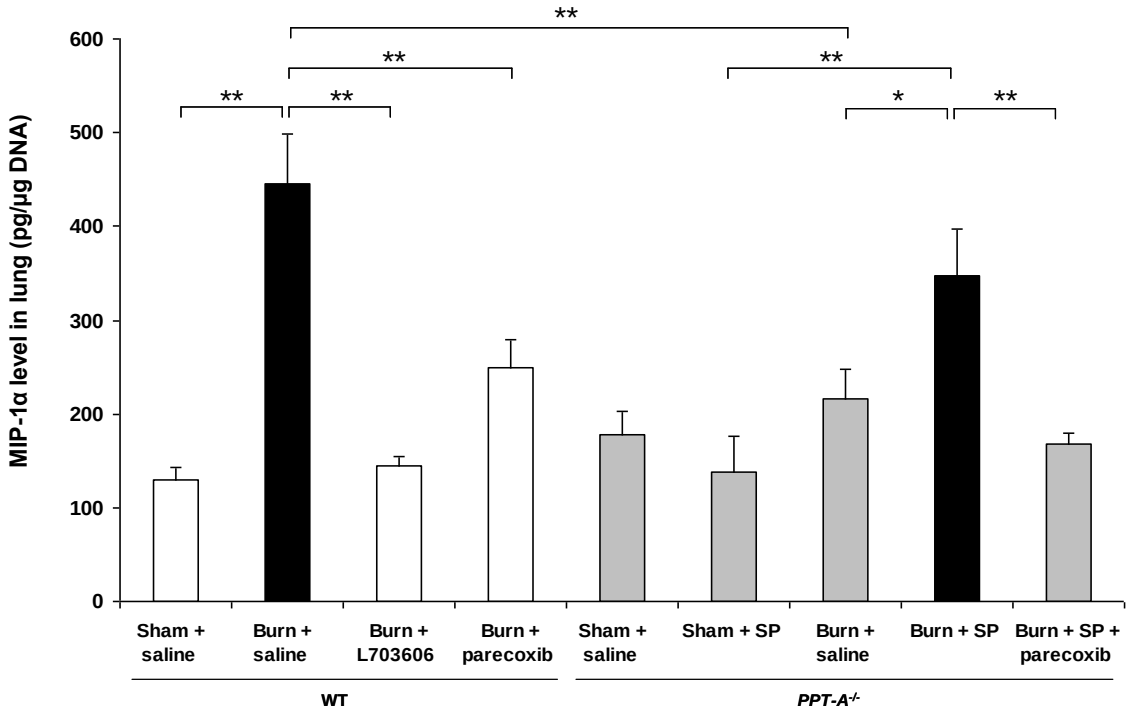


Figure 6.5 Significant reductions in pulmonary cytokines and chemokines after administration of L703606 and parecoxib after burn. Levels of IL-1β (A), IL-6 (B), TNF-α (C), MIP-2 (D) and MIP-1α (E) at 2h in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT mice, L703606 (12mg/kg, i.p., 1h before sham or burn injury) or parecoxib (30mg/kg, i.v., 20min post burn) was administered. *PPT-A*$^{-/-}$ mice injected with SP were also subsequently administered parecoxib (30mg/kg, i.v., 20min post burn). Results shown are the mean values ± SEM (n = 4-7 mice per group). *, $p<0.05$; **, $p<0.01$.

A

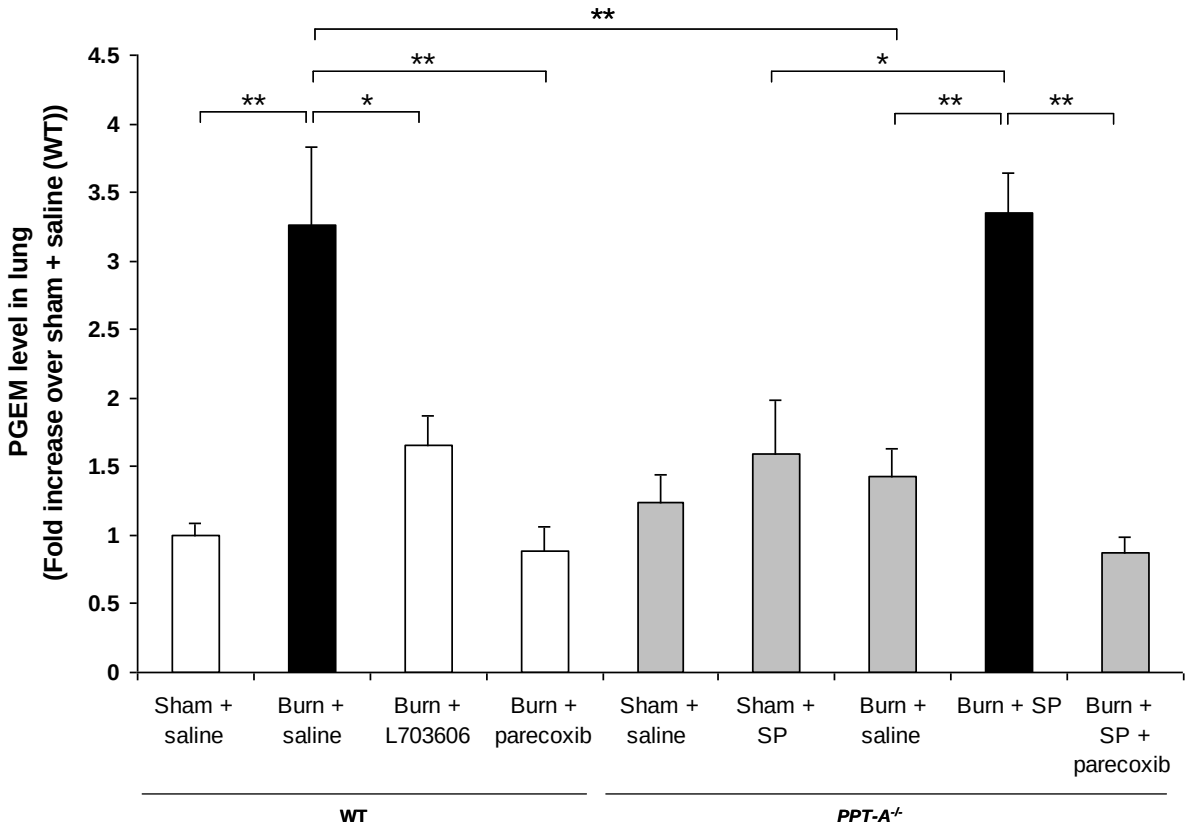
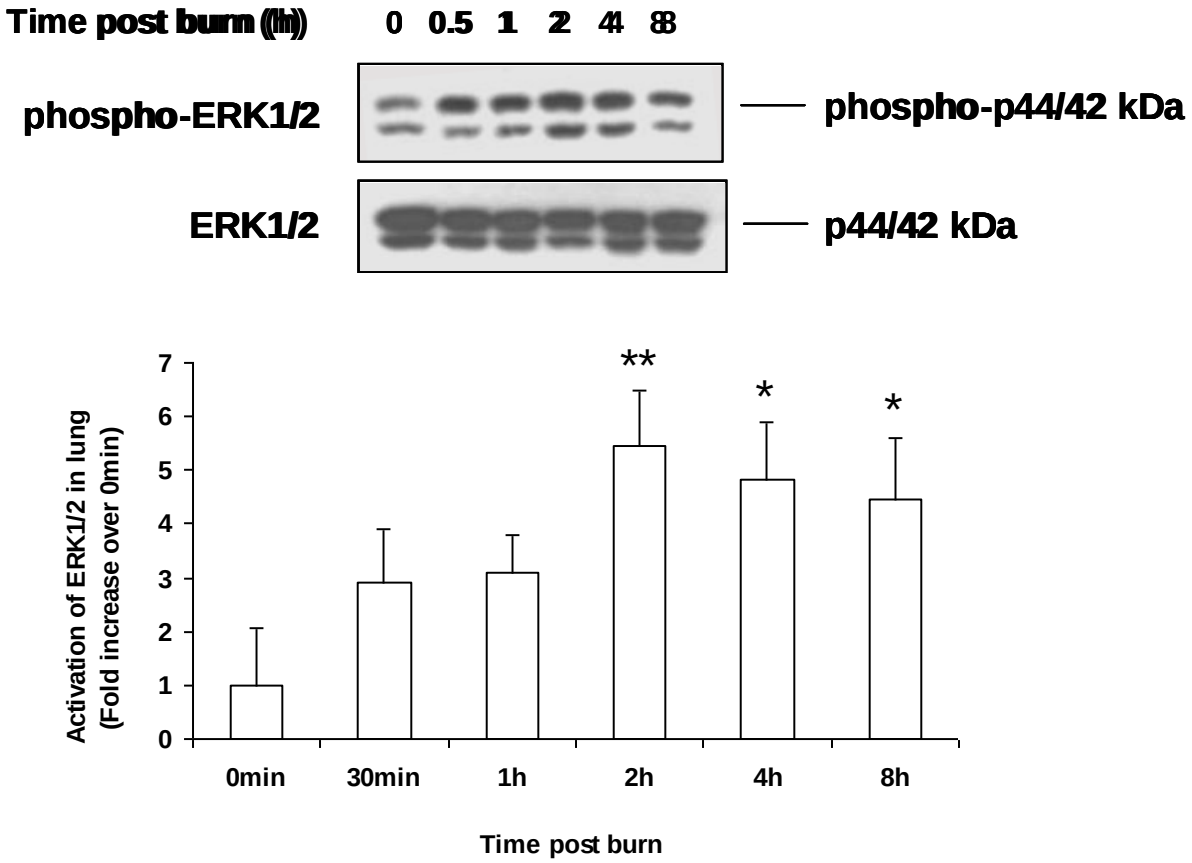
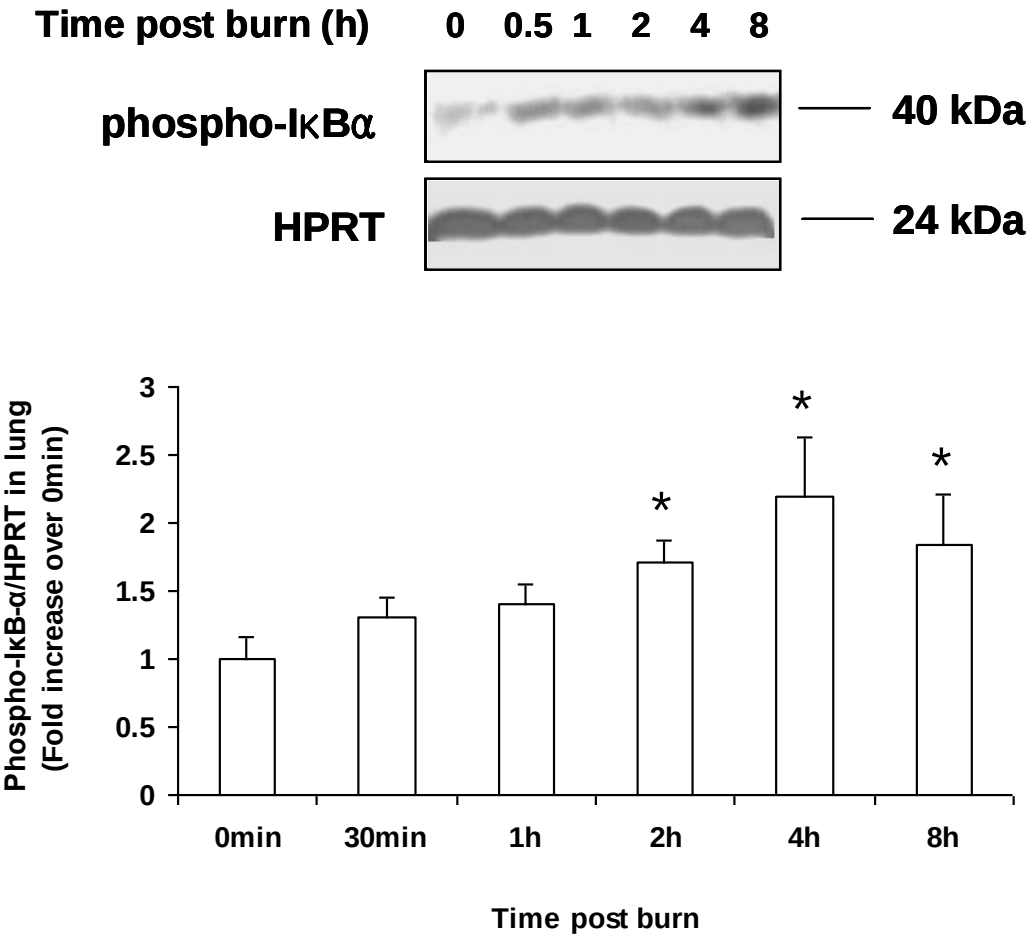


Figure 6.6 Elevation in lung PGEM levels upon activation of SP-NK1R signaling and up-regulation of COX-2 expression following burn injury. (A) Levels of PGEM measured by ELISA at 2h in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT mice, L703606 (12mg/kg, i.p., 1h before sham or burn injury) or parecoxib (30mg/kg, i.v., 20min post burn) was administered. *PPT-A*$^{-/-}$ mice injected with SP were also subsequently administered parecoxib (30mg/kg, i.v., 20min post burn). Results shown are the mean values ± SEM (n = 5-8 mice per group). *, $p<0.05$; **, $p<0.01$.

A


Time post burn (h)
0 0.5 1 2 4 8
phospho-ERK1/2
phospho-p44/42 kDa
ERK1/2
p44/42 kDa
Activation of ERK1/2 in lung
(Fold increase over 0min)
0
1
2
3
4
5
6
7
**
*
*
0min
30min
1h
2h
4h
8h
Time post burn

B


Time post burn (h)
0 0.5 1 2 4 8
phospho-IκBα
40 kDa
HPRT
24 kDa
Phospho-IκB-α/HPRT in lung
(Fold increase over 0min)
3
2.5
2
1.5
1
0.5
0
*
*
*
0min
30min
1h
2h
4h
8h
Time post burn

C

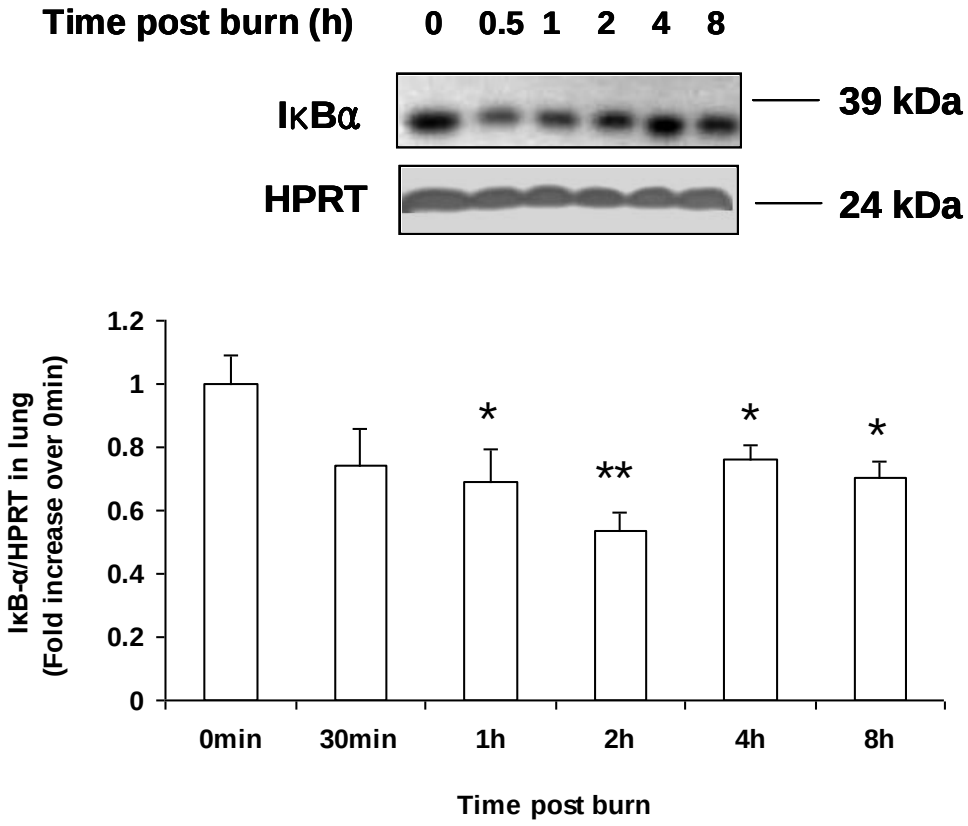


Figure 6.7 Time course study of ERK1/2 activation, phosphorylation and degradation of IκBα levels after burn injury. (A) phospho-ERK, (B) phospho-IκBα, and (C) IκBα expression levels, measured by western blot, in lung of WT mice at 0min, 30min, 1h, 2h, 4h and 8h after burn injury. (n = 4 mice per group).

A

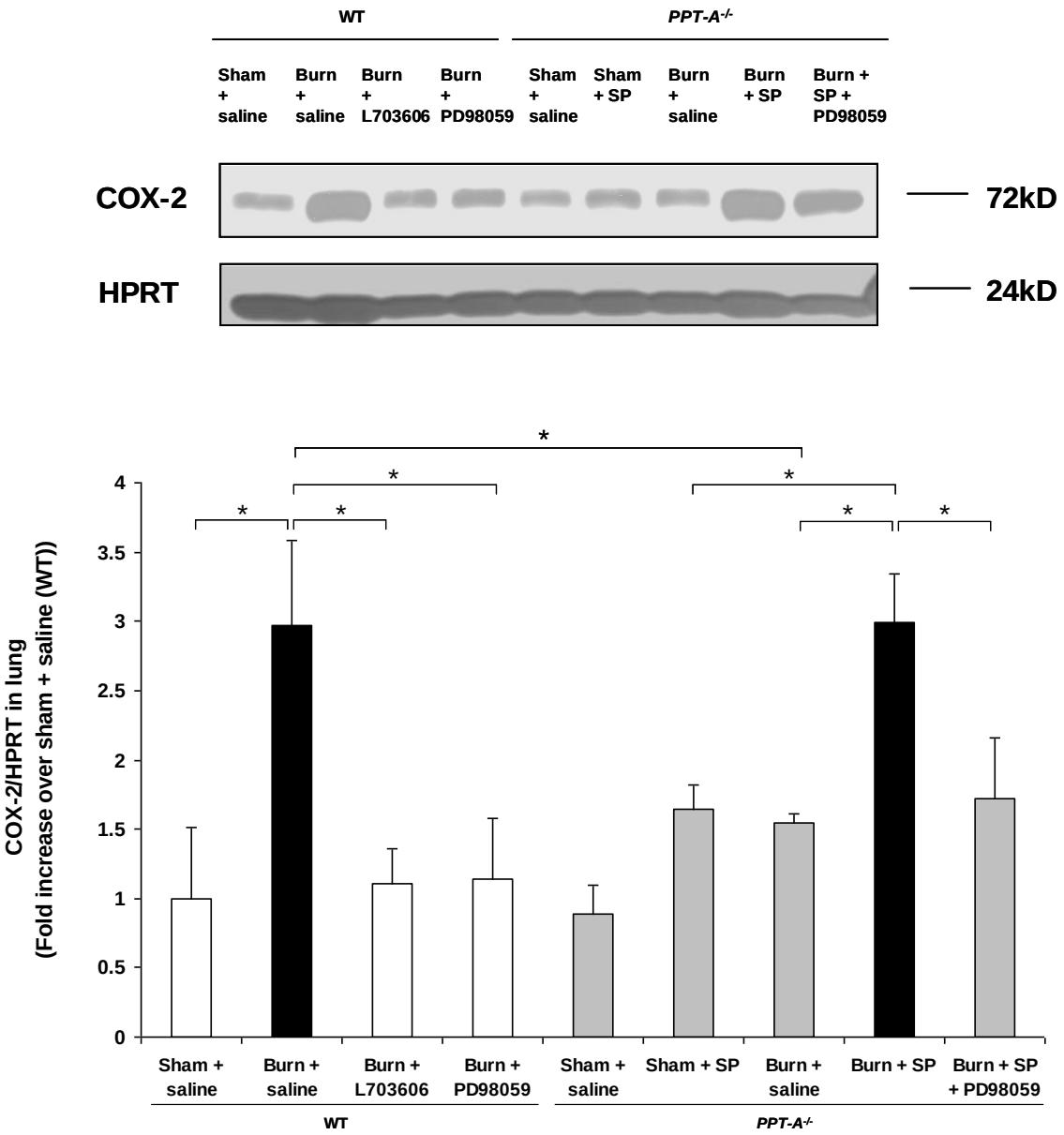
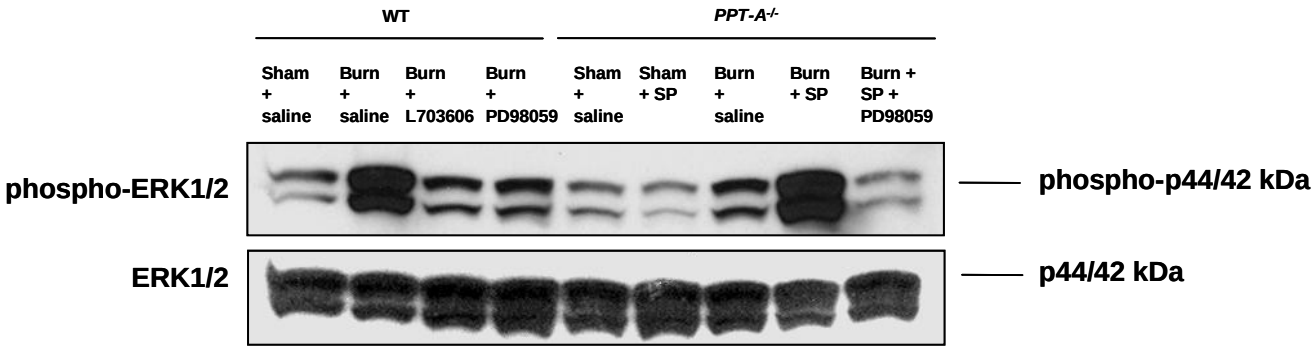


Figure 6.8 Activation of SP-NK1R signaling and ERK1/2 pathway leads to increased COX-2 levels after burn injury. (A) Western blot results showing COX-2 expression levels at 2h in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT or *PPT-A*$^{-/-}$ mice injected with SP, L703606 (12mg/kg, i.p.) or PD98059 (10mg/kg, i.p.) was administered 1h before sham or burn injury. (n = 4 mice per group).

A



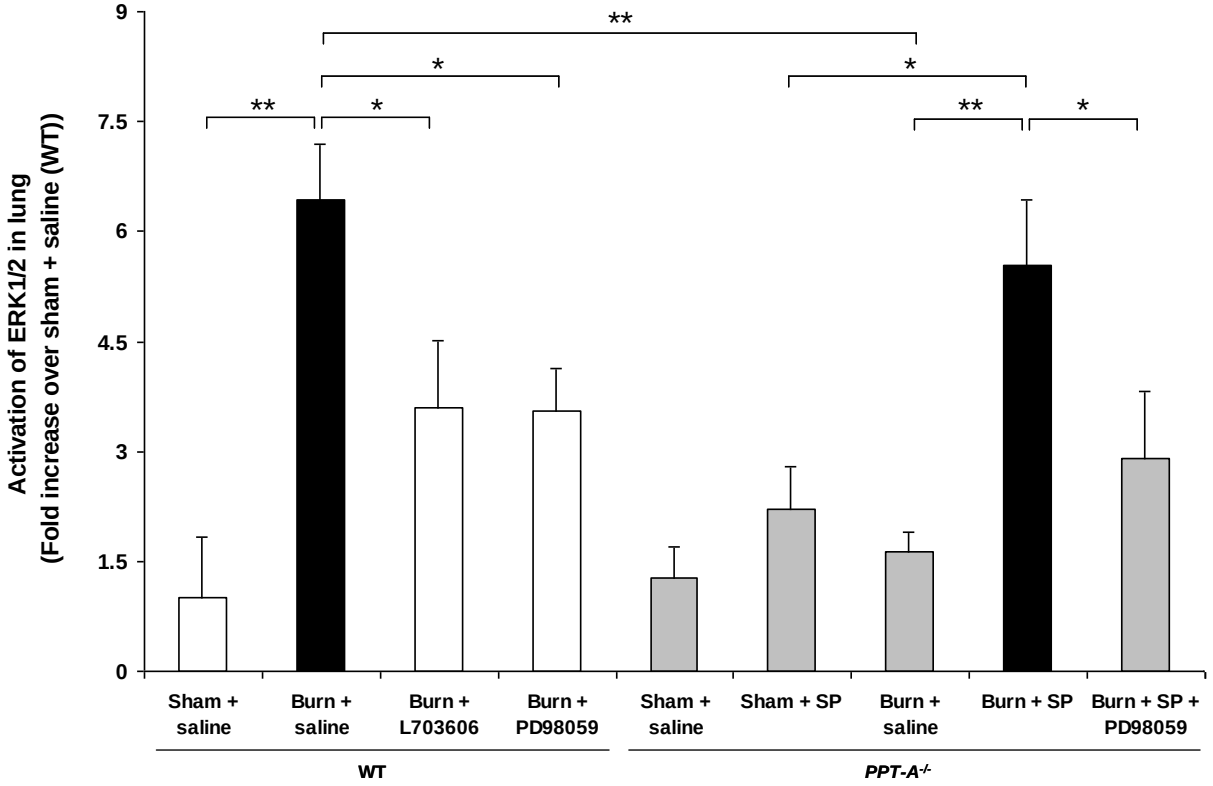
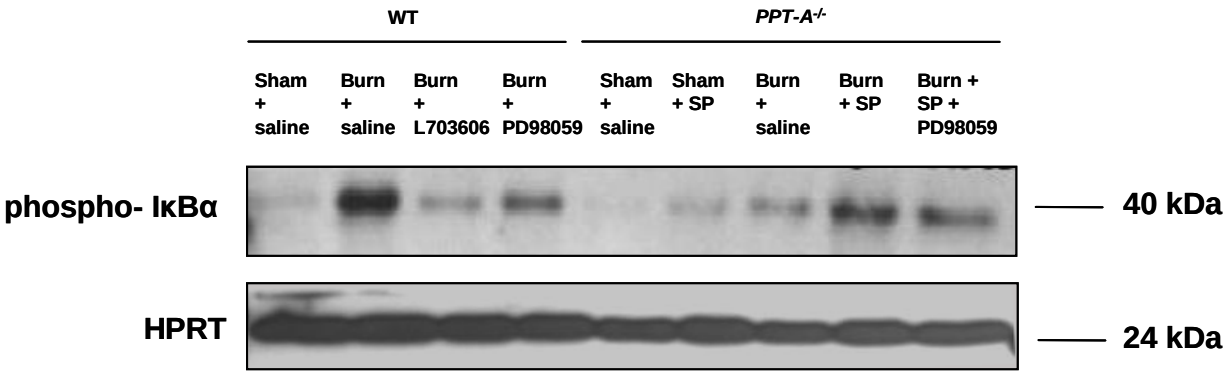
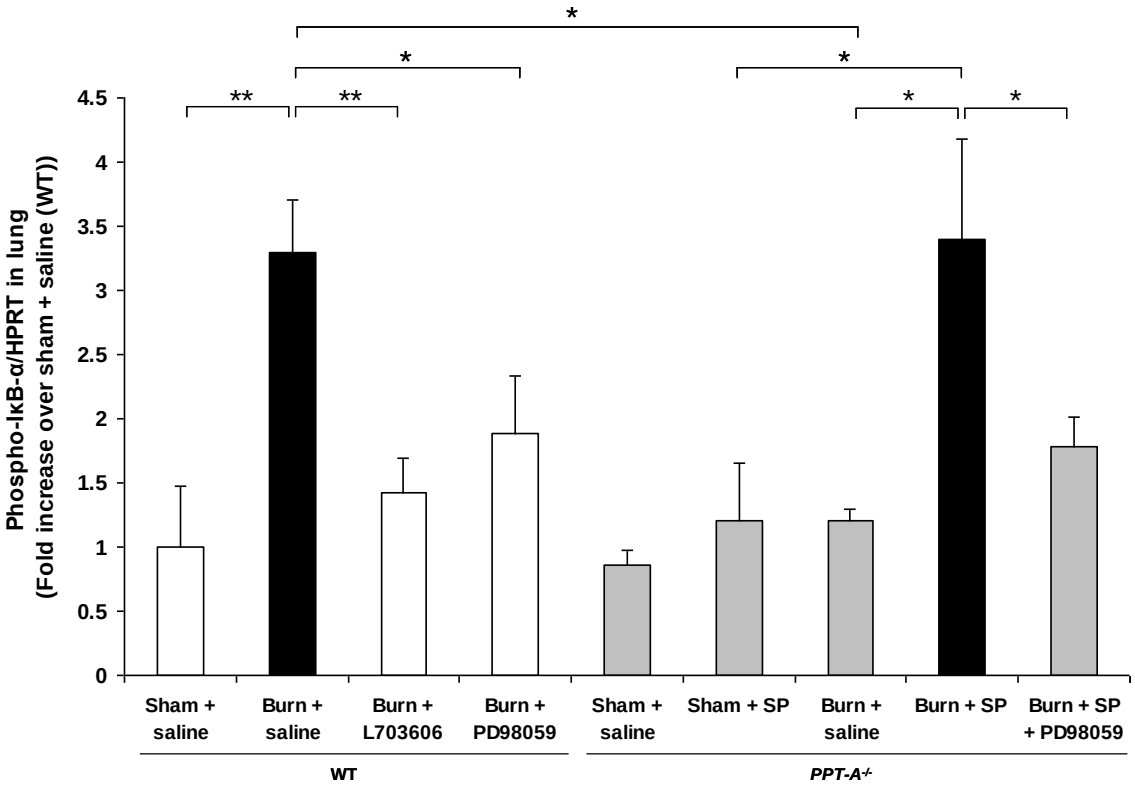


Figure 6.9 SP-NK1R signaling induces the activation of ERK1/2 pathway following burn injury. (A) Western blot results showing ERK1/2 expression levels at 2h in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT or *PPT-A*$^{-/-}$ mice injected with SP, L703606 (12mg/kg, i.p.) or PD98059 (10mg/kg, i.p.) was administered 1h before sham or burn injury. (n = 4 mice per group).

A


WT
PPT-A-/-
Sham + saline
Burn + saline
Burn + L703606
Burn + PD98059
Sham + saline
Sham + SP
Burn + saline
Burn + SP
Burn + SP + PD98059
phospho- IκBα
40 kDa
HPRT
24 kDa


Phospho-IκB-α/HPRT in lung
(Fold increase over sham + saline (WT))
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
**
*
*
*
*
*
Sham + saline
Burn + saline
Burn + L703606
Burn + PD98059
Sham + saline
Sham + SP
Burn + saline
Burn + SP
Burn + SP + PD98059
WT
PPT-A-/-

B

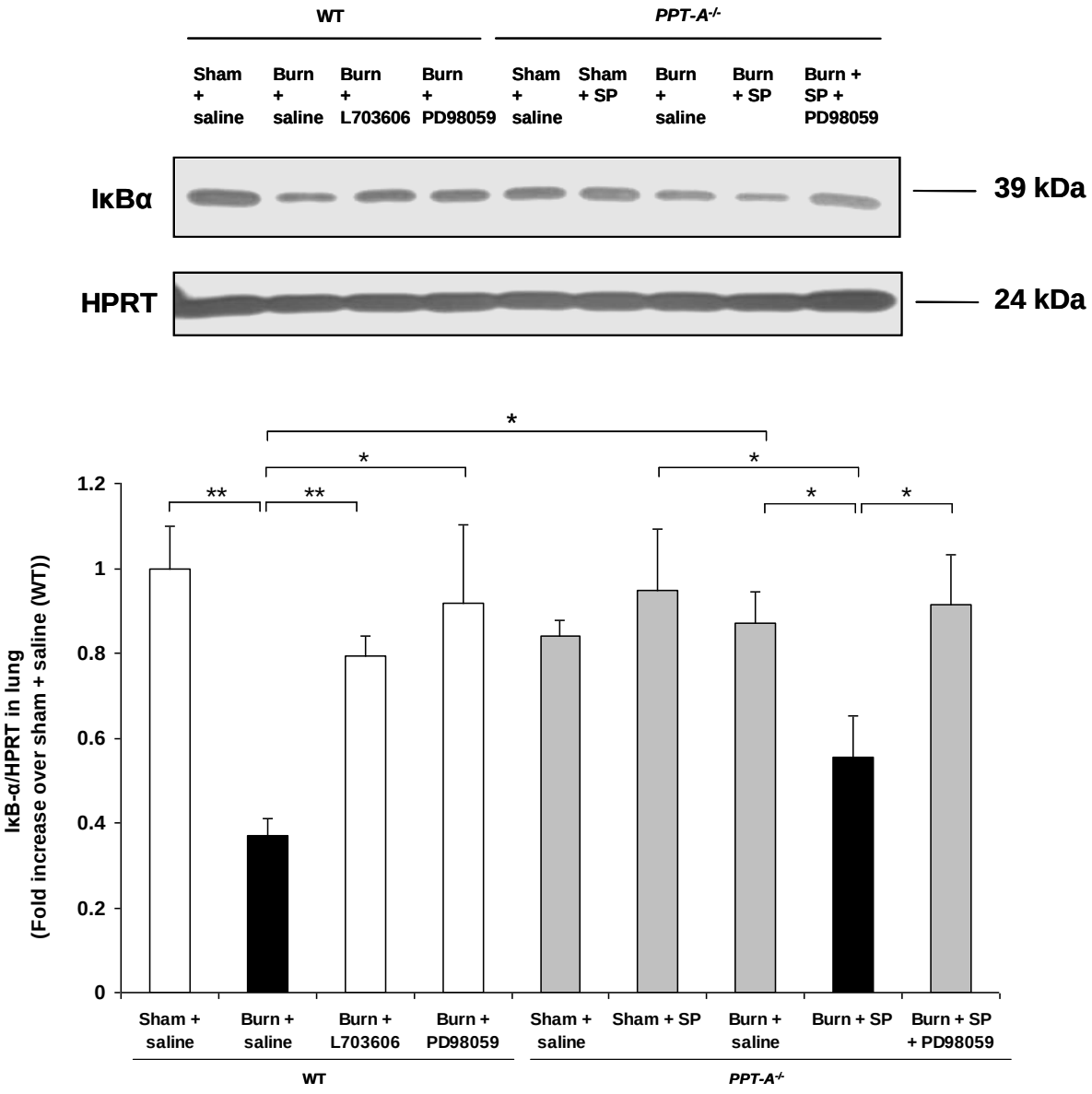


Figure 6.10 SP-NK1R signaling and ERK1/2 pathway incites phosphorylation and degradation of IκBα levels after burn injury. (A) phospho-IκBα and (B) IκBα expression levels, measured by western blot, at 2h in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT or *PPT-A*$^{-/-}$ mice injected with SP, L703606 (12mg/kg, i.p.) or PD98059 (10mg/kg, i.p.) was administered 1h before sham or burn injury. (n = 4 mice per group).

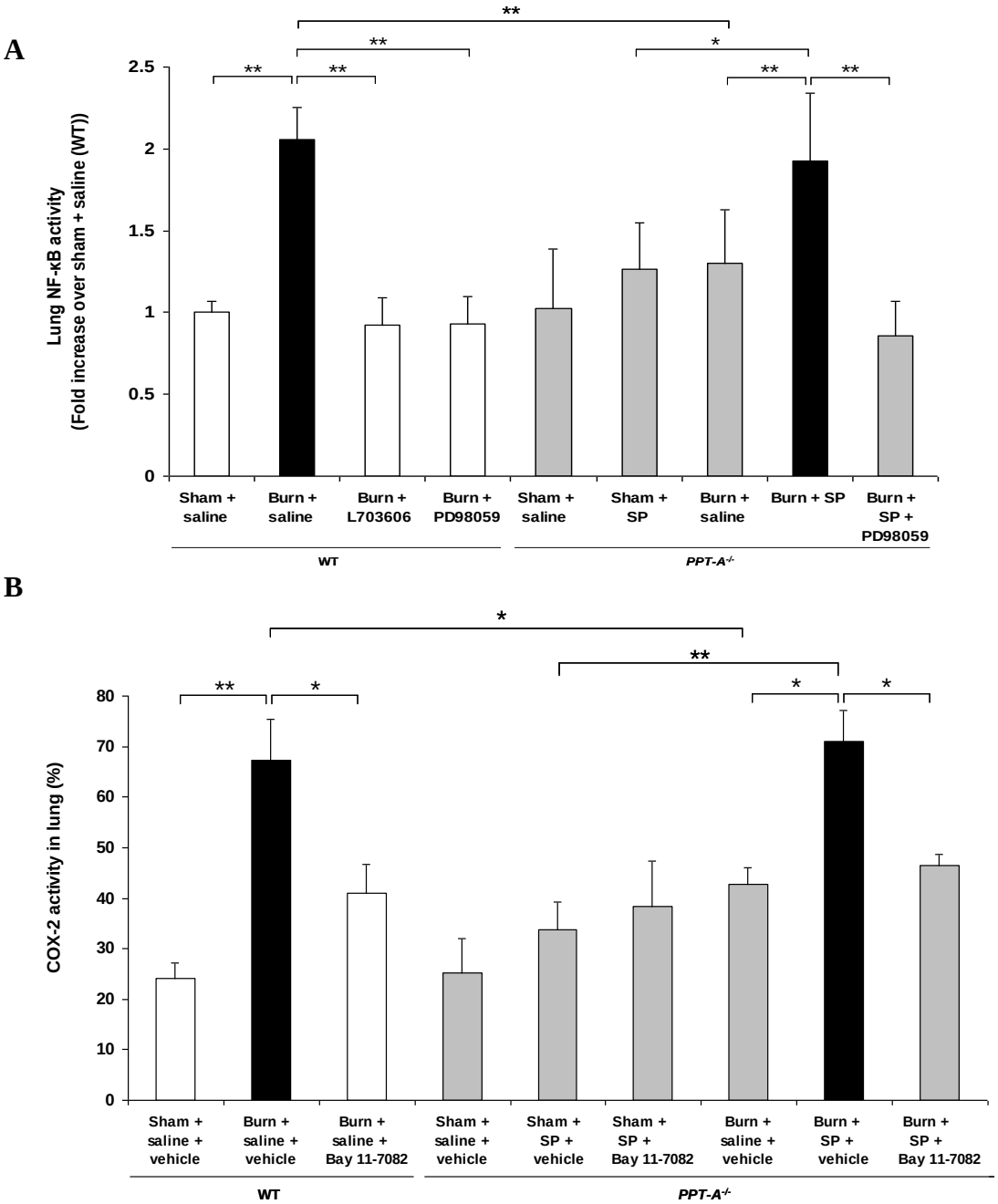


Figure 6.11 Effect of SP-NK1R signaling and ERK1/2 pathway on NF-κB activation following burn injury. (A) NF-κB activation levels, measured by ELISA and (B) COX-2 activity levels at 2h in lung of WT, *PPT-A*$^{-/-}$, and *PPT-A*$^{-/-}$ mice injected with SP (0.12µg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT or *PPT-A*$^{-/-}$ mice injected with SP, L703606 (12mg/kg, i.p.), PD98059 (10mg/kg, i.p.) or Bay 11-7082 (20mg/kg, i.p.) or vehicle, was administered 1h, 1h and 30min before burn injury respectively. (n = 3-9 mice per group). *, $p<0.05$; **, $p<0.01$.

Chapter VII General Discussion, Conclusions and Future Directions

7.1 Significance of results

Burn injury accompanied with severe ALI and respiratory dysfunction is a common clinical disorder in hospitals associated with high morbidity and mortality. However, the early triggers of remote ALI after severe local burn injury remains poorly understood. Therefore, defining the endogenous triggers that initiate and further mediate the early onset of systemic inflammation that leads to remote ALI represents an important goal, with immediate diagnostic, prognostic and therapeutic significance.

There is accumulating evidence that neuropeptides and their receptors are essential mediators of inflammation. The activation of peripheral sensory innervation and neurogenic inflammatory events has been demonstrated to lead to the development and progression of multiple inflammatory diseases (Groneberg et al., 2006; O'Connor et al., 2004). This phenomenon termed, 'neurogenic inflammation', refers to inflammatory responses which results from the release of molecules from primary sensory nerve fibers (Akazawa et al., 2009). The neuropeptide, SP, for instance, has been well established to exert an extensive range of pro-inflammatory neural effects *in vitro* and *in vivo*, modulating many immune and non immune effector cell types (Carolan and Casale, 1993; Harrison and Geppetti, 2001). Studies have also shown that SP via activation of NK1R mediates direct ALI and increased vascular permeability after cigarette (De Swert et al., 2009), oil (Li et al., 2008), and fire (Wong et al., 2004) smoke exposure. However,

the role of SP in instigating indirect and remote ALI after severe local burn injury, even in the absence of inhalational injury has not yet been investigated. Therefore, in the present study, we investigated the role of SP in a mouse model of burn-induced ALI.

The major findings in our study show that male Balb/c mice subjected to 30% total body surface area full thickness exhibited significantly increased endogenous SP production in WT mice, and biological activity of SP-NK1R signaling. The enhanced SP production further resulted in significant elevation of pro-inflammatory cytokines, chemokines, endothelial adhesion molecules and cells counts of neutrophils, monocytes, and lymphocytes in the circulation; concurrent with disruption of pulmonary permeability barrier, excessive neutrophil infiltration and severe ALI. Additionally, decreased neutral endopeptidase and elevated matrix metalloproteinase-9 were evident. Notably, disruption of respiratory function further demonstrated a critical role of SP in lungs after burn injury. More importantly, these effects were significantly attenuated upon administration of L703606, a specific NK1R antagonist and in *PPT-A* gene deficient mice, which encodes for SP; while exogenous administration of SP to *PPT-A*$^{-/-}$ mice restored the inflammatory response and ALI. Additionally, administration of SP analogue peptide in burn-injured WT or *PPT-A*$^{-/-}$ mice failed to aggravate ALI, further verifying the pro-inflammatory role of SP.

In the next part of this study, we investigated the molecular mechanisms by which SP induces remote ALI following severe local burn injury. Our results indicate that SP can work in conjunction with other important inflammatory mediators, such as COX-2, to

incite severe ALI after burn. Specifically, we show that SP acts via NK1R-ERK1/2-NFκB-COX-2 pathway to induce significant up-regulation of PGEM which may further contribute to ALI following severe local burn injury. Furthermore, pharmacological blockade by administration of L703606 and PD98059 in WT mice and treatment of *PPT-A*$^{-/-}$ mice injected with exogenous SP with parecoxib or PD98059, resulted in significant reductions of lung COX-2, PGEM levels and attenuation of severe ALI after burn. Thereby, indicating that SP-NK1R signaling is an important regulator of inflammation after burn which also significantly influences the activity of COX-2.

In conclusion, we provide for the first time, compelling evidence of the multiple roles of SP in instigating heightened inflammatory responses that contribute to the pathophysiology of remote ALI and disruption of breathing function, early after severe local burn injury. More importantly, our findings also indicate that the inhibition or absence of a single endogenous factor, SP, provides early protection against burn-induced ALI. Taken together, an in-depth study of SP and its multiple roles in up-regulating inflammatory pathways, signaling molecules and other prominent mediators of inflammation such as COX-2 and PGE_2 may provide approaches for new forms of therapeutic interventions for the treatment and prevention of an acute pulmonary inflammatory cascade following severe injury in critically ill patients. A summary of the findings in the present study is illustrated in Figure 7.1.

7.2 Conclusions and Future directions

In summary, the important contributions of this study have addressed previously unknown areas. Here, our work presents for the first time substantial evidences of:

- The early ability of SP to incite remote ALI and lung function disruption after severe local burn injury
- SP working via other important inflammatory mediators, such as COX-2 to induce ALI after burn
- the mechanism by which SP acts to induce ALI after burn

Thereby, results of this study may highlight potential novel approaches, useful for the development of new treatments targeted at resolving the early inflammatory responses in critically burn-injured patients. By doing so, preventing the onset of early ALI will prove to have more practical benefit to the patient than efforts to treat ARDS once it has ensued.

Nevertheless, much more research is required to fully elucidate the complex interactions of inflammatory molecules such as SP in the multifaceted pathophysiology of burn injury. Thereby, it is important to understand the simultaneous responses of various systems in response to SP after burn injury. For example, the intricate crosstalk of SP with the immune system could be examined by looking at the effect of SP binding to NK1R present on T cells, macrophages, neutrophils, and monocytes, which are important for understanding how the nervous system influences the immune response after burn injury. In this case, bone marrow derived chimeras could be used to determine the importance of SP-NK1R dependent signaling on the bone marrow derived cells in contributing to the

pathophysiology of burn injury. Futhermore, investigations on the role of SP in the cardiovascular pathology after burn would be an important area as heart failure is a major component of mortality in burn patients. For instance, studies on the responses of parenchymal cells of the cardiovascular system could be investigated.Understanding the interplay between the respiratory, cardiovascular and immune systems would help to understand how early interventions can be developed for the early treatment of patients with severe burns. More efforts may also be made to investigate candidate signaling molecules in inflammatory pathways that can lead to the initiation of ALI such as G-coupled receptor associated proteins (e.g. $G\alpha_q$ and $G\alpha_s$); upstream kinases that mediate the ERK1/2-NFκB pathway including protein kinases, phospholipases, small GTPases and calcium; other members of the MAPK family such as p38 MAPKs and JNK/SAPK family and other potential pathways underlying the pro-inflammatory effects of SP after burn injury. For instance, one interesting and potential pathway which could be examined is the role a more recent and novel protein family, the inflammasomes, in burn injury. Inflammasomes are muti-protein complexes that trigger the maturation of pro-inflammatory cytokines like IL-1β to engage innate immune responses (Schroder and Tschopp, 2010). Studies have shown that the inflammasome, Nlrp3, mediates the development of sterile inflammatory responses (Iyer et al., 2009; Yamasaki et al., 2009). Furthermore, SP has been shown to activate inflammasomes that express NK1R in a rat fracture model (Li et al., 2009). However, to our understanding, it appears that the roles of SP and inflammasomes have not been investigated after burn. Finally, investigations in large animals and human patients would be important to further verify results based on small animals and cell culture systems.

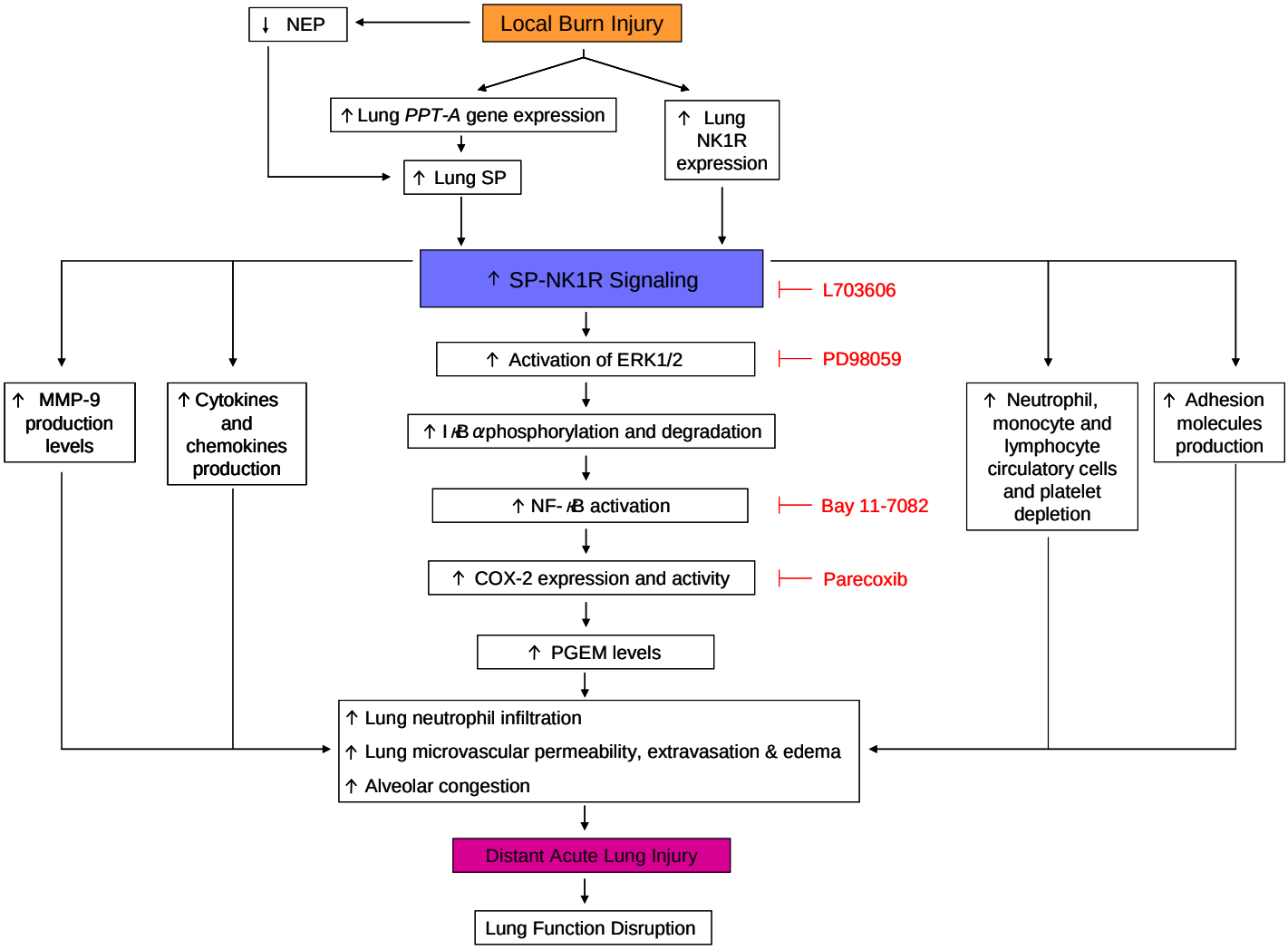


Figure 7.1 Flowchart summarizing the pro-inflammatory effects of SP in ALI following severe local burn injury. Burn injury results in increased *PPT-A* gene expression, an overproduction of endogenous SP and elevated NK1R expression levels, along with a decrease in NEP levels. Together, these contribute to the increased activatation of SP-NK1R signaling which stimulates the phosphorylation of ERK1/2, leading to the phosphorylation and degradation of IκBα as well as the activation of NF-κB. As such, COX-2 and PGEM levels are augmented. SP-NK1R signaling also leads to increased cytokines, chemokines, MMP-9, adhesion molecules, neutrophil, monocyte and lymphocyte cells in blood, and depletion of platelet numbers. The accumulation of all these events, leads to pulmonary vascular leakage, edema, alveolar congestion and ALI, which in turn disrupts lung function. Usage of L703606, PD98059, Bay 11-7082 and parecoxib, which are specific NK1R, MEK-1, NF-κB and COX-2 antagonists respectively confirm these signaling events. ⊢ indicates inhibition.

Bibliography

Aberle DR, Wiener-Kronish JP, Webb WR, Matthay MA. 1988. Hydrostatic versus increased permeability pulmonary edema: diagnosis based on radiographic criteria in critically ill patients. Radiology 168(1):73-79.

Abraham E, Laterre PF, Garbino J, Pingleton S, Butler T, Dugernier T, Margolis B, Kudsk K, Zimmerli W, Anderson P, Reynaert M, Lew D, Lesslauer W, Passe S, Cooper P, Burdeska A, Modi M, Leighton A, Salgo M, Van der Auwera P. 2001. Lenercept (p55 tumor necrosis factor receptor fusion protein) in severe sepsis and early septic shock: a randomized, double-blind, placebo-controlled, multicenter phase III trial with 1,342 patients. Crit Care Med 29(3):503-510.

Adams HR, Baxter CR, Izenberg SD. 1984. Decreased contractility and compliance of the left ventricle as complications of thermal trauma. Am Heart J 108(6):1477-1487.

Adams HR, Baxter CR, Parker JL. 1982. Contractile function of heart muscle from burned guinea pigs. Circ Shock 9(1):63-73.

Adams HR, Baxter CR, Parker JL, Senning R. 1981. Development of acute burn shock in unresuscitated guinea pigs. Circ Shock 8(6):613-625.

Adcock IM, Peters M, Gelder C, Shirasaki H, Brown CR, Barnes PJ. 1993. Increased tachykinin receptor gene expression in asthmatic lung and its modulation by steroids. J Mol Endocrinol 11(1):1-7.

Akazawa T, Kwatra SG, Goldsmith LE, Richardson MD, Cox EA, Sampson JH, Kwatra MM. 2009. A constitutively active form of neurokinin 1 receptor and neurokinin 1 receptor-mediated apoptosis in glioblastomas. J Neurochem 109(4):1079-1086.

Ansel JC, Brown JR, Payan DG, Brown MA. 1993. Substance P selectively activates TNF-alpha gene expression in murine mast cells. J Immunol 150(10):4478-4485.

Antonelli M, Conti G, Rocco M, Bufi M, De Blasi RA, Vivino G, Gasparetto A, Meduri GU. 1998. A comparison of noninvasive positive-pressure ventilation and conventional mechanical ventilation in patients with acute respiratory failure. N Engl J Med 339(7):429-435.

Arturson G. 1996. Pathophysiology of the burn wound and pharmacological treatment. The Rudi Hermans Lecture, 1995. Burns 22(4):255-274.

Ashbaugh DG, Bigelow DB, Petty TL, Levine BE. 1967. Acute respiratory distress in adults. Lancet 2(7511):319-323.

Atkinson JJ, Senior RM. 2003. Matrix metalloproteinase-9 in lung remodeling. Am J Respir Cell Mol Biol 28(1):12-24.

Azzolina A, Bongiovanni A, Lampiasi N. 2003. Substance P induces TNF-alpha and IL-6 production through NF kappa B in peritoneal mast cells. Biochim Biophys Acta 1643(1-3):75-83.

Backstrom JR, Tokes ZA. 1995. The 84-kDa form of human matrix metalloproteinase-9 degrades substance P and gelatin. J Neurochem 64(3):1312-1318.

Baeuerle PA, Baltimore D. 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. Cell 53(2):211-217.

Baluk P, Bertrand C, Geppetti P, McDonald DM, Nadel JA. 1996. NK1 receptor antagonist CP-99,994 inhibits cigarette smoke-induced neutrophil and eosinophil adhesion in rat tracheal venules. Exp Lung Res 22(4):409-418.

Banwell PE, Tyler MP, Watts AM, Roberts AH, McGrouther DA. 1999. Burn depth estimation: use of laser doppler flowmetry. Plast Reconstr Surg 103(1):334-335.

Bao Z, Guan S, Cheng C, Wu S, Wong SH, Kemeny DM, Leung BP, Wong WS. 2009. A novel antiinflammatory role for andrographolide in asthma via inhibition of the nuclear factor-kappaB pathway. Am J Respir Crit Care Med 179(8):657-665.

Barnes PJ, Karin M. 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336(15):1066-1071.

Barret JP. 2002. Cost-containment and outcome measures. In: Herndon D, editor. Total burn care. London, England: Saunders. p 740-746.

Barton GM. 2008. A calculated response: control of inflammation by the innate immune system. J Clin Invest 118(2):413-420.

Baue AE, Durham R, Faist E. 1998. Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle? Shock 10(2):79-89.

Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg R. 1994. The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. Am J Respir Crit Care Med 149(3 Pt 1):818-824.

Bertrand C, Geppetti P, Graf PD, Foresi A, Nadel JA. 1993. Involvement of neurogenic inflammation in antigen-induced bronchoconstriction in guinea pigs. Am J Physiol 265(5 Pt 1):L507-511.

Bessey PQ, Jiang ZM, Johnson DJ, Smith RJ, Wilmore DW. 1989. Posttraumatic skeletal muscle proteolysis: the role of the hormonal environment. World J Surg 13(4):465-470; discussion 471.

Betsuyaku T, Fukuda Y, Parks WC, Shipley JM, Senior RM. 2000. Gelatinase B is required for alveolar bronchiolization after intratracheal bleomycin. Am J Pathol 157(2):525-535.

Bhatia M, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J. 2000a. Inflammatory mediators in acute pancreatitis. J Pathol 190(2):117-125.

Bhatia M, Brady M, Zagorski J, Christmas SE, Campbell F, Neoptolemos JP, Slavin J. 2000b. Treatment with neutralising antibody against cytokine induced neutrophil chemoattractant (CINC) protects rats against acute pancreatitis associated lung injury. Gut 47(6):838-844.

Bhatia M, Moochhala S. 2004. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. J Pathol 202(2):145-156.

Bhatia M, Neoptolemos JP, Slavin J. 2001. Inflammatory mediators as therapeutic targets in acute pancreatitis. Curr Opin Investig Drugs 2(4):496-501.

Bhatia M, Saluja AK, Hofbauer B, Frossard JL, Lee HS, Castagliuolo I, Wang CC, Gerard N, Pothoulakis C, Steer ML. 1998a. Role of substance P and the neurokinin 1 receptor in acute pancreatitis and pancreatitis-associated lung injury. Proc Natl Acad Sci U S A 95(8):4760-4765.

Bhatia M, Saluja AK, Hofbauer B, Lee HS, Frossard JL, Steer ML. 1998b. The effects of neutrophil depletion on a completely noninvasive model of acute pancreatitis-associated lung injury. Int J Pancreatol 24(2):77-83.

Bhatia M, Slavin J, Cao Y, Basbaum AI, Neoptolemos JP. 2003. Preprotachykinin-A gene deletion protects mice against acute pancreatitis and associated lung injury. Am J Physiol Gastrointest Liver Physiol 284(5):G830-836.

Biffl WL, Moore EE, Moore FA, Peterson VM. 1996. Interleukin-6 in the injured patient. Marker of injury or mediator of inflammation? Ann Surg 224(5):647-664.

Bird TA, Schooley K, Dower SK, Hagen H, Virca GD. 1997. Activation of nuclear transcription factor NF-kappaB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. J Biol Chem 272(51):32606-32612.

Blakeney PE, Fauerbach JA, Meyer III WJ, Thomas CR. 2002. Psychosocial recovery and reintegration of patients with burn injuries. In: Herndon D, editor. Total burn care. London, England: Saunder. p 783-797.

Bohuslav J, Chen LF, Kwon H, Mu Y, Greene WC. 2004. p53 induces NF-kappaB activation by an IkappaB kinase-independent mechanism involving phosphorylation of p65 by ribosomal S6 kinase 1. J Biol Chem 279(25):26115-26125.

Bone RC. 1992. Toward an epidemiology and natural history of SIRS (systemic inflammatory response syndrome). Jama 268(24):3452-3455.

Bongianni F, Mutolo D, Cinelli E, Pantaleo T. 2008. Neurokinin receptor modulation of respiratory activity in the rabbit. Eur J Neurosci 27(12):3233-3243.

Borson DB. 1991. Roles of neutral endopeptidase in airways. Am J Physiol 260(4 Pt 1):L212-225.

Bothwell W, Verburg M, Wynalda M, Daniels EG, Fitzpatrick FA. 1982. A radioimmunoassay for the unstable pulmonary metabolites of prostaglandin E1 and E2: an indirect index of their in vivo disposition and pharmacokinetics. J Pharmacol Exp Ther 220(2):229-235.

Bowden JJ, Garland AM, Baluk P, Lefevre P, Grady EF, Vigna SR, Bunnett NW, McDonald DM. 1994. Direct observation of substance P-induced internalization of neurokinin 1 (NK1) receptors at sites of inflammation. Proc Natl Acad Sci U S A 91(19):8964-8968.

Bozic CR, Lu B, Hopken UE, Gerard C, Gerard NP. 1996. Neurogenic amplification of immune complex inflammation. Science 273(5282):1722-1725.

Braunstein G, Fajac I, Lacronique J, Frossard N. 1991. Clinical and inflammatory responses to exogenous tachykinins in allergic rhinitis. Am Rev Respir Dis 144(3 Pt 1):630-635.

Brimijoin S, Lundberg JM, Brodin E, Hokfelt T, Nilsson G. 1980. Axonal transport of substance P in the vagus and sciatic nerves of the guinea pig. Brain Res 191(2):443-457.

Brogden KA, Guthmiller JM, Salzet M, Zasloff M. 2005. The nervous system and innate immunity: the neuropeptide connection. Nat Immunol 6(6):558-564.

Browne G, Byrne C, Brown B, Pennock M, Streiner D, Roberts R, Eyles P, Truscott D, Dabbs R. 1985. Psychosocial adjustment of burn survivors. Burns Incl Therm Inj 12(1):28-35.

Brun-Buisson C. 2000. The epidemiology of the systemic inflammatory response. Intensive Care Med 26 Suppl 1:S64-74.

Burns JA, Issekutz TB, Yagita H, Issekutz AC. 2001. The alpha 4 beta 1 (very late antigen (VLA)-4, CD49d/CD29) and alpha 5 beta 1 (VLA-5, CD49e/CD29) integrins mediate beta 2 (CD11/CD18) integrin-independent neutrophil recruitment to endotoxin-induced lung inflammation. J Immunol 166(7):4644-4649.

Cadier MA, Shakespeare PG. 1995. Burns in octogenarians. Burns 21(3):200-204.

Calvo CF, Chavanel G, Senik A. 1992. Substance P enhances IL-2 expression in activated human T cells. J Immunol 148(11):3498-3504.

Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ, Basbaum AI. 1998. Primary afferent tachykinins are required to experience moderate to intense pain. Nature 392(6674):390-394.

Carlsson A. 2001. A paradigm shift in brain research. Science 294(5544):1021-1024.

Carolan EJ, Casale TB. 1993. Effects of neuropeptides on neutrophil migration through noncellular and endothelial barriers. J Allergy Clin Immunol 92(4):589-598.

Carpagnano GE, Spanevello A, Palladino GP, Gramiccioni C, Ruggieri C, Carpagnano F, Foschino Barbaro MP. 2009. Cigarette smoke and increased COX-2 and survivin levels in exhaled breath condensate of lung cancer patients: How hot is the link? Lung Cancer.

Carr MJ, Hunter DD, Jacoby DB, Undem BJ. 2002. Expression of tachykinins in nonnociceptive vagal afferent neurons during respiratory viral infection in guinea pigs. Am J Respir Crit Care Med 165(8):1071-1075.

Cascieri MA, Ber E, Fong TM, Sadowski S, Bansal A, Swain C, Seward E, Frances B, Burns D, Strader CD. 1992. Characterization of the binding of a potent, selective, radioiodinated antagonist to the human neurokinin-1 receptor. Mol Pharmacol 42(3):458-463.

Castell JV, Gomez-Lechon MJ, David M, Andus T, Geiger T, Trullenque R, Fabra R, Heinrich PC. 1989. Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. FEBS Lett 242(2):237-239.

Chang L, Karin M. 2001. Mammalian MAP kinase signalling cascades. Nature 410(6824):37-40.

Chang MM, Leeman SE, Niall HD. 1971. Amino-acid sequence of substance P. Nat New Biol 232(29):86-87.

Chang W, Chen J, Schlueter CF, Hoyle GW. 2009. Common pathways for activation of proinflammatory gene expression by G protein-coupled receptors in primary lung epithelial and endothelial cells. Exp Lung Res 35(4):324-343.

Chen L, Fischle W, Verdin E, Greene WC. 2001. Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science 293(5535):1653-1657.

Chen LF, Greene WC. 2003. Regulation of distinct biological activities of the NF-kappaB transcription factor complex by acetylation. J Mol Med 81(9):549-557.

Chen LW, Hsu CM, Cha MC, Chen JS, Chen SC. 1999. Changes in gut mucosal nitric oxide synthase (NOS) activity after thermal injury and its relation with barrier failure. Shock 11(2):104-110.

Chen Y, Chen P, Hanaoka M, Droma Y, Kubo K. 2008. Enhanced levels of prostaglandin E2 and matrix metalloproteinase-2 correlate with the severity of airflow limitation in stable COPD. Respirology 13(7):1014-1021.

Chen Z, Hedner J, Hedner T. 1990. Substance P in the ventrolateral medulla oblongata regulates ventilatory responses. J Appl Physiol 68(6):2631-2639.

Chen Z, Hedner T, Hedner J. 1988. Hypoventilation and apnoea induced by the substance P antagonist [D-Pro2,D-Trp7,9]-SP in the ventrolateral rat medulla. Acta Physiol Scand 134(1):153-154.

Church D, Elsayed S, Reid O, Winston B, Lindsay R. 2006. Burn wound infections. Clin Microbiol Rev 19(2):403-434.

Clapham DE. 2003. TRP channels as cellular sensors. Nature 426(6966):517-524.

Cohen J. 2002. The immunopathogenesis of sepsis. Nature 420(6917):885-891.

Coussens LM, Tinkle CL, Hanahan D, Werb Z. 2000. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell 103(3):481-490.

Cuesta MC, Quintero L, Pons H, Suarez-Roca H. 2002. Substance P and calcitonin gene-related peptide increase IL-1 beta, IL-6 and TNF alpha secretion from human peripheral blood mononuclear cells. Neurochem Int 40(4):301-306.

Cuzzocrea S, Mazzon E, Sautebin L, Dugo L, Serraino I, De Sarro A, Caputi AP. 2002. Protective effects of Celecoxib on lung injury and red blood cells modification induced by carrageenan in the rat. Biochem Pharmacol 63(4):785-795.

Dancey DR, Hayes J, Gomez M, Schouten D, Fish J, Peters W, Slutsky AS, Stewart TE. 1999. ARDS in patients with thermal injury. Intensive Care Med 25(11):1231-1236.

De Swert KO, Bracke KR, Demoor T, Brusselle GG, Joos GF. 2009. Role of the tachykinin NK1 receptor in a murine model of cigarette smoke-induced pulmonary inflammation. Respir Res 10:37.

Deitch EA. 1992. Multiple organ failure. Pathophysiology and potential future therapy. Ann Surg 216(2):117-134.

Delgado AV, McManus AT, Chambers JP. 2003. Production of tumor necrosis factor-alpha, interleukin 1-beta, interleukin 2, and interleukin 6 by rat leukocyte subpopulations after exposure to substance P. Neuropeptides 37(6):355-361.

DeLorme MP, Moss OR. 2002. Pulmonary function assessment by whole-body plethysmography in restrained versus unrestrained mice. J Pharmacol Toxicol Methods 47(1):1-10.

Demling R, LaLonde C, Heron P. 1994. Initial effect of smoke inhalation injury on oxygen consumption (response to positive pressure ventilation). Surgery 115(5):563-570.

Demling RH. 1985. Burns. N Engl J Med 313(22):1389-1398.

Demling RH, Seigne P. 2000. Metabolic management of patients with severe burns. World J Surg 24(6):673-680.

Denis M, Guojian L, Widmer M, Cantin A. 1994. A mouse model of lung injury induced by microbial products: implication of tumor necrosis factor. Am J Respir Cell Mol Biol 10(6):658-664.

DeRose V, Robbins RA, Snider RM, Spurzem JR, Thiele GM, Rennard SI, Rubinstein I. 1994. Substance P increases neutrophil adhesion to bronchial epithelial cells. J Immunol 152(3):1339-1346.

Devillier P, Acker GM, Advenier C, Marsac J, Regoli D, Frossard N. 1992. Activation of an epithelial neurokinin NK-1 receptor induces relaxation of rat trachea through release of prostaglandin E2. J Pharmacol Exp Ther 263(2):767-772.

Di Maria GU, Bellofiore S, Geppetti P. 1998. Regulation of airway neurogenic inflammation by neutral endopeptidase. Eur Respir J 12(6):1454-1462.

Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA, Jr., O'Connor JV. 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J Exp Med 163(6):1433-1450.

Dinarello CA, Okusawa S, Gelfand JA. 1989. Interleukin-1 induces a shock-like state in rabbits: synergism with tumor necrosis factor and the effect of ibuprofen. Prog Clin Biol Res 299:203-215.

Drews J. 2000. Drug discovery: a historical perspective. Science 287(5460):1960-1964.

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE. 1998. Cyclooxygenase in biology and disease. Faseb J 12(12):1063-1073.

Duffield-Lillico AJ, Boyle JO, Zhou XK, Ghosh A, Butala GS, Subbaramaiah K, Newman RA, Morrow JD, Milne GL, Dannenberg AJ. 2009. Levels of prostaglandin E metabolite and leukotriene E(4) are increased in the urine of smokers: evidence that celecoxib shunts arachidonic acid into the 5-lipoxygenase pathway. Cancer Prev Res (Phila Pa) 2(4):322-329.

Dunnick CA, Gibran NS, Heimbach DM. 1996. Substance P has a role in neurogenic mediation of human burn wound healing. J Burn Care Rehabil 17(5):390-396.

Dusser DJ, Djokic TD, Borson DB, Nadel JA. 1989. Cigarette smoke induces bronchoconstrictor hyperresponsiveness to substance P and inactivates airway neutral endopeptidase in the guinea pig. Possible role of free radicals. J Clin Invest 84(3):900-906.

Eichenholz PW, Eichacker PQ, Hoffman WD, Banks SM, Parrillo JE, Danner RL, Natanson C. 1992. Tumor necrosis factor challenges in canines: patterns of cardiovascular dysfunction. Am J Physiol 263(3 Pt 2):H668-675.

Elling CE, Nielsen SM, Schwartz TW. 1995. Conversion of antagonist-binding site to metal-ion site in the tachykinin NK-1 receptor. Nature 374(6517):74-77.

Enkhbaatar P, Traber DL. 2004. Pathophysiology of acute lung injury in combined burn and smoke inhalation injury. Clin Sci (Lond) 107(2):137-143.

Espiritu RF, Pittet JF, Matthay MA, Goetzl EJ. 1992. Neuropeptides in pulmonary edema fluid of adult respiratory distress syndrome. Inflammation 16(5):509-517.

Falcone AE, Spadaro JA. 1986. Inhibitory effects of electrically activated silver material on cutaneous wound bacteria. Plast Reconstr Surg 77(3):455-459.

Feldman JL, Del Negro CA. 2006. Looking for inspiration: new perspectives on respiratory rhythm. Nat Rev Neurosci 7(3):232-242.

Fischer A, McGregor GP, Saria A, Philippin B, Kummer W. 1996. Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation. J Clin Invest 98(10):2284-2291.

FitzGerald GA, Patrono C. 2001. The coxibs, selective inhibitors of cyclooxygenase-2. N Engl J Med 345(6):433-442.

Folkers K, Hakanson R, Horig J, Xu JC, Leander S. 1984. Biological evaluation of substance P antagonists. Br J Pharmacol 83(2):449-456.

Folkers K, Rosell S, Chu JY, Lu LA, Tang PF, Ljungqvist A. 1986. Design and synthesis of antagonists of substance P. Acta Chem Scand B 40(4):295-302.
Folkers K, Rosell S, Xu JC, Bjorkroth U, Lu YA, Liu YZ. 1983. Antagonists of substance P from emphasis on position 11. Acta Chem Scand B 37(7):623-627.
Fong TM, Huang RR, Strader CD. 1992. Localization of agonist and antagonist binding domains of the human neurokinin-1 receptor. J Biol Chem 267(36):25664-25667.
Fortner CN, Breyer RM, Paul RJ. 2001. EP2 receptors mediate airway relaxation to substance P, ATP, and PGE2. Am J Physiol Lung Cell Mol Physiol 281(2):L469-474.
Francis BE, Swain C, Sabin V, Burns HD. 1994. Radioiodinated L-703,606: a potent, selective antagonist to the human NK1 receptor. Appl Radiat Isot 45(1):97-103.
Fujimi S, MacConmara MP, Maung AA, Zang Y, Mannick JA, Lederer JA, Lapchak PH. 2006. Platelet depletion in mice increases mortality after thermal injury. Blood 107(11):4399-4406.
Fukunaga K, Kohli P, Bonnans C, Fredenburgh LE, Levy BD. 2005. Cyclooxygenase 2 plays a pivotal role in the resolution of acute lung injury. J Immunol 174(8):5033-5039.
Funk CD. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294(5548):1871-1875.
Gallicchio M, Benetti E, Rosa AC, Fantozzi R. 2009. Tachykinin receptor modulation of cyclooxygenase-2 expression in human polymorphonuclear leucocytes. Br J Pharmacol 156(3):486-496.
Gallicchio M, Rosa AC, Benetti E, Collino M, Dianzani C, Fantozzi R. 2006. Substance P-induced cyclooxygenase-2 expression in human umbilical vein endothelial cells. Br J Pharmacol 147(6):681-689.
Geiger T, Andus T, Klapproth J, Hirano T, Kishimoto T, Heinrich PC. 1988. Induction of rat acute-phase proteins by interleukin 6 in vivo. Eur J Immunol 18(5):717-721.
George A, Bang RL, Lari AR, Gang RK. 2001. Acute thrombocytopenic crisis following burns complicated by staphylococcal septicaemia. Burns 27(1):84-88.
Gerard NP, Garraway LA, Eddy RL, Jr., Shows TB, Iijima H, Paquet JL, Gerard C. 1991. Human substance P receptor (NK-1): organization of the gene, chromosome localization, and functional expression of cDNA clones. Biochemistry 30(44):10640-10646.
Gerding RL, Emerman CL, Effron D, Lukens T, Imbembo AL, Fratianne RB. 1990. Outpatient management of partial-thickness burns: Biobrane versus 1% silver sulfadiazine. Ann Emerg Med 19(2):121-124.
Ghosh S, Hayden MS. 2008. New regulators of NF-kappaB in inflammation. Nat Rev Immunol 8(11):837-848.
Ghosh S, Karin M. 2002. Missing pieces in the NF-kappaB puzzle. Cell 109 Suppl:S81-96.
Giannoudis PV, Smith RM, Banks RE, Windsor AC, Dickson RA, Guillou PJ. 1998. Stimulation of inflammatory markers after blunt trauma. Br J Surg 85(7):986-990.
Giroir BP, Horton JW, White DJ, McIntyre KL, Lin CQ. 1994. Inhibition of tumor necrosis factor prevents myocardial dysfunction during burn shock. Am J Physiol 267(1 Pt 2):H118-124.

Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maunder RJ, Kunkel SL, Walz A, Hudson LD, Martin TR. 1996. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. Am J Respir Crit Care Med 154(3 Pt 1):602-611.

Grady EF, Garland AM, Gamp PD, Lovett M, Payan DG, Bunnett NW. 1995. Delineation of the endocytic pathway of substance P and its seven-transmembrane domain NK1 receptor. Mol Biol Cell 6(5):509-524.

Graham GJ, Stevens JM, Page NM, Grant AD, Brain SD, Lowry PJ, Gibbins JM. 2004. Tachykinins regulate the function of platelets. Blood 104(4):1058-1065.

Granstrom E, Hamberg M, Hansson G, Kindahl H. 1980. Chemical instability of 15-keto-13,14-dihydro-PGE2: the reason for low assay reliability. Prostaglandins 19(6):933-957.

Gray PA, Janczewski WA, Mellen N, McCrimmon DR, Feldman JL. 2001. Normal breathing requires preBotzinger complex neurokinin-1 receptor-expressing neurons. Nat Neurosci 4(9):927-930.

Grbic JT, Mannick JA, Gough DB, Rodrick ML. 1991. The role of prostaglandin E2 in immune suppression following injury. Ann Surg 214(3):253-262; discussion 262-263.

Greengard P. 2001. The neurobiology of slow synaptic transmission. Science 294(5544):1024-1030.

Grimm MC, Ben-Baruch A, Taub DD, Howard OM, Wang JM, Oppenheim JJ. 1998. Opiate inhibition of chemokine-induced chemotaxis. Ann N Y Acad Sci 840:9-20.

Grimsholm O, Guo Y, Ny T, Rantapaa-Dahlqvist S, Forsgren S. 2007. Are neuropeptides important in arthritis? Studies on the importance of bombesin/GRP and substance P in a murine arthritis model. Ann N Y Acad Sci 1110:525-538.

Groneberg DA, Eynott PR, Doring F, Dinh QT, Oates T, Barnes PJ, Chung KF, Daniel H, Fischer A. 2002. Distribution and function of the peptide transporter PEPT2 in normal and cystic fibrosis human lung. Thorax 57(1):55-60.

Groneberg DA, Harrison S, Dinh QT, Geppetti P, Fischer A. 2006. Tachykinins in the respiratory tract. Curr Drug Targets 7(8):1005-1010.

Groneberg DA, Nickolaus M, Springer J, Doring F, Daniel H, Fischer A. 2001. Localization of the peptide transporter PEPT2 in the lung: implications for pulmonary oligopeptide uptake. Am J Pathol 158(2):707-714.

Groneberg DA, Quarcoo D, Frossard N, Fischer A. 2004. Neurogenic mechanisms in bronchial inflammatory diseases. Allergy 59(11):1139-1152.

Gross ND, Boyle JO, Morrow JD, Williams MK, Moskowitz CS, Subbaramaiah K, Dannenberg AJ, Duffield-Lillico AJ. 2005. Levels of prostaglandin E metabolite, the major urinary metabolite of prostaglandin E2, are increased in smokers. Clin Cancer Res 11(16):6087-6093.

Gueugniaud PY, Carsin H, Bertin-Maghit M, Petit P. 2000. Current advances in the initial management of major thermal burns. Intensive Care Med 26(7):848-856.

Guo CJ, Lai JP, Luo HM, Douglas SD, Ho WZ. 2002. Substance P up-regulates macrophage inflammatory protein-1beta expression in human T lymphocytes. J Neuroimmunol 131(1-2):160-167.

Guyton AC, Hall JE. 2000. Textbook of Medical Physiology. Philadelphia.: W.B. Saunders. . 1064

p.
Hack CE, Hart M, van Schijndel RJ, Eerenberg AJ, Nuijens JH, Thijs LG, Aarden LA. 1992. Interleukin-8 in sepsis: relation to shock and inflammatory mediators. Infect Immun 60(7):2835-2842.
Hahn EL, Gamelli RL. 2000. Prostaglandin E2 synthesis and metabolism in burn injury and trauma. J Trauma 49(6):1147-1154.
Hallberg B, Rayter SI, Downward J. 1994. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. J Biol Chem 269(6):3913-3916.
Han J, Lee JD, Bibbs L, Ulevitch RJ. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265(5173):808-811.
Harmar A, Schofield JG, Keen P. 1980. Cycloheximide-sensitive synthesis of substance P by isolated dorsal root ganglia. Nature 284(5753):267-269.
Harris BH, Gelfand JA. 1995. The immune response to trauma. Semin Pediatr Surg 4(2):77-82.
Harrison S, Geppetti P. 2001. Substance p. Int J Biochem Cell Biol 33(6):555-576.
Hartford CE. 2002. Care of outpatient burns. In: Herndon D, editor. Total burn care. London, England: Saunders. p 40-50.
Hartmannsgruber MWB, Mozingo DW, Layon AJ. 2000. Thermal injuries: pathophysiology and anesthetic considerations, 2nd ed. In: Kirby RR, Gravenstein N, Gravenstein JS, Lobato EB, editors. Clinical anesthesia practice. Philadelphia Saunders
Hayashi H, Koike H, Kurata Y, Imanishi N, Tojo SJ. 1999. Protective effects of sialyl Lewis X and anti-P-selectin antibody against lipopolysaccharide-induced acute lung injury in rabbits. Eur J Pharmacol 370(1):47-56.
Hayden MS, West AP, Ghosh S. 2006. NF-kappaB and the immune response. Oncogene 25(51):6758-6780.
Heard SO, Perkins MW, Fink MP. 1992. Tumor necrosis factor-alpha causes myocardial depression in guinea pigs. Crit Care Med 20(4):523-527.
Hedner J, Hedner T, Wessberg P, Jonason J. 1984. Interaction of substance P with the respiratory control system in the rat. J Pharmacol Exp Ther 228(1):196-201.
Hedner J, Mueller RA, Hedner T, McCown TJ, Breese GR. 1985. A centrally elicited respiratory stimulant effect by bombesin in the rat. Eur J Pharmacol 115(1):21-29.
Heggers JP, Hawkins H, Edgar P, Villarreal C, Herndon D. 2002. Treatment of infections in burns. In: Herndon D, editor. Total burn care. London, England: Saunders. p 120-169.
Heideman M, Bengtsson A. 1992. The immunologic response to thermal injury. World J Surg 16(1):53-56.
Hemington-Gorse SJ. 2005. A comparison of laser Doppler imaging with other measurement techniques to assess burn depth. J Wound Care 14(4):151-153.
Herndon DN, Spies M. 2001. Modern burn care. Semin Pediatr Surg 10(1):28-31.
Hettiaratchy S, Dziewulski P. 2004a. ABC of burns. Introduction. Bmj 328(7452):1366-1368.
Hettiaratchy S, Dziewulski P. 2004b. ABC of burns: pathophysiology and types of burns. Bmj 328(7453):1427-1429.
Hinz B, Brune K. 2002. Cyclooxygenase-2--10 years later. J Pharmacol Exp Ther 300(2):367-375.

Ho WZ, Kaufman D, Uvaydova M, Douglas SD. 1996. Substance P augments interleukin-10 and tumor necrosis factor-alpha release by human cord blood monocytes and macrophages. J Neuroimmunol 71(1-2):73-80.

Ho WZ, Lai JP, Zhu XH, Uvaydova M, Douglas SD. 1997. Human monocytes and macrophages express substance P and neurokinin-1 receptor. J Immunol 159(11):5654-5660.

Hoesel LM, Mattar AF, Arbabi S, Niederbichler AD, Ipaktchi K, Su GL, Westfall MV, Wang SC, Hemmila MR. 2009. Local wound p38 MAPK inhibition attenuates burn-induced cardiac dysfunction. Surgery 146(4):775-785; discussion 785-776.

Hoesel LM, Niederbichler AD, Schaefer J, Ipaktchi KR, Gao H, Rittirsch D, Pianko MJ, Vogt PM, Sarma JV, Su GL, Arbabi S, Westfall MV, Wang SC, Hemmila MR, Ward PA. 2007. C5a-blockade improves burn-induced cardiac dysfunction. J Immunol 178(12):7902-7910.

Hokfelt T, Bartfai T, Bloom F. 2003. Neuropeptides: opportunities for drug discovery. Lancet Neurol 2(8):463-472.

Holst B, Hastrup H, Raffetseder U, Martini L, Schwartz TW. 2001. Two active molecular phenotypes of the tachykinin NK1 receptor revealed by G-protein fusions and mutagenesis. J Biol Chem 276(23):19793-19799.

Hong JL, Lee LY. 1996. Cigarette smoke-induced bronchoconstriction: causative agents and role of thromboxane receptors. J Appl Physiol 81(5):2053-2059.

Hooper NM. 1994. Families of zinc metalloproteases. FEBS Lett 354(1):1-6.

Horton JW. 2004. Left ventricular contractile dysfunction as a complication of thermal injury. Shock 22(6):495-507.

Horton JW, Maass DL, White J, Sanders B. 2001. Hypertonic saline-dextran suppresses burn-related cytokine secretion by cardiomyocytes. Am J Physiol Heart Circ Physiol 280(4):H1591-1601.

Howarth PH, Djukanovic R, Wilson JW, Holgate ST, Springall DR, Polak JM. 1991. Mucosal nerves in endobronchial biopsies in asthma and non-asthma. Int Arch Allergy Appl Immunol 94(1-4):330-333.

Humphrey JM. 2003. Medicinal chemistry of selective neurokinin-1 antagonists. Curr Top Med Chem 3(12):1423-1435.

Hunt JL, Purdue GF. 1992. The elderly burn patient. Am J Surg 164(5):472-476.

Hunter DD, Myers AC, Undem BJ. 2000. Nerve growth factor-induced phenotypic switch in guinea pig airway sensory neurons. Am J Respir Crit Care Med 161(6):1985-1990.

Ichinose M, Nakajima N, Takahashi T, Yamauchi H, Inoue H, Takishima T. 1992. Protection against bradykinin-induced bronchoconstriction in asthmatic patients by neurokinin receptor antagonist. Lancet 340(8830):1248-1251.

Inoue H, Ando K, Wakisaka N, Matsuzaki K, Aihara M, Kumagai N. 2001. Effects of nitric oxide synthase inhibitors on vascular hyperpermeability with thermal injury in mice. Nitric Oxide 5(4):334-342.

Ivanov AI, Scheck AC, Romanovsky AA. 2003. Expression of genes controlling transport and catabolism of prostaglandin E2 in lipopolysaccharide fever. Am J Physiol Regul Integr Comp Physiol 284(3):R698-706.

Iwamoto I, Nakagawa N, Yamazaki H, Kimura A, Tomioka H, Yoshida S. 1993. Mechanism for substance P-induced activation of human neutrophils and eosinophils. Regul Pept 46(1-2):228-230.

Iyer SS, Pulskens WP, Sadler JJ, Butter LM, Teske GJ, Ulland TK, Eisenbarth SC, Florquin S, Flavell RA, Leemans JC, Sutterwala FS. 2009. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. Proc Natl Acad Sci U S A 106(48):20388-20393.

Jaffe JH. 1990. Drug addiction and drug abuse. In: Gilman AG, Rall TW, Nies AS, Taylor P, editors. Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition. New York: Pergamon Press. p 522-573.

Jandera V, Hudson DA, de Wet PM, Innes PM, Rode H. 2000. Cooling the burn wound: evaluation of different modalites. Burns 26(3):265-270.

Jeng JC, Bridgeman A, Shivnan L, Thornton PM, Alam H, Clarke TJ, Jablonski KA, Jordan MH. 2003. Laser Doppler imaging determines need for excision and grafting in advance of clinical judgment: a prospective blinded trial. Burns 29(7):665-670.

Jia Y, Lee LY. 2007. Role of TRPV receptors in respiratory diseases. Biochim Biophys Acta 1772(8):915-927.

Jones S, Gibbins JM. 2008. The neurokinin 1 receptor: a potential new target for anti-platelet therapy? Curr Opin Pharmacol 8(2):114-119.

Joos GF, Pauwels RA. 2000. Pro-inflammatory effects of substance P: new perspectives for the treatment of airway diseases? Trends Pharmacol Sci 21(4):131-133.

Kalia M, Fuxe K, Agnati LF, Hokfelt T, Harfstrand A. 1984. Somatostatin produces apnea and is localized in medullary respiratory nuclei: a possible role in apneic syndromes. Brain Res 296(2):339-344.

Kandel ER. 2001. The molecular biology of memory storage: a dialogue between genes and synapses. Science 294(5544):1030-1038.

Kavelaars A, Broeke D, Jeurissen F, Kardux J, Meijer A, Franklin R, Gelfand EW, Heijnen CJ. 1994. Activation of human monocytes via a non-neurokinin substance P receptor that is coupled to Gi protein, calcium, phospholipase D, MAP kinase, and IL-6 production. J Immunol 153(8):3691-3699.

Kiernan R, Bres V, Ng RW, Coudart MP, El Messaoudi S, Sardet C, Jin DY, Emiliani S, Benkirane M. 2003. Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. J Biol Chem 278(4):2758-2766.

King TC, Zimmerman JM. 1965. First-Aid Cooling of the Fresh Burn. Surg Gynecol Obstet 120:1271-1273.

Kobayashi M, Herndon DN, Pollard RB, Suzuki F. 1995. CD4+ contrasuppressor T cells improve the resistance of thermally injured mice infected with HSV. J Leukoc Biol 58(2):159-167.

Koon HW, Zhao D, Zhan Y, Rhee SH, Moyer MP, Pothoulakis C. 2006. Substance P stimulates cyclooxygenase-2 and prostaglandin E2 expression through JAK-STAT activation in human colonic epithelial cells. J Immunol 176(8):5050-5059.

Koon HW, Zhao D, Zhan Y, Simeonidis S, Moyer MP, Pothoulakis C. 2005. Substance P-stimulated interleukin-8 expression in human colonic epithelial cells involves protein kinase Cdelta activation. J Pharmacol Exp Ther 314(3):1393-1400.

Kowal-Vern A, Walenga JM, Hoppensteadt D, Sharp-Pucci M, Gamelli RL. 1994. Interleukin-2 and interleukin-6 in relation to burn wound size in the acute phase of thermal injury. J Am Coll Surg 178(4):357-362.

Krakauer T. 2004. Molecular therapeutic targets in inflammation: cyclooxygenase and NF-kappaB. Curr Drug Targets Inflamm Allergy 3(3):317-324.

Kramer GC, Lund T, Herndon D. 2002. Total Burn Care. In: Herndon D, editor. Pathophysiology of burn shock and burn edema. London, England Saunders. p 78-87.

Kramer N, Meyer TJ, Meharg J, Cece RD, Hill NS. 1995. Randomized, prospective trial of noninvasive positive pressure ventilation in acute respiratory failure. Am J Respir Crit Care Med 151(6):1799-1806.

Kuebler WM. 2006. Selectins revisited: the emerging role of platelets in inflammatory lung disease. J Clin Invest 116(12):3106-3108.

Kuo HP, Lin HC, Hwang KH, Wang CH, Lu LC. 2000. Lipopolysaccharide enhances substance P-mediated neutrophil adherence to epithelial cells and cytokine release. Am J Respir Crit Care Med 162(5):1891-1897.

Kupper TS, Baker CC, Ferguson TA, Green DR. 1985. A burn induced Ly-2 suppressor T cell lowers resistance to bacterial infection. J Surg Res 38(6):606-612.

Kwong K, Wu ZX, Kashon ML, Krajnak KM, Wise PM, Lee LY. 2001. Chronic smoking enhances tachykinin synthesis and airway responsiveness in guinea pigs. Am J Respir Cell Mol Biol 25(3):299-305.

Kyriakis JM, Avruch J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81(2):807-869.

Labarca C, Paigen K. 1980. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102(2):344-352.

Lamb JP, Sparrow MP. 2002. Three-dimensional mapping of sensory innervation with substance p in porcine bronchial mucosa: comparison with human airways. Am J Respir Crit Care Med 166(9):1269-1281.

Lansdown AB. 2002. Silver. I: Its antibacterial properties and mechanism of action. J Wound Care 11(4):125-130.

Lansdown AB, Williams A, Chandler S, Benfield S. 2005. Silver absorption and antibacterial efficacy of silver dressings. J Wound Care 14(4):155-160.

Lappi-Blanco E, Kaarteenaho-Wiik R, Maasilta PK, Anttila S, Paakko P, Wolff HJ. 2006. COX-2 is widely expressed in metaplastic epithelium in pulmonary fibrous disorders. Am J Clin Pathol 126(5):717-724.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 372(6508):739-746.

Leff JA, Bodman ME, Cho OJ, Rohrbach S, Reiss OK, Vannice JL, Repine JE. 1994. Post-insult treatment with interleukin-1 receptor antagonist decreases oxidative lung injury in rats given intratracheal interleukin-1. Am J Respir Crit Care Med 150(1):109-112.

Leser HG, Gross V, Scheibenbogen C, Heinisch A, Salm R, Lausen M, Ruckauer K, Andreesen R, Farthmann EH, Scholmerich J. 1991. Elevation of serum

interleukin-6 concentration precedes acute-phase response and reflects severity in acute pancreatitis. Gastroenterology 101(3):782-785.

Li PC, Chen WC, Chang LC, Lin SC. 2008. Substance P acts via the neurokinin receptor 1 to elicit bronchoconstriction, oxidative stress, and upregulated ICAM-1 expression after oil smoke exposure. Am J Physiol Lung Cell Mol Physiol 294(5):L912-920.

Li WW, Guo TZ, Liang D, Shi X, Wei T, Kingery WS, Clark JD. 2009. The NALP1 inflammasome controls cytokine production and nociception in a rat fracture model of complex regional pain syndrome. Pain 147(1-3):277-286.

Lieb K, Fiebich BL, Berger M, Bauer J, Schulze-Osthoff K. 1997. The neuropeptide substance P activates transcription factor NF-kappa B and kappa B-dependent gene expression in human astrocytoma cells. J Immunol 159(10):4952-4958.

Lilly CM, Bai TR, Shore SA, Hall AE, Drazen JM. 1995. Neuropeptide content of lungs from asthmatic and nonasthmatic patients. Am J Respir Crit Care Med 151(2 Pt 1):548-553.

Liu XS, Luo ZH, Yang ZC, Li AN. 1996. Clinical significance of the alterations of plasma prostaglandin E2 (PGE2) in severely burned patients. Burns 22(4):298-302.

Lotz M, Vaughan JH, Carson DA. 1988. Effect of neuropeptides on production of inflammatory cytokines by human monocytes. Science 241(4870):1218-1221.

Lu B, Figini M, Emanueli C, Geppetti P, Grady EF, Gerard NP, Ansell J, Payan DG, Gerard C, Bunnett N. 1997. The control of microvascular permeability and blood pressure by neutral endopeptidase. Nat Med 3(8):904-907.

Lu B, Gerard NP, Kolakowski LF, Jr., Bozza M, Zurakowski D, Finco O, Carroll MC, Gerard C. 1995. Neutral endopeptidase modulation of septic shock. J Exp Med 181(6):2271-2275.

Lundberg JM, Saria A. 1983. Capsaicin-induced desensitization of airway mucosa to cigarette smoke, mechanical and chemical irritants. Nature 302(5905):251-253.

Luster AD. 1998. Chemokines--chemotactic cytokines that mediate inflammation. N Engl J Med 338(7):436-445.

Macdonald SG, Dumas JJ, Boyd ND. 1996. Chemical cross-linking of the substance P (NK-1) receptor to the alpha subunits of the G proteins Gq and G11. Biochemistry 35(9):2909-2916.

Maclouf J, Grassi J, Pradelles P. 1987. Development of enzyme-immunoassay techniques for the measurement of eicosanoids. In: Walden TLJ, Hughes HN, editors. Prostaglandin and lipid metabolism in radiation injury. Rockville: Plenum Press. p 355-364.

Maggi CA, Patacchini R, Feng DM, Folkers K. 1991. Activity of spantide I and II at various tachykinin receptors and NK-2 tachykinin receptor subtypes. Eur J Pharmacol 199(1):127-129.

Makhija R, Kingsnorth AN. 2002. Levels of the chemokines growth-related oncogene alpha and epithelial neutrophil-activating protein 78 are raised in patients with severe acute pancreatitis (Br J Surg 2002; 89: 566-72). Br J Surg 89(9):1194.

Malaver E, Romaniuk MA, D'Atri LP, Pozner RG, Negrotto S, Benzadon R, Schattner M. 2009. NF-kappaB inhibitors impair platelet activation responses. J Thromb Haemost 7(8):1333-1343.

Manske JM, Sullivan EL, Andersen SM. 1995. Substance P mediated stimulation of cytokine levels in cultured murine bone marrow stromal cells. Adv Exp Med Biol 383:53-64.

Mantyh PW. 1991. Substance P and the inflammatory and immune response. Ann N Y Acad Sci 632:263-271.

Marano MA, Moldawer LL, Fong Y, Wei H, Minei J, Yurt R, Cerami A, Lowry SF. 1988. Cachectin/TNF production in experimental burns and Pseudomonas infection. Arch Surg 123(11):1383-1388.

Marko P, Layon AJ, Caruso L, Mozingo DW, Gabrielli A. 2003. Burn injuries. Curr Opin Anaesthesiol 16(2):183-191.

Marriott I, Mason MJ, Elhofy A, Bost KL. 2000. Substance P activates NF-kappaB independent of elevations in intracellular calcium in murine macrophages and dendritic cells. J Neuroimmunol 102(2):163-171.

Marshall WG, Jr., Dimick AR. 1983. The natural history of major burns with multiple subsystem failure. J Trauma 23(2):102-105.

Martins MA, Shore SA, Drazen JM. 1991. Capsaicin-induced release of tachykinins: effects of enzyme inhibitors. J Appl Physiol 70(5):1950-1956.

Masson I, Mathieu J, Nolland XB, De Sousa M, Chanaud B, Strzalko S, Chancerelle Y, Kergonou JF, Giroud JP, Florentin I. 1998. Role of nitric oxide in depressed lymphoproliferative responses and altered cytokine production following thermal injury in rats. Cell Immunol 186(2):121-132.

Matos IM, Souza DG, Seabra DG, Freire-Maia L, Teixeira MM. 1999. Effects of tachykinin NK1 or PAF receptor blockade on the lung injury induced by scorpion venom in rats. Eur J Pharmacol 376(3):293-300.

Matthay MA, Zimmerman GA. 2005. Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management. Am J Respir Cell Mol Biol 33(4):319-327.

McCampbell B, Wasif N, Rabbitts A, Staiano-Coico L, Yurt RW, Schwartz S. 2002. Diabetes and burns: retrospective cohort study. J Burn Care Rehabil 23(3):157-166.

McCown TJ, Hedner JA, Towle AC, Breese GR, Mueller RA. 1986. Brainstem localization of a thyrotropin-releasing hormone-induced change in respiratory function. Brain Res 373(1-2):189-196.

Meakins JL. 1990. Etiology of multiple organ failure. J Trauma 30(12 Suppl):S165-168.

Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. 1995. Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. Chest 108(5):1303-1314.

Miller-Graziano CL, Fink M, Wu JY, Szabo G, Kodys K. 1988. Mechanisms of altered monocyte prostaglandin E2 production in severely injured patients. Arch Surg 123(3):293-299.

Milligan ED, Sloane EM, Langer SJ, Cruz PE, Chacur M, Spataro L, Wieseler-Frank J, Hammack SE, Maier SF, Flotte TR, Forsayeth JR, Leinwand LA, Chavez R, Watkins LR. 2005. Controlling neuropathic pain by adeno-associated virus driven production of the anti-inflammatory cytokine, interleukin-10. Mol Pain 1:9.

Minutoli L, Antonuccio P, Romeo C, Nicotina PA, Bitto A, Arena S, Polito F, Altavilla D, Turiaco N, Cutrupi A, Zuccarello B, Squadrito F. 2005. Evidence for a role of

mitogen-activated protein kinase 3/mitogen-activated protein kinase in the development of testicular ischemia-reperfusion injury. Biol Reprod 73(4):730-736.
Mizgerd JP, Meek BB, Kutkoski GJ, Bullard DC, Beaudet AL, Doerschuk CM. 1996. Selectins and neutrophil traffic: margination and Streptococcus pneumoniae-induced emigration in murine lungs. J Exp Med 184(2):639-645.
Monafo WW. 1996. Initial management of burns. N Engl J Med 335(21):1581-1586.
Monafo WW, Bessey PQ. 2002. Total Burn Care. In: Herndon D, editor. Wound Care. London, England: Saunders. p 109-119.
Monafo WW, West MA. 1990. Current treatment recommendations for topical burn therapy. Drugs 40(3):364-373.
Morita I. 2002. Distinct functions of COX-1 and COX-2. Prostaglandins Other Lipid Mediat 68-69:165-175.
Muckart DJ, Bhagwanjee S. 1997. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference definitions of the systemic inflammatory response syndrome and allied disorders in relation to critically injured patients. Crit Care Med 25(11):1789-1795.
Mulligan MS, Miyasaka M, Ward PA. 1996. Protective effects of combined adhesion molecule blockade in models of acute lung injury. Proc Assoc Am Physicians 108(3):198-208.
Mulligan MS, Smith CW, Anderson DC, Todd RF, 3rd, Miyasaka M, Tamatani T, Issekutz TB, Ward PA. 1993. Role of leukocyte adhesion molecules in complement-induced lung injury. J Immunol 150(6):2401-2406.
Murakami K, Enkhbaatar P, Yu YM, Traber LD, Cox RA, Hawkins HK, Tompkins RG, Herndon D, Traber DL. 2007. L-arginine attenuates acute lung injury after smoke inhalation and burn injury in sheep. Shock 28(4):477-483.
Nadel JA. 1990. Decreased neutral endopeptidases: possible role in inflammatory diseases of airways. Lung 168 Suppl:123-127.
Nadel JA. 1991. Neutral endopeptidase modulates neurogenic inflammation. Eur Respir J 4(6):745-754.
Nadel JA, Borson DB. 1991. Modulation of neurogenic inflammation by neutral endopeptidase. Am Rev Respir Dis 143(3 Pt 2):S33-36.
Nakajima Y, Tsuchida K, Negishi M, Ito S, Nakanishi S. 1992. Direct linkage of three tachykinin receptors to stimulation of both phosphatidylinositol hydrolysis and cyclic AMP cascades in transfected Chinese hamster ovary cells. J Biol Chem 267(4):2437-2442.
Nathan C. 2002. Points of control in inflammation. Nature 420(6917):846-852.
Nawa H, Kotani H, Nakanishi S. 1984. Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. Nature 312(5996):729-734.
Nguyen TT, Gilpin DA, Meyer NA, Herndon DN. 1996. Current treatment of severely burned patients. Ann Surg 223(1):14-25.
Nieber K, Baumgarten CR, Rathsack R, Furkert J, Oehme P, Kunkel G. 1992. Substance P and beta-endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic asthma. J Allergy Clin Immunol 90(4 Pt 1):646-652.
Niederbichler AD, Westfall MV, Su GL, Donnerberg J, Usman A, Vogt PM, Ipaktchi KR, Arbabi S, Wang SC, Hemmila MR. 2006. Cardiomyocyte function after burn

injury and lipopolysaccharide exposure: single-cell contraction analysis and cytokine secretion profile. Shock 25(2):176-183.

Norman JG, Fink GW, Franz MG. 1995. Acute pancreatitis induces intrapancreatic tumor necrosis factor gene expression. Arch Surg 130(9):966-970.

O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. 2004. The role of substance P in inflammatory disease. J Cell Physiol 201(2):167-180.

Ofeigsson OJ. 1965. Water Cooling: First-Aid Treatment for Scalds and Burns. Surgery 57:391-400.

Ogle CK, Guo X, Wu JZ, Ogle JD. 1993. Production of cytokines and PGE2 and cytotoxicity of stimulated bone marrow macrophages after thermal injury and cytotoxicity of stimulated U-937 macrophages. Inflammation 17(5):583-594.

Ogle CK, Mao JX, Wu JZ, Ogle JD, Alexander JW. 1994. The 1994 Lindberg Award. The production of tumor necrosis factor, interleukin-1, interleukin-6, and prostaglandin E2 by isolated enterocytes and gut macrophages: effect of lipopolysaccharide and thermal injury. J Burn Care Rehabil 15(6):470-477.

Ohara M, Sawa T, Kurahashi K, Wiener-Kronish JP, Doshi V, Kudoh I, Gropper MA. 1998. Induction of cyclooxygenase-2 in alveolar macrophages after acid aspiration: selective cyclooxygenase-2 blockade reduces interleukin-6 production. Anesthesiology 88(4):1014-1022.

Ollerenshaw SL, Jarvis D, Sullivan CE, Woolcock AJ. 1991. Substance P immunoreactive nerves in airways from asthmatics and nonasthmatics. Eur Respir J 4(6):673-682.

Opal SM, Fisher CJ, Jr., Dhainaut JF, Vincent JL, Brase R, Lowry SF, Sadoff JC, Slotman GJ, Levy H, Balk RA, Shelly MP, Pribble JP, LaBrecque JF, Lookabaugh J, Donovan H, Dubin H, Baughman R, Norman J, DeMaria E, Matzel K, Abraham E, Seneff M. 1997. Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group. Crit Care Med 25(7):1115-1124.

Padi SS, Jain NK, Singh S, Kulkarni SK. 2004. Pharmacological profile of parecoxib: a novel, potent injectable selective cyclooxygenase-2 inhibitor. Eur J Pharmacol 491(1):69-76.

Pagani FD, Baker LS, Hsi C, Knox M, Fink MP, Visner MS. 1992. Left ventricular systolic and diastolic dysfunction after infusion of tumor necrosis factor-alpha in conscious dogs. J Clin Invest 90(2):389-398.

Papp A, Valtonen P. 2006. Tissue substance P levels in acute experimental burns. Burns 32(7):842-845.

Park GY, Christman JW. 2006. Involvement of cyclooxygenase-2 and prostaglandins in the molecular pathogenesis of inflammatory lung diseases. Am J Physiol Lung Cell Mol Physiol 290(5):L797-805.

Park WY, Goodman RB, Steinberg KP, Ruzinski JT, Radella F, 2nd, Park DR, Pugin J, Skerrett SJ, Hudson LD, Martin TR. 2001. Cytokine balance in the lungs of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 164(10 Pt 1):1896-1903.

Parker JC, Townsley MI. 2004. Evaluation of lung injury in rats and mice. Am J Physiol Lung Cell Mol Physiol 286(2):L231-246.

Parks WC, Shapiro SD. 2001. Matrix metalloproteinases in lung biology. Respir Res 2(1):10-19.

Patel L, Lindley C. 2003. Aprepitant--a novel NK1-receptor antagonist. Expert Opin Pharmacother 4(12):2279-2296.

Paterson HM, Murphy TJ, Purcell EJ, Shelley O, Kriynovich SJ, Lien E, Mannick JA, Lederer JA. 2003. Injury primes the innate immune system for enhanced Toll-like receptor reactivity. J Immunol 171(3):1473-1483.

Payan DG, Goetzl EJ. 1985. Modulation of lymphocyte function by sensory neuropeptides. J Immunol 135(2 Suppl):783s-786s.

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22(2):153-183.

Pease JE, Sabroe I. 2002. The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. Am J Respir Med 1(1):19-25.

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. 1997. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem 272(34):21096-21103.

Pinter E, Brown B, Hoult JR, Brain SD. 1999. Lack of evidence for tachykinin NK1 receptor-mediated neutrophil accumulation in the rat cutaneous microvasculature by thermal injury. Eur J Pharmacol 369(1):91-98.

Piper PJ. 1974. Release and metabolism of prostaglandins in lung tissue. Pol J Pharmacol Pharm 26(1):61-72.

Piper PJ, Vane JR, Wyllie JH. 1970. Inactivation of prostaglandins by the lungs. Nature 225(5233):600-604.

Pizzichini MM, Pizzichini E, Clelland L, Efthimiadis A, Pavord I, Dolovich J, Hargreave FE. 1999. Prednisone-dependent asthma: inflammatory indices in induced sputum. Eur Respir J 13(1):15-21.

Poulsen TD, Freund KG, Arendrup K, Nyhuus P, Pedersen OD. 1991. Polyurethane film (Opsite) vs. impregnated gauze (Jelonet) in the treatment of outpatient burns: a prospective, randomized study. Burns 17(1):59-61.

Pruitt BA, Goodwin CW, Mason D. 2002. Total Burn Care. In: Herndon D, editor. Epidemiological, demographic and outcome characteristics of burn injury. London, England: Saunders. p 16-30.

Ptacek JT, Patterson DR, Heimbach DM. 2002. Inpatient depression in persons with burns. J Burn Care Rehabil 23(1):1-9.

Puneet P, Hegde A, Ng SW, Lau HY, Lu J, Moochhala SM, Bhatia M. 2006. Preprotachykinin-A gene products are key mediators of lung injury in polymicrobial sepsis. J Immunol 176(6):3813-3820.

Puneet P, Moochhala S, Bhatia M. 2005. Chemokines in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 288(1):L3-15.

Putensen C, Wrigge H. 2000. Ventilator-associated systemic inflammation in acute lung injury. Intensive Care Med 26(10):1411-1413.

Quanjer PH, Lebowitz MD, Gregg I, Miller MR, Pedersen OF. 1997. Peak expiratory flow: conclusions and recommendations of a Working Party of the European Respiratory Society. Eur Respir J Suppl 24:2S-8S.

Ram M, Sherer Y, Shoenfeld Y. 2006. Matrix metalloproteinase-9 and autoimmune diseases. J Clin Immunol 26(4):299-307.
Rameshwar P. 1997. Substance P: a regulatory neuropeptide for hematopoiesis and immune functions. Clin Immunol Immunopathol 85(2):129-133.
Rameshwar P, Ganea D, Gascon P. 1993. In vitro stimulatory effect of substance P on hematopoiesis. Blood 81(2):391-398.
Rameshwar P, Ganea D, Gascon P. 1994. Induction of IL-3 and granulocyte-macrophage colony-stimulating factor by substance P in bone marrow cells is partially mediated through the release of IL-1 and IL-6. J Immunol 152(8):4044-4054.
Rameshwar P, Gascon P. 1995. Substance P (SP) mediates production of stem cell factor and interleukin-1 in bone marrow stroma: potential autoregulatory role for these cytokines in SP receptor expression and induction. Blood 86(2):482-490.
Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. 2002. Six at six: interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. Shock 17(6):463-467.
Reutershan J, Ley K. 2004. Bench-to-bedside review: acute respiratory distress syndrome - how neutrophils migrate into the lung. Crit Care 8(6):453-461.
Robertson RP. 1995. Molecular regulation of prostaglandin synthesis Implications for endocrine systems. Trends Endocrinol Metab 6(9-10):293-297.
Robson MC. 2002. Overview of burn reconstruction. In: Herndon D, editor. Total burn care. London, England: Saunder. p 620-627.
Rodriguez JL, Miller CG, Garner WL, Till GO, Guerrero P, Moore NP, Corridore M, Normolle DP, Smith DJ, Remick DG. 1993. Correlation of the local and systemic cytokine response with clinical outcome following thermal injury. J Trauma 34(5):684-694; discussion 694-685.
Roebuck KA, Finnegan A. 1999. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. J Leukoc Biol 66(6):876-888.
Roques BP, Noble F, Dauge V, Fournie-Zaluski MC, Beaumont A. 1993. Neutral endopeptidase 24.11: structure, inhibition, and experimental and clinical pharmacology. Pharmacol Rev 45(1):87-146.
Rost K, Fleischer F, Nieber K. 2006. [Neurokinin 1 receptor antagonists--between hope and disappointment]. Med Monatsschr Pharm 29(6):200-205.
Roth JJ, Hughes WB. 2004. The essential burn unit handbook. St. Louis, Mo.: Quality Medical Publishing
Roux PP, Blenis J. 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68(2):320-344.
Saffle JR, Sullivan JJ, Tuohig GM, Larson CM. 1993. Multiple organ failure in patients with thermal injury. Crit Care Med 21(11):1673-1683.
Saranto JR, Rubayi S, Zawacki BE. 1983. Blisters, cooling, antithromboxanes, and healing in experimental zone-of-stasis burns. J Trauma 23(10):927-933.
Saria A. 1984. Substance P in sensory nerve fibres contributes to the development of oedema in the rat hind paw after thermal injury. Br J Pharmacol 82(1):217-222.
Savino W, Dardenne M. 1995. Immune-neuroendocrine interactions. Immunol Today 16(7):318-322.

Sawada Y, Urushidate S, Yotsuyanagi T, Ishita K. 1997. Is prolonged and excessive cooling of a scalded wound effective? Burns 23(1):55-58.

Schroder K, Tschopp J. 2010. The inflammasomes. Cell 140(6):821-832.

Schuster VL. 1998. Molecular mechanisms of prostaglandin transport. Annu Rev Physiol 60:221-242.

Schwacha MG, Ayala A, Chaudry IH. 2000. Insights into the role of gammadelta T lymphocytes in the immunopathogenic response to thermal injury. J Leukoc Biol 67(5):644-650.

Schwacha MG, Ayala A, Cioffi WG, Bland KI, Chaudry IH. 1999. Role of protein kinase C in cyclic AMP-mediated suppression of T-lymphocyte activation following burn injury. Biochim Biophys Acta 1455(1):45-53.

Schwacha MG, Chung CS, Ayala A, Bland KI, Chaudry IH. 2002. Cyclooxygenase 2-mediated suppression of macrophage interleukin-12 production after thermal injury. Am J Physiol Cell Physiol 282(2):C263-270.

Schwacha MG, Somers SD. 1998a. Thermal injury-induced immunosuppression in mice: the role of macrophage-derived reactive nitrogen intermediates. J Leukoc Biol 63(1):51-58.

Schwacha MG, Somers SD. 1998b. Thermal injury induces macrophage hyperactivity through pertussis toxin-sensitive and -insensitive pathways. Shock 9(4):249-255.

Serra MC, Bazzoni F, Della Bianca V, Greskowiak M, Rossi F. 1988. Activation of human neutrophils by substance P. Effect on oxidative metabolism, exocytosis, cytosolic Ca2+ concentration and inositol phosphate formation. J Immunol 141(6):2118-2124.

Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK, Collins T. 1999. Transcriptional activation by NF-kappaB requires multiple coactivators. Mol Cell Biol 19(9):6367-6378.

Sheridan RL, Ryan CM, Yin LM, Hurley J, Tompkins RG. 1998. Death in the burn unit: sterile multiple organ failure. Burns 24(4):307-311.

Sherwood ER, Traber DL. 2002. The systemic inflammatory response syndrome. In: Herndon D, editor. Total burn care. London, England: Saunders. p 257-270.

Shipp MA, Stefano GB, Switzer SN, Griffin JD, Reinherz EL. 1991. CD10 (CALLA)/neutral endopeptidase 24.11 modulates inflammatory peptide-induced changes in neutrophil morphology, migration, and adhesion proteins and is itself regulated by neutrophil activation. Blood 78(7):1834-1841.

Siler TM, Swierkosz JE, Hyers TM, Fowler AA, Webster RO. 1989. Immunoreactive interleukin-1 in bronchoalveolar lavage fluid of high-risk patients and patients with the adult respiratory distress syndrome. Exp Lung Res 15(6):881-894.

Simeonidis S, Castagliuolo I, Pan A, Liu J, Wang CC, Mykoniatis A, Pasha A, Valenick L, Sougioultzis S, Zhao D, Pothoulakis C. 2003. Regulation of the NK-1 receptor gene expression in human macrophage cells via an NF-kappa B site on its promoter. Proc Natl Acad Sci U S A 100(5):2957-2962.

Siney L, Brain SD. 1996. Involvement of sensory neuropeptides in the development of plasma extravasation in rat dorsal skin following thermal injury. Br J Pharmacol 117(6):1065-1070.

Sio SW, Moochhala S, Lu J, Bhatia M. 2010. Early protection from burn-induced acute lung injury by deletion of preprotachykinin-A gene. Am J Respir Crit Care Med 181(1):36-46.

Sizemore N, Leung S, Stark GR. 1999. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol 19(7):4798-4805.

Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL. 1991. Pre-Botzinger complex: a brainstem region that may generate respiratory rhythm in mammals. Science 254(5032):726-729.

Smith WL. 1989. The eicosanoids and their biochemical mechanisms of action. Biochem J 259(2):315-324.

Sokawa M, Manafo W, Deitz F, Flynn D. 1981. The relationship between experimental fluid therapy and wound edema in scald wounds. Ann Surg 193(2):237-244.

Solway J, Kao BM, Jordan JE, Gitter B, Rodger IW, Howbert JJ, Alger LE, Necheles J, Leff AR, Garland A. 1993. Tachykinin receptor antagonists inhibit hyperpnea-induced bronchoconstriction in guinea pigs. J Clin Invest 92(1):315-323.

Steinman L. 2004. Elaborate interactions between the immune and nervous systems. Nat Immunol 5(6):575-581.

Sternberg EM. 2006. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. Nat Rev Immunol 6(4):318-328.

Stevenson JM, Gamelli RL, Shankar R. 2003. Wound Healing: Methods and Protocols (Methods in Molecular Medicine) DiPietro LA, Burns AL, editors. Totowa, New Jersey: Humana Press Inc. 95-106 p.

Still JM, Jr., Belcher K, Law EJ. 1993. Experience with polymicrobial sepsis in a regional burn unit. Burns 19(5):434-436.

Strieter RM. 2002. Interleukin-8: a very important chemokine of the human airway epithelium. Am J Physiol Lung Cell Mol Physiol 283(4):L688-689.

Strohm C, Barancik T, Bruhl ML, Kilian SA, Schaper W. 2000. Inhibition of the ER-kinase cascade by PD98059 and UO126 counteracts ischemic preconditioning in pig myocardium. J Cardiovasc Pharmacol 36(2):218-229.

Sturiale S, Barbara G, Qiu B, Figini M, Geppetti P, Gerard N, Gerard C, Grady EF, Bunnett NW, Collins SM. 1999. Neutral endopeptidase (EC 3.4.24.11) terminates colitis by degrading substance P. Proc Natl Acad Sci U S A 96(20):11653-11658.

Sulpizio AC, Pullen MA, Edwards RM, Brooks DP. 2004. The effect of acute angiotensin-converting enzyme and neutral endopeptidase 24.11 inhibition on plasma extravasation in the rat. J Pharmacol Exp Ther 309(3):1141-1147.

Swann K, Berger J, Sprague SM, Wu Y, Lai Q, Jimenez DF, Barone CM, Ding Y. 2007. Peripheral thermal injury causes blood-brain barrier dysfunction and matrix metalloproteinase (MMP) expression in rat. Brain Res 1129(1):26-33.

Szarek JL, Spurlock B, Gruetter CA, Lemke S. 1998. Substance P and capsaicin release prostaglandin E2 from rat intrapulmonary bronchi. Am J Physiol 275(5 Pt 1):L1006-1012.

Tak PP, Firestein GS. 2001. NF-kappaB: a key role in inflammatory diseases. J Clin Invest 107(1):7-11.

Takano H, Yanagisawa R, Ichinose T, Sadakane K, Yoshino S, Yoshikawa T, Morita M. 2002. Diesel exhaust particles enhance lung injury related to bacterial endotoxin

through expression of proinflammatory cytokines, chemokines, and intercellular adhesion molecule-1. Am J Respir Crit Care Med 165(9):1329-1335.

Takashima Y. 1997. Blood platelets in severely injured burned patients. Burns 23(7-8):591-595.

Takemura M, Quarcoo D, Niimi A, Dinh QT, Geppetti P, Fischer A, Chung KF, Groneberg DA. 2008. Is TRPV1 a useful target in respiratory diseases? Pulm Pharmacol Ther 21(6):833-839.

Takeyama M, Nagai S, Mori K, Ikawa K, Satake N, Izumi T. 1996. Substance P-like immunoreactive substance in bronchoalveolar lavage fluids from patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. Sarcoidosis Vasc Diffuse Lung Dis 13(1):33-37.

Talley JJ, Bertenshaw SR, Brown DL, Carter JS, Graneto MJ, Kellogg MS, Koboldt CM, Yuan J, Zhang YY, Seibert K. 2000. N-[[(5-methyl-3-phenylisoxazol-4-yl)-phenyl]sulfonyl]propanamide, sodium salt, parecoxib sodium: A potent and selective inhibitor of COX-2 for parenteral administration. J Med Chem 43(9):1661-1663.

Tamaoki J, Tagaya E, Kawatani K, Nakata J, Endo Y, Nagai A. 2004. Airway mucosal thickening and bronchial hyperresponsiveness induced by inhaled beta 2-agonist in mice. Chest 126(1):205-212.

Tanabe T, Tohnai N. 2002. Cyclooxygenase isozymes and their gene structures and expression. Prostaglandins Other Lipid Mediat 68-69:95-114.

Tasaka S, Hasegawa N, Ishizaka A. 2002. Pharmacology of acute lung injury. Pulm Pharmacol Ther 15(2):83-95.

Teagarden DL, Sandeep N. 2007. Case Study: Parecoxib: A Prodrug of Valdecoxib. . In: Stella VJ, Borchardt RT, Hageman MJ, Oliyai R, Maag H, Tilley JW, editors. Biotechnology: Pharmaceutical Aspects, Prodrugs: Challenges and Rewards (Part 1). St. Louis, MO: Springer New York. p 1335-1346.

Teodorczyk-Injeyan JA, Sparkes BG, Mills GB, Peters WJ, Falk RE. 1986. Impairment of T cell activation in burn patients: a possible mechanism of thermal injury-induced immunosuppression. Clin Exp Immunol 65(3):570-581.

Tomaki M, Ichinose M, Miura M, Hirayama Y, Yamauchi H, Nakajima N, Shirato K. 1995. Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. Am J Respir Crit Care Med 151(3 Pt 1):613-617.

Tomashefski JF, Jr. 1990. Pulmonary pathology of the adult respiratory distress syndrome. Clin Chest Med 11(4):593-619.

Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ, 3rd, Zentella A, Albert JD, et al. 1986. Shock and tissue injury induced by recombinant human cachectin. Science 234(4775):470-474.

Turnage RH, Nwariaku F, Murphy J, Schulman C, Wright K, Yin H. 2002. Mechanisms of pulmonary microvascular dysfunction during severe burn injury. World J Surg 26(7):848-853.

Uddman R, Hakanson R, Luts A, Sundler F. 1997. Autonomic control of the respiratory system. In: Barnes PJ, editor. Distribution of neuropeptides in airways. London: Harvard Academic.

Ulbrich H, Eriksson EE, Lindbom L. 2003. Leukocyte and endothelial cell adhesion molecules as targets for therapeutic interventions in inflammatory disease. Trends Pharmacol Sci 24(12):640-647.

Van Rensen EL, Hiemstra PS, Rabe KF, Sterk PJ. 2002. Assessment of microvascular leakage via sputum induction: the role of substance P and neurokinin A in patients with asthma. Am J Respir Crit Care Med 165(9):1275-1279.

Varedi M, Jeschke MG, Englander EW, Herndon DN, Barrow RE. 2001. Serum TGF-beta in thermally injured rats. Shock 16(5):380-382.

Veronesi B, Carter JD, Devlin RB, Simon SA, Oortgiesen M. 1999. Neuropeptides and capsaicin stimulate the release of inflammatory cytokines in a human bronchial epithelial cell line. Neuropeptides 33(6):447-456.

Versteeg HH, van Bergen en Henegouwen PM, van Deventer SJ, Peppelenbosch MP. 1999. Cyclooxygenase-dependent signalling: molecular events and consequences. FEBS Lett 445(1):1-5.

Vindenes HA, Ulvestad E, Bjerknes R. 1998. Concentrations of cytokines in plasma of patients with large burns: their relation to time after injury, burn size, inflammatory variables, infection, and outcome. Eur J Surg 164(9):647-656.

Von Euler US, Gaddum JH. 1931. An unidentified depressor substance in certain tissue extracts. J Physiol 72:74-87.

Wagner F, Fink R, Hart R, Dancygier H. 1987. Substance P enhances interferon-gamma production by human peripheral blood mononuclear cells. Regul Pept 19(5-6):355-364.

Ware LB, Matthay MA. 2000. The acute respiratory distress syndrome. N Engl J Med 342(18):1334-1349.

Watkins LR, Maier SF. 1999. Implications of immune-to-brain communication for sickness and pain. Proc Natl Acad Sci U S A 96(14):7710-7713.

Wenninger JM, Pan LG, Klum L, Leekley T, Bastastic J, Hodges MR, Feroah T, Davis S, Forster HV. 2004. Small reduction of neurokinin-1 receptor-expressing neurons in the pre-Botzinger complex area induces abnormal breathing periods in awake goats. J Appl Physiol 97(5):1620-1628.

Wheeler AP, Bernard GR. 2007. Acute lung injury and the acute respiratory distress syndrome: a clinical review. Lancet 369(9572):1553-1564.

Wiechman SA, Ptacek JT, Patterson DR, Gibran NS, Engrav LE, Heimbach DM. 2001. Rates, trends, and severity of depression after burn injuries. J Burn Care Rehabil 22(6):417-424.

Williams R, Zou X, Hoyle GW. 2007. Tachykinin-1 receptor stimulates proinflammatory gene expression in lung epithelial cells through activation of NF-kappaB via a G(q)-dependent pathway. Am J Physiol Lung Cell Mol Physiol 292(2):L430-437.

Wischmeyer PE, Lynch J, Liedel J, Wolfson R, Riehm J, Gottlieb L, Kahana M. 2001. Glutamine administration reduces Gram-negative bacteremia in severely burned patients: a prospective, randomized, double-blind trial versus isonitrogenous control. Crit Care Med 29(11):2075-2080.

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. 2000. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 80(5):617-653.

Wolfe JH, Wu AV, O'Connor NE, Saporoschetz I, Mannick JA. 1982. Anergy, immunosuppressive serum, and impaired lymphocyte blastogenesis in burn patients. Arch Surg 117(10):1266-1271.

Wong SS, Sun NN, Lantz RC, Witten ML. 2004. Substance P and neutral endopeptidase in development of acute respiratory distress syndrome following fire smoke inhalation. Am J Physiol Lung Cell Mol Physiol 287(4):L859-866.

Wu KK. 1996. Cyclooxygenase 2 induction: molecular mechanism and pathophysiologic roles. J Lab Clin Med 128(3):242-245.

Wyka KA, Mathews, P. J., Clark, W. F. . 2002. Foundations of Respiratory Care. New York: Delmar Cengage Learning. 1032 p.

Wyllie JH, Piper PJ, Vane JR. 1969. Fate of prostaglandins in the lungs. Br J Surg 56(8):623.

Yamada Y, Endo S, Inada K. 1996. Plasma cytokine levels in patients with severe burn injury--with reference to the relationship between infection and prognosis. Burns 22(8):587-593.

Yamamoto Y, Gaynor RB. 2001. Role of the NF-kappaB pathway in the pathogenesis of human disease states. Curr Mol Med 1(3):287-296.

Yamaoka J, Kawana S. 2007. Rapid changes in substance P signaling and neutral endopeptidase induced by skin-scratching stimulation in mice. J Dermatol Sci 48(2):123-132.

Yamasaki K, Muto J, Taylor KR, Cogen AL, Audish D, Bertin J, Grant EP, Coyle AJ, Misaghi A, Hoffman HM, Gallo RL. 2009. NLRP3/cryopyrin is necessary for interleukin-1beta (IL-1beta) release in response to hyaluronan, an endogenous trigger of inflammation in response to injury. J Biol Chem 284(19):12762-12771.

Yokoyama T, Vaca L, Rossen RD, Durante W, Hazarika P, Mann DL. 1993. Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. J Clin Invest 92(5):2303-2312.

Yonekawa K, Harlan JM. 2005. Targeting leukocyte integrins in human diseases. J Leukoc Biol 77(2):129-140.

Yowler CJ, Fratianne RB. 2000. Current status of burn resuscitation. Clin Plast Surg 27(1):1-10.

Zhang H, Moochhala SM, Bhatia M. 2008. Endogenous hydrogen sulfide regulates inflammatory response by activating the ERK pathway in polymicrobial sepsis. J Immunol 181(6):4320-4331.

Zhang H, Zhi L, Moochhala SM, Moore PK, Bhatia M. 2007. Endogenous hydrogen sulfide regulates leukocyte trafficking in cecal ligation and puncture-induced sepsis. J Leukoc Biol 82(4):894-905.

Zhang YL, Dong C. 2005. MAP kinases in immune responses. Cell Mol Immunol 2(1):20-27.

Zhong H, May MJ, Jimi E, Ghosh S. 2002. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol Cell 9(3):625-636.