1-13-2015 12:00 AM

Surfactant and Matrix Metalloproteinase 3 in the Pathogenesis of Acute Lung Injury

Valeria Puntorieri, *The University of Western Ontario*

Supervisor: Dr. Jim Lewis, *The University of Western Ontario*
Joint Supervisor: Dr. Ruud Veldhuizen, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology

SURFACTANT AND MATRIX METALLOPROTEINASE 3 IN THE PATHOGENESIS OF ACUTE LUNG INJURY

(Thesis format: Integrated-Article)

by

Valeria Puntorieri

Graduate Program in Physiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

ABSTRACT:

Acute Lung Injury (ALI) is a **pulmonary inflammatory disorder** resulting in respiratory failure that is initiated by a number of different insults to the lung. Despite very high mortality, there are still no effective pharmacological therapies for this disease, and the main supportive treatment, **mechanical ventilation (MV)**, can further lung injury and inflammation, contributing to ALI progression.

The overall objective of this work was, therefore, to broaden the knowledge of ALI pathophysiology in an attempt to improve outcomes for this disorder. To this end, the roles of two key players in the disease process were evaluated, namely: i) **lung surfactant**, a material essential for minimizing the work of breathing and for pulmonary immuno-modulation, and ii) **matrix metalloproteinase 3** (MMP-3), protease involved in the **inflammatory response** associated with ALI. The experimental approach consisted of exposing mice to different models of ALI, in order to investigate: i) the effects of exogenous surfactant administration on lung inflammation and injury progression associated with MV, ii) the role of MMP-3 in the pulmonary inflammatory response associated with ALI, and iii) the potential interactions between MMP-3-related inflammatory changes, surfactant function, and pulmonary mechanics in ALI.

The results demonstrated that exogenous surfactant treatment did not impact inflammatory outcomes of ALI that are associated, clinically, with mortality. Further research is therefore required to improve such potential therapy. The data also illustrated the contribution of MMP-3 to the pulmonary inflammation associated with ALI, specifically in female mice. Furthermore, the complexity of the interactions between lung inflammation, surfactant function, and mechanics of the lung was demonstrated.

Overall, this evidence underscored the challenges faced in the treatment of ALI; nonetheless, a broader knowledge of ALI complex pathophysiology will be beneficial to the design of new therapies and the improvement of ALI outcomes.

KEYWORDS:

Acute Lung Injury, lung inflammation, cytokines, chemokines, mechanical ventilation, lung surfactant, exogenous surfactant, matrix metalloproteinase-3, lipopolysaccharide, acid-induced lung injury, respiratory mechanics.

CO-AUTHORSHIP STATEMENT:

Chapters 2, 3, and 4 describe experimental studies performed by Valeria Puntorieri under the supervision of Dr. Jim Lewis and Dr. Ruud Veldhuizen.

Both Dr. Ruud Veldhuizen and Dr. Jim Lewis provided intellectual contribution to all of the studies described in this thesis, participating in experimental design, data analysis and interpretation, and manuscript review.

Lynda McCaig provided general assistance with animal work in all of the studies.

Li-Juan Yao helped with the Milliplex assays and cell counting in Chapters 2 and 3.

In Chapter 2, Josh Qua Hiansen provided assistance with the collection of lavage and perfusate samples and with data analysis. He also performed the analysis of surfactant function on the captive bubble surfactometer.

In Chapter 3, the contribution of Dr. Cory Yamashita was intellectual in nature, and consisted of the participation in experimental design, data interpretation, and manuscript review.

In Chapter 4, Scott Milos analyzed the surfactant samples on the constrained sessile drop surfactometer. Stephanie Aigbe performed measurements of surfactant pool sizes in lavage, while Dr. Chris Howlett prepared and analyzed the histological sections.

ACKNOWLEDGMENTS:

I can hardly express how much gratitude I have towards the Lung Lab for welcoming me as a lab member years ago, and for what has truly been a life-changing experience. The Lung Lab has been my family away from home, and the most scientifically stimulating environment I had the pleasure to join. I would also like to thank the Department of Physiology and Pharmacology, as well as the members of my advisory committee, Dr. Gedas Cepinskas, Dr. Sean Gill, and Dr. Donglin Bai, for the support and insight they provided throughout the years. My supervisors, Dr. Ruud Veldhuizen and Dr. Jim Lewis, are two outstanding scientists who have shown, time and again, to have the best interest of their students and collaborators at heart. My thanks for the all the support I received as part of the Lung Lab begin with them.

Rudy, your mentorship, help, and friendship have been indispensable throughout these years. I admire your scientific rigor, dedication, and your humble attitude, all of which are, for me, an example to follow. Thank you for the enjoyable chats about science, and for your words of encouragement during my moments of anxiety and stress. You have taught me more than good science and research by sharing some ‘life insights’ over a beer at the Grad Club, and by encouraging me to push past my boundaries. Thank you for always being there, even through difficult times, during all these years.

Jim, over the years I have had the pleasure to know what a great scientist and wonderful person you are. I truly admire your commitment to science and medicine, and I can only wish to become half as knowledgeable as you are. Thank you for giving me this unique opportunity, and for taking the time to provide me with feedback and guidance on my work throughout the years.

I would also like to thank Dr. Cory Yamashita: you are such a perfect match for the lab, and I am glad I had the chance to work with you. Thank you, Cory, for the scientific discussions, the ideas, and the feedback you have shared with me. Even though you were not my official supervisor, you have made yourself available on multiple occasions, and that has really meant a lot to me.

My experience in the lab would not have been the same without the indispensable technical and moral support of two fantastic women: Lynda McCaig and Li-Juan Yao. What would I have done without you? You both have been excellent teachers, and I only have deep admiration for all your competence and knowledge. I hope you realize how much respect and gratitude I have for you both. Lynda, your whole family has adopted me from the very beginning, making my transition into this new environment so far away from home, so much easier. You have never left me alone, and I truly appreciate all of your kindness and friendship. Thank you for playing such an essential role throughout my experience, on so many fronts of my life. Also, life in the lab would have not been as smooth and as fun without Yao Meister around. Thank you, Yao, for keeping your promise not to retire before I had finished my experiments. I am grateful for all your precious help. I will remember the joy you shared with me over the picture of your grandkids, and our great swimming nights at the pool.

Additionally, my thanks go out to all of the past and present members of the Lung Lab. Josh Qua Hiansen, Brandon Banaschewski, Scott Milos, Stephanie Aigbe, and many more of you have made this experience easier not only through your help around the lab, but also through sharing lots of good times and good memories that I will always keep with me.

I am also glad I had the opportunity to meet and spend time with such amazing people as Rosige Zeiten, Shannon Seney, Christine Beamish, among many others: thank you for the chats and the house parties; in one way or another, you all helped to bring balance into my life. I would especially like to thank Lenny (a.k.a. Leonardo) Guizzetti – you have been and still are such a great friend! Thank you for listening to me when I needed to vent my frustrations, and for the great evenings spent with you and Heather. A special thanks also goes to Erica Martin-Conte, for being such an amazing friend throughout the years despite the geographical distance separating us, and for all the support (moral and material!) that you have given me. It meant a lot.

A lot of gratitude goes to Chris Langlois. You are an incredible man. You have been an invaluable support, and I do not think I could have made it through these last few months without your encouragement, your understanding, and your attention. Thank you for your patience. I hope you know how much you (and the cats) mean to me.

Lastly, a few words for my family. I do not know where to begin to thank you all. You have always accepted my choices, even when they seemed a bit radical and crazy, and have always unconditionally supported me. Despite the distance, you have made sure to be close to me with your thoughts and words when I needed them the most, and encouraged me to persevere during the toughest times. I hope I will always be worthy of all your love. Mamma, Papa', Patrizia, Donatella, Salvatore, Sandro, Giulia, Claudio, Luca, Maria Sofia: GRAZIE.

Chapter 2: The authors would like to thank bLES Biochemical Inc. London, Ontario for generously providing bLES, and Shannon Seney for her help with the Milliplex cytokine/ chemokine analysis.

Chapter 3: The authors would like to thank Arian Gholami for technical assistance with cell counting, and Shannon Seney for her help with the Milliplex cytokine/ chemokine analysis.

Chapter 4: The authors would like to thank Wenjia Zhou and Manon Need for assistance with the surfactant analysis.

TABLE OF CONTENTS:

LIST OF TABLES:

LIST OF FIGURES:

LIST OF APPENDICES:

LIST OF ABBREVIATIONS:

ALI	acute lung injury
AM	alveolar macrophages
ANOVA	analysis of variance
ARDS	acute respiratory distress syndrome
bLES	bovine lipid extract surfactant
BMDM	bone marrow derived macrophage
CBS	captive bubble surfactometer
Crs	compliance of whole respiratory system
Cst	quasi-static compliance
DPPC	dipalmitoylphosphatidylcholine
E_2	17β-estradiol
ECM	extracellular matrix
ELISA	enzyme-linked immune-sorbent assay
Ers	elastance of whole respiratory system
Est	quasi-static elastance
F_IO_2	fraction of inspired oxygen
FOT	forced oscillation technique
G	tissue damping
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
H	tissue elastance
H&E	hematoxylin & eosin
HCl	hydrochloric acid
IC	inspiratory capacity
IL-10	interleukin-10
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8

IP-10	interferon-γ-induced protein-10
IPML	isolated perfused mouse lung
KC	keratinocyte chemoattractant
LA	large aggregate
LIX	lipopolysaccharide-induced CXC chemokine
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
MCP-1	monocyte chemotactic protein-1
MIP-2	macrophage inflammatory protein-2
MMP	matrix metalloproteinase
MMP-3	matrix metalloproteinase-3
MOF	multiple organ failure
MV	mechanical ventilation
NRDS	neonatal respiratory distress syndrome
PaO_2	partial pressure of oxygen
PC	phosphatidylcholine
PEEP	positive end expiratory pressure
PGE_2	prostaglandin E_2
PIP	peak inspiratory pressure
PL	phospholipid
PMN	polymorphonuclear
PV	pressure-volume
R_N	airway resistance (Newtonian resistance)
RR	respiratory rate
Rrs	resistance of whole respiratory system
SA	small aggregate
SEM	standard error of the mean
SP-A	surfactant protein A
SP-B	surfactant protein B
SP-C	surfactant protein C
SP-D	surfactant protein D

TIMPs	tissue inhibitor of metalloproteinases
TLC	total lung capacity
TLR-4	toll-like receptor 4
TNF-α	tumor necrosis factor-α
TS	total surfactant
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2
VALI	ventilation-associated lung injury
VFD	ventilator-free days
VILI	ventilation-induced lung injury
Vt	tidal volume

CHAPTER 1:

General introduction and literature review

1.1. General overview

Most of the metabolic processes taking place in the human body require oxygen and generate carbon dioxide as a waste product that needs to be eliminated. The lung is the organ responsible for exchange of gases, providing oxygen to the blood for delivery to all systems in the body, and allowing for carbon dioxide removal to the external environment [1]. This process, essential for life, can become impaired as a result of a variety of diseases affecting the lung, such as asthma, chronic bronchitis, fibrosis and, central to this thesis, acute lung injury.

Acute Lung Injury/Acute Respiratory Distress Syndrome (ALI/ARDS) is a pulmonary disorder with a complex pathophysiology and no proven therapeutic option available for its treatment [2]. Throughout the years, understanding of this disorder has been hampered by the multiplicity of lung insults potentially causing ALI/ARDS, the wide range of patient population, which includes both pediatric and adult subjects, and a complex disease progression [3, 4]. Two aspects of its pathophysiology and treatment support, however, are common to all patients with ALI/ARDS: i) the use of mechanical ventilation, necessary to support impaired gas exchange, and ii) the development of an overwhelming pulmonary inflammatory response.

As explained more extensively throughout this doctoral thesis, persisting and overwhelming lung inflammation negatively affects lung function, and has been shown to correlate with poor prognosis and outcome in patients with ALI/ARDS [5, 6]. This scenario is further aggravated by the effects of mechanical ventilation on the injured lung. Mechanical ventilation has been shown to alter lung surfactant [7], a substance lining the inner pulmonary surface, with biophysical and immune-modulatory properties essential for lung function [8, 9]. Ventilation-induced impairment of lung surfactant leads to impaired lung function [10], which can often be rescued by administration of exogenous surfactant [11]. The effects of mechanical ventilation, however, are not limited to surfactant, as ventilation can further pulmonary inflammation, and participate in the progression toward the development of an inflammatory response in the systemic circulation [12–14]. These events can, ultimately, affect distal organ function causing multi-organ failure, the major cause of death in ALI/ARDS patients [15, 16].

In light of these issues, the first scientific problem addressed in **chapter two** is related to **the assessment of a lung-targeted strategy aimed at mitigating the effects of ventilation on the inflamed lung**. The strategy of interest is exogenous surfactant administration. The objective of chapter two is to determine whether exogenous surfactant administration can mitigate ventilation-induced pulmonary and systemic inflammation, in different mouse models of ALI/ARDS.

The second aspect highlighted by the lack of suitable treatment is the need for a better understanding of ALI/ARDS pathophysiology, specifically focused on **identifying key mediators in the pulmonary inflammatory response** that may serve as future potential therapeutic targets. Among the multiple mediators involved in ALI/ARDS, **chapter 3** focuses on the protease matrix metalloproteinase-3 for its role in inflammation and inflammatory diseases [17]. The objective of chapter three is to assess the role of matrix metalloproteinase-3 in the development of the pulmonary inflammation associated with ALI/ARDS. The results of matrix metalloproteinase-3 contribution to inflammation in two different mouse models of lung injury prompted the investigation of further aspects of disease development.

Chapter four focuses on the examination of the **potential role of matrix metalloproteinase-3 in the interplay between pulmonary inflammation and lung function.** The objective of chapter four is in fact to investigate whether such protease, mediator of lung inflammation, can affect the surfactant system and overall lung function.

The remaining of this first chapter provides general information on lung structure and function, and illustrates in greater detail the pathophysiology of ALI/ARDS, touching on the inflammatory response, lung surfactant, and matrix metalloproteinase-3, before closing with a brief description of the animal models available for the study of this disorder.

1.2. Lung function and structure

The primary function of the lung is gas exchange. During breathing, oxygen entering the lungs diffuses into the blood, while carbon dioxide diffuses from the blood into the lung to be exhaled in the external environment. Lung function is facilitated by a number of anatomical features, namely the presence of an extremely large pulmonary surface area available for diffusion, close proximity of the inhaled air to blood vessels at the alveolar level, and the very low thickness of the alveolo-capillary barrier through which oxygen and carbon dioxide diffuse [1].

Such anatomical features are the final result of a tree-like structure that starts at the nose and mouth, and proceeds within the thoracic cavity via a semi-flexible tube, the trachea, which divides into left and right main primary bronchi. Each bronchus branches multiple times into progressively narrower and shorter bronchi/bronchioles down to the terminal bronchioles, thereby generating a very large number of conducting airways. The process of subsequent divisions continues further into the distal regions of the lung, until millions of individual lung units, known as alveoli, generate a very large surface area suitable for gas exchange. Diffusion of oxygen and carbon dioxide is also favored by the vast network of capillaries wrapped around the alveoli, with the capillary endothelial cells laying in very close proximity to the epithelial cells of the alveoli [1].

As mentioned, epithelial cells form the alveolar wall; precisely, alveoli are mainly made of flat, squamous type I alveolar epithelial cells and some cuboidal type II epithelial cells. The type II cells produce and secrete pulmonary surfactant at the air-liquid interface [18]. Surfactant, a protein-lipid mixture, is very important for reducing the surface tension in the alveoli, thereby optimizing lung compliance and facilitating the work of breathing [8]. In addition to type I and type II epithelial cells, some resident alveolar macrophages (AM) are found within the alveolar space, where they act as a first line defense against pathogens and participate in surfactant metabolism [19, 20].

1.3. Lung mechanics

In addition to the structure of the lung, the process of ventilation is important for gas exchange. Ventilation refers to the process by which a volume of air, known as tidal volume, enters and leaves the lung with each breathing cycle. The mechanics of this process are closely linked to the properties of the lungs and those of the thoracic cavity in which the lungs are located. Within this enclosed space, the base of the lungs comes in contact with the diaphragm, a dome-shaped muscle that separates the lungs from the abdominal contents. Pleural membranes surround the outer surface of each lung and line the inside of the chest wall, forming a thin intra-pleural space that is normally filled with a small volume of fluid. The intra-pleural space provides connection between the lung and the chest wall, and given the natural tendency of the chest wall to expand and the lung to collapse, this space has a slightly sub-atmospheric pressure or, in other words, a negative pressure [1].

During inspiration, contraction of the inspiratory muscles (diaphragm and external intercostals) causes an increase in the volume of the thoracic cavity. With the increase in volume, the negative intra-pleural pressure becomes more negative leading to the expansion of the lung and a fall in alveolar pressure to a slightly sub-atmospheric value. This pressure gradient promotes flow of air from the atmosphere to the alveoli. In tidal breathing (non-exertional), expiration, unlike inspiration, is a passive process resulting from the relaxation of the inspiratory muscles, with subsequent decrease in the thoracic and lung volumes. These changes affect the alveolar pressure, now slightly greater than atmospheric pressure, providing the driving force necessary for air to flow from the alveoli back to the atmosphere [1].

The work of breathing can be affected by two main factors: the resistance to airflow within the conducting airways and the distensibility of the lung tissue. In healthy subjects, the medium to larger airways offer a negligible degree of resistance to the airflow. Narrowing of these airways, however, as a result of bronchial constriction or obstruction such as in asthma, will increase the resistance and impair airflow [21].

The distensibility of the lung tissue, known as compliance, refers to the ability of the lung to inflate and stretch during inspiration. Lung compliance is an indicator of the stiffness of the lungs, and it is defined as volume change per unit pressure change [1]. Two principal factors can influence compliance: the elastic properties of the lung and the surface tension of the alveolar lining fluid. The elastic properties can be described essentially as the tendency of the lung to recoil to the resting volume after distention. Lung elasticity arises from the elastin and collagen fibers in the pulmonary tissue, and any alteration of these fibers can cause changes in lung compliance [1]. As mentioned, compliance is also influenced by the surface tension arising from attractive forces between water molecules at the air-liquid interface within the alveoli. In healthy lungs, surface tension has very low values attributable to the presence of a material lining the alveoli called pulmonary surfactant. Secreted by alveolar type II cells at the air-liquid interface, lung surfactant is a protein-lipid mixture that lowers surface tension, thereby stabilizing the alveoli and reducing the work of breathing [8, 22]. Whole lung compliance is therefore strongly affected by the presence of a functioning surfactant system. The relationship between an impaired surfactant system and consequently poor lung compliance is most evident in a disease called the neonatal respiratory distress syndrome, where preterm infants are born with insufficient amounts of surfactant and struggle to breath [23]. Alterations of the surfactant system in a mature lung can occur in the acute respiratory distress syndrome, which is the focus of this thesis.

A more detailed overview of surfactant composition, function and alterations during disease will be provided in the following sections of this thesis.

1.4. Lung insults: the Acute Lung Injury / Acute Respiratory Distress Syndrome (ALI/ARDS) paradigm.

1.4.1. ALI/ARDS definition

This thesis focuses on the clinical problem of acute lung injury (ALI) and on ALI's more severe form, the acute respiratory distress syndrome (ARDS). An adult respiratory-distress

syndrome was first described in 1967 by Ashbaugh in twelve adults presenting with acute onset of rapid breathing, hypoxia, and poor lung distensibility [24]. The extensive research that followed improved the knowledge of this disease's pathophysiology, leading to a renaming of the disease to acute respiratory distress syndrome and a clear clinical definition in 1994 by the American-European Consensus Conference. ARDS and ALI were defined as acute in onset and characterized by bilateral radiologic infiltrates with no evidence of heart failure, and hypoxemia, as determined by the ratio of arterial partial pressure of oxygen (PaO_2) to fraction of inspired oxygen (F_IO_2). Specifically, ALI was defined by a $PaO_2/ F_IO_2 \leq 300$mmHg, while the cut off for the more severe ARDS was a $PaO_2/ F_IO_2 \leq$ 200mmHg [25]. In more recent years, some of the limitations of this definition have been addressed with the updated "Berlin definition", which has removed the term ALI and instead distinguishes between three mutually exclusive ARDS subgroups (mild, moderate, or severe ARDS) based on the severity of the hypoxemia [26]. While this new definition constitutes an improvement to the clinical practice allowing for better stratification of patients in clinical trials, it poses some challenges in its application to animal models of lung injury since there is no reference to underlying pathophysiology. The terminology acute lung injury, instead, traditionally includes a broader spectrum of the disease, encompassing both patients and experimental models. For this reason, the term ALI will be used to refer to the acute lung injury/acute respiratory distress syndrome throughout the remainder of this thesis.

A broadly accepted definition of this disease has allowed for the collection of useful epidemiological information. Recent estimates suggest an ALI incidence of approximately 60-80 new cases per 100,000 person-years in the United States [4, 27]. Importantly, mortality from ALI is still very high at 40%, with distal organ failure, rather than respiratory failure, being the main cause of death for these patients [15, 16, 28].

1.4.2. ALI overview

Despite a relatively simple clinical definition based on physiological parameters, ALI is a complex pulmonary disorder characterized by decreased compliance, persistent and elevated lung inflammation, a high mortality, and no available therapeutic options [29–31].

After many years of clinical and basic research, many aspects of ALI pathophysiology are still elusive and challenge the development of effective pharmacological therapies. To add to the complexity, disease progression is associated with the development of systemic inflammation and multi-organ failure which is, as previously mentioned, the most common cause of death in ALI [15, 32].

Affecting patients of all ages, ALI is initiated by a variety of lung insults of different origin [2]. For example, potential threats and pathogens can come from the external environment, to which the lung is continuously exposed [33, 34]. On the other hand, the lung receives the entire cardiac output through its vasculature and can therefore be affected indirectly by damage-associated molecules or invading organisms found in the pulmonary circulation [33, 34]. In most cases, the lung can manage and effectively clear such threats. On occasion, however, the host response to a lung insult can become maladaptive. As a result, patients with ALI present with lung edema, altered pulmonary surfactant, decreased compliance with an associated hypoxemia, and a sustained pulmonary inflammatory response [2, 35, 36].

The progressive hypoxemia affecting these patients ultimately requires the use of mechanical ventilation (MV), the main supportive treatment for this disorder. Even though essential, MV can contribute to lung injury and inflammation, thereby promoting ALI progression [12]. The role of MV in ALI became strongly evident in a large, multi-center randomized clinical trial conducted in 2000 [37]. In this study, the ARDS Network assessed the effect of ventilation using different tidal volumes in patients with ALI. Patients received MV with either a ‘conventional’ tidal volume (Vt=12mL/kg predicted body weight) or a low tidal volume (Vt=6mL/kg predicted body weight) strategy. The trial was stopped early due to the significantly lower mortality in the low Vt group (31.0%) compared to the group receiving conventional Vt ventilation (39.8%) [37]. The underlying pathophysiological mechanism responsible for such outcome was suggested to stem from the effects of MV on the inflammatory response in ALI. An earlier clinical study by Ranieri et al. had in fact shown that, at 36 hours post randomization, more injurious ventilation strategies caused greater increases in both pulmonary and systemic inflammation in ALI patients, compared to concentrations at study entry and in patients ventilated with lung-protective MV [12].

Overall, these clinical studies have highlighted that MV is a potential contributor to disease progression, by enhancing lung inflammation and leading to a systemic inflammatory response and peripheral organ failure. As such, MV could represent an ultimately effective target in ALI treatment.

Additionally, this evidence shifted the focus from oxygenation, the traditional "clinical outcome" of patients with ALI/ARDS, to inflammation, as it became clear that a persistent, excessive lung inflammatory response is the culprit for disease progression [5, 6]. In this respect, Meduri et al. demonstrated that at the onset of ARDS, non-survivors had significantly higher pulmonary inflammatory mediators (i.e. IL6, IL8, TNF-α) levels than survivors, stressing the association between lung inflammation and disease outcome [6].

Lastly, the evidence of a central role of inflammation in ALI would suggest that a lung-targeted treatment, aimed at reducing pulmonary and systemic inflammation, could be extremely beneficial and more effective than strategies merely aimed at improving oxygenation. In this sense, **exogenous surfactant** administration is a **lung-targeted treatment** whose role in affecting the inflammatory response associated with ALI has been insufficiently characterized. Moreover, the study of key **mediators of pulmonary inflammatio**n (such as, for example, **matrix metalloproteinase-3**) could help identify new potential therapeutic targets for this disorder.

1.4.3. Treatment of ALI

Treatment of ALI is based on supporting gas exchange through MV, careful monitoring and stabilization of these critically ill patients, and management of the initiating insult when possible [38]. Unfortunately, no pharmacological treatment is yet available for this disorder, and even though many therapies have been promising experimentally, they have failed to improve outcomes clinically [39]. Among these, exogenous surfactant administration led to exciting improvements in oxygenation and compliance for ALI patients in Phase 2 trials and smaller Phase 3 trials [40, 41]. This treatment, however, did not appear to improve mortality in more recent, larger Phase 3 trials, possibly due to surfactant administration occurring too late in the paradigm of ALI development [40]. A

more in depth description of surfactant in ALI pathogenesis and treatment will be described in section 1.6.

To date, the only approach shown to reduce mortality in ALI is the use of low tidal volume/ protective MV, likely due to lower pulmonary and systemic inflammation elicited by this strategy compared to higher Vt ventilation [37]. Since ALI is characterized by a persistent, excessive pulmonary inflammatory response [42, 43], anti-inflammatory treatments such as corticosteroids, statins and activated protein C could theoretically decrease mortality. The results from clinical trials however have been disappointing, showing no clear benefits of such treatments [44, 45]. It is therefore imperative to expand our knowledge of ALI pathophysiology and the associated inflammatory response, as well as to re-examine some of its treatments, in order to effectively interfere with disease progression and improve mortality.

In summary, examination of the clinical studies performed to date suggest that ALI, although defined by physiological criteria, is a complex inflammatory disease in which its essential therapeutic intervention (MV) can actually contribute to disease progression. Despite the complex pathophysiology of ALI, recent animal and clinical studies have started to provide insight into the development of this disease. The current state of knowledge, and areas requiring further research, are described below with a specific focus on inflammation.

1.4.4. Development of ALI: the ALI paradigm

The current model for ALI development and progression, based on clinical, *in vivo*, and *in vitro* studies, is shown in figure 1.1. Briefly, the model illustrates how multiple insults (or hits) to a normal lung can lead to the development of ALI, and exemplifies the disease progression from lung injury to multi-organ failure, which is the main cause of death for ALI patients. More detailed information on the different steps in the multiple hit paradigm of ALI is provided in the upcoming sections.

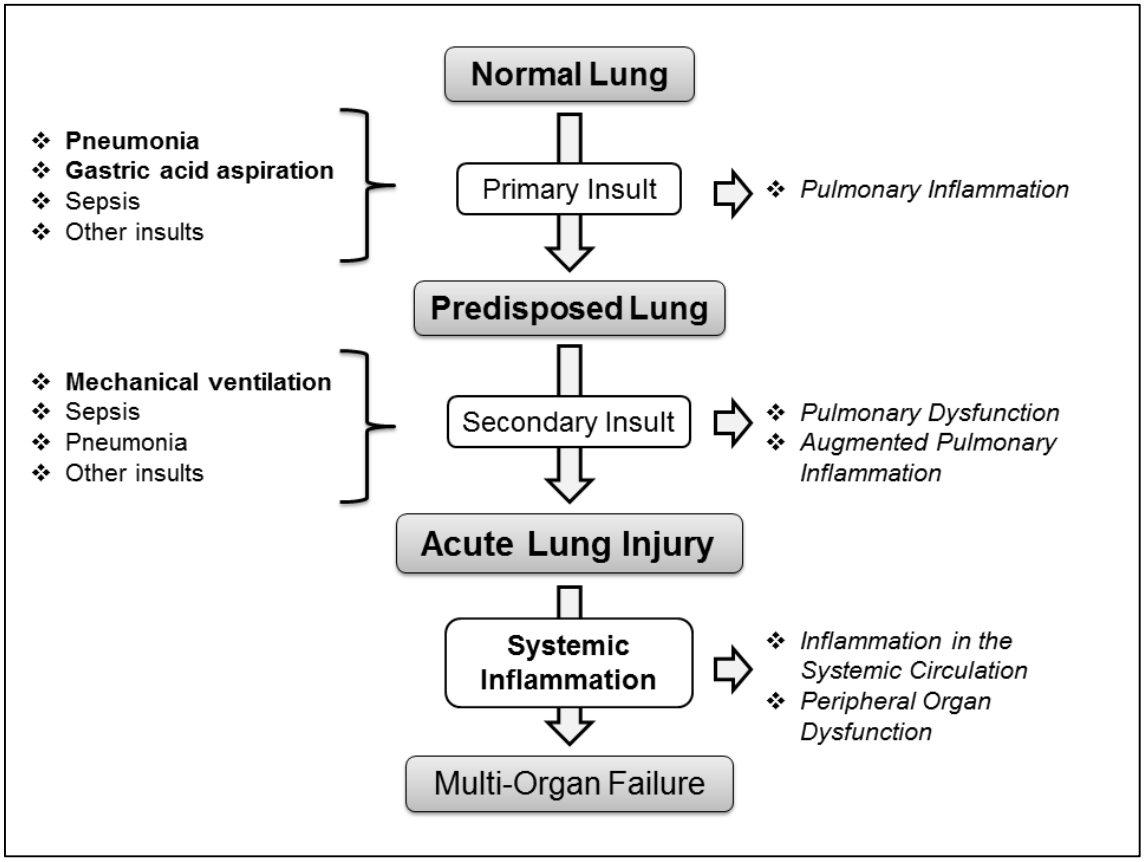

Figure 1.1: Multiple hit paradigm of ALI development.

1.4.4.1. Primary insults and ALI pathogenesis

In the multiple hit paradigm of ALI, a normal lung is first exposed to an initiating or *primary insult*, which can be classified as either direct or indirect (Fig. 1.1) [2]. Indirect insults such as sepsis or trauma primarily affect the pulmonary vasculature, given the presence in the circulation of pathogens and inflammatory molecules from various potential sources. On the other hand, insults such as *pneumonia* or *gastric acid aspiration* represent direct injuries to the lung, and are the main focus of this thesis [2]. Numerous animal studies have shown that such direct injuries cause greater damage to the alveolar epithelium, and a more robust inflammatory response within the alveolar space compared to indirect insults [33, 46]. Overall, the response of the lung to any insult is a rather

complicated process involving a multitude of soluble mediators, multiple cells types, and a complex integration of intracellular pathways. For this reason, a simplification of the major pathophysiological steps involved in this disease is necessary to better understand ALI.

Following the initial lung insult, the disruption of the alveolo-capillary barrier allows the abnormal leakage of a protein-rich edema into the alveolar space [29, 47]. This, in turn, inhibits surfactant function [48] and leads to a profound decrease in lung compliance [49]. Importantly, alveolar macrophages and, to some extent, epithelial cells respond to the injurious event by mounting an inflammatory response within the alveolar space [50]. Up-regulation of pulmonary cytokines and chemokines results in the recruitment of polymorphonuclear neutrophils (PMNs), cells of the innate immune system that are first responders in tissue injury and infection [51]. While this initial inflammatory response is a homeostatic process important for injury resolution, the severity and persistence of inflammation in ALI can cause tissue injury, impair effective resolution, and correlate with poor outcomes. Occurrence of the latter processes may be promoted if the lung is exposed to a secondary insult.

1.4.4.2. Secondary insult, systemic inflammation and multi-organ failure

The elevated pulmonary inflammation, alveolar flooding and surfactant alterations that follow a primary insult make the lung susceptible (*predisposed lung*) to the effects of other *secondary insults*, such as sepsis, trauma or, most commonly, mechanical ventilation (Fig. 1.1). As mentioned earlier, decreased compliance and hypoxemia are hallmarks of ALI [25], and MV is necessary to support gas exchange. The contribution of MV as a secondary insult has been extensively highlighted in both clinical studies and animal models of lung injury [12, 14, 52, 53], many of which have also shed light on the three different ways MV participates in ALI progression.

First, MV contributes to lung surfactant inactivation, with consequent alveolar collapse and worsening lung compliance [7, 54]. Second, due to the overstretching of the more aerated and compliant alveolar units, MV causes increased release of inflammatory mediators,

thereby exacerbating lung injury [55–57]. Third, ventilation itself is an important contributor in ALI progression toward *systemic inflammation* (Fig. 1.1) [13, 58].

A vast body of experimental evidence has in fact shown that MV promotes the de-compartmentalization of pulmonary inflammatory mediators into the systemic circulation, and that exacerbation of pulmonary inflammation by MV enhances the development of systemic inflammation [58–60]. Importantly, the severity of the systemic inflammatory response correlates with mortality in ALI [37]. Inflammatory molecules in the circulation are, in fact, biologically active and exert a pathogenic role on extra-pulmonary organs (i.e., liver and kidneys) ultimately leading to *multi-organ failure* [14, 52, 61–63]. An interesting study from our lab has in fact demonstrated that circulating inflammatory mediators released from injured lungs can activate distal cell populations, namely mouse liver endothelial cells and leukocytes, leading to a pro-inflammatory and pro-adhesive phenotype in these cells [64].

Since inflammation is a crucial component of ALI pathophysiology, an overview of the inflammatory response associated with ALI is necessary, before addressing in greater details the specific variables manipulated for the study of lung injury in this thesis.

1.5. Inflammation and inflammatory mediators in acute lung injury

As mentioned above, inflammation plays a central role in ALI. Inflammation is a complex, highly regulated adaptive response to tissue injury or infection, mounted to re-establish homeostatic conditions. The inflammatory response involves a variety of cellular and soluble mediators cooperatively working to eliminate the detrimental stimuli, thereby progressing toward phases of resolution and tissue repair [65]. Sometimes, however, for reasons that are not yet clear, the inflammatory process persists, becomes maladaptive, and may lead to organ injury and dysfunction as in the case of ALI.

1.5.1. Cellular components in the inflammatory process

Among the different cell types participating in the inflammatory process, neutrophils are particularly relevant in the settings of ALI. Neutrophils are recruited to the injured lung and migrate into the air space shortly after a primary insult [51]. These cells are essential players in the innate immune response to injury and/or infection, and within the alveolar environment these cells can contribute to the development of ALI through the release of pro-inflammatory cytokines and production of reactive oxygen species [51, 66]. Moreover, activated neutrophils can secrete potent proteolytic enzymes, such as elastase, collagenase (i.e., matrix metalloproteinase-8) and gelatinases (i.e., matrix metalloproteinase-9), potentially responsible for alterations of the lung extracellular matrix [66]. The pathogenic role of neutrophils in ALI has been shown in animal models of lung injury [67, 68]; moreover, pulmonary accumulation and persistence of neutrophils appear to correlate with disease severity in patients with ALI [69]. Nonetheless, the evidence that neutropenic patients can develop lung injury as well [70], suggests that other cell populations may be involved in ALI. In fact, important contributors to this elaborate inflammatory response are also the parenchymal cells, namely endothelial cells, alveolar epithelial cells, fibroblasts, and the alveolar macrophages [50].

As a first line of defense, the alveolar macrophages phagocytose pathogens and dead cells, can secrete anti-microbial peptides, and release proteases such as matrix metalloproteinases (including matrix metalloproteinase-3), thereby orchestrating the inflammatory and immune response, and contributing to the later reparative phase [71]. Additionally, alveolar macrophages can also release a variety of soluble mediators of inflammation, proteinaceous and/or lipidic in nature, responsible for many of the pathophysiological events occurring in ALI, including neutrophils recruitment [71].

Migration and influx of leukocytes to the injured lung is also facilitated by the activated endothelium, which expresses surface adhesion molecules necessary for cell to cell interaction [72]. Epithelial cells participate to the immune response in ALI through the secretion of collectins (SP-A, SP-D) associated with surfactant and, additionally, via the release of inflammatory mediators (i.e. IL-1β, IL-8, TNF-α) in response to multiple stimuli, including stretch associated with MV [73–76].

1.5.2. Overview of soluble inflammatory mediators

In addition to the cellular component, soluble mediators are a second key pathological feature in the development and progression of ALI. Following insults to the lung (Fig. 1.1), a broad variety of pro- and anti-inflammatory molecules are released from the aforementioned cellular sources within the lung and in the bloodstream. For the sake of brevity, only inflammatory signals relevant to this thesis will be reviewed in this section. Mediators of interest include: i) cytokines, ii) chemokines, and iii) lipid mediators. A list of such mediators with an indication of their main respective biological functions is given in Table 1.1.

Inflammatory Mediators	Biological Activity
Cytokines	
G-CSF	Granulocyte survival/growth
GM-CSF	Host defense; granulocyte/monocyte/AM survival & growth
IL-1β	Pro-inflammatory; fever; neutrophil migration
IL-6	Pro-inflammatory; acute-phase response; leukocytes growth/differentiation
IL-10	Dual role; pro- and anti- inflammatory
IL-13	Anti-inflammatory; asthma and allergic disease
TNF-α	Pro-inflammatory; hypotension/shock; cell cytotoxicity; fever
Chemokines (alternative name)	
Eotaxin (CCL11)	Pro-inflammatory; chemoattractant for eosinophils & basophils; allergic airways inflammation
IP-10 (CXCL10)	Pro-inflammatory; chemoattractant for activated T cells
KC (CXCL1)	Pro-inflammatory; chemoattractant for neutrophils
LIX (CXCL5)	Pro-inflammatory; chemoattractant & activator of neutrophils
MCP-1 (CCL2)	Pro-inflammatory; chemoattractant for monocytes
MIP-2 (CXCL2)	Pro-inflammatory; neutrophils chemoattractant/activator
Lipid Mediators	
8-Isoprostane	Marker of oxidative stress
Leukotriene B_4	Pro-inflammatory; neutrophils chemoattractant/activator
Prostaglandin E_2	Inflammation; vascular tone & permeability
Thromboxane A_2	Pro-inflammatory; neutrophils chemoattractant/activator

Table 1.1: Summary of inflammatory mediators involved in ALI and most relevant in this thesis. KC, MIP-2 are considered murine equivalent of human IL-8. For further information, see Bathia M. *et al.* [43], Puneet P. *et al.* [77], and "Principles of internal medicine", Harrison, 15th edition [78]. AM, alveolar macrophages.
G-CSF = granulocyte colony-stimulating factor, GM-CSF = granulocyte-macrophage CSF, IL-6 = interleukin-6, IP-10 = interferon-γ-induced protein 10, KC = keratinocyte chemoattractant, LIX = lipopolysaccharide-induced CXC chemokine, MCP-1 = monocyte chemotactic protein-1, MIP-2 = macrophage inflammatory protein 2 and TNF-α = tumor necrosis factor-alpha.

1.5.2.1. Cytokines and chemokines

Cytokines and chemokines are small proteins secreted by immune and non-immune cells. Once released in the extracellular environment, cytokines will affect the activity and function of other, target cells [65]. Chemokines work as chemoattractant and activators of leukocytes, and are generally classified based on the position of the first two cysteine residues at the N-terminal: CC chemokines for adjacent residues, CXC if an amino acid separates them [77]. Together, cytokines and chemokines coordinate the inflammatory response through cell activation, changes in gene expression, and recruitment of inflammatory cells to the site of injury. These events are extremely significant in the pathogenesis of ALI, where increases of chemokine levels in alveolar fluid lead to massive recruitment and infiltration of neutrophils, potentially contributing to lung dysfunction [42, 66]. In the multiple hit paradigm of ALI development, changes in pulmonary cytokine and chemokine levels are induced at first by a primary insult of variable nature [2]; invariably, however, MV is applied to the predisposed lung contributing to overwhelming cytokine release. Numerous experimental studies confirm the exacerbation of lung inflammation by MV and point out the ventilation-induced de-compartmentalization of mediators such as TNF-α, IL-8, MCP-1 in the systemic circulation, with consequent development of distal organ failure [13, 58, 60, 61, 79]. It appears, therefore, that alterations of the inflammatory milieu resulting from MV can worsen ALI outcomes. The pathogenic role of cytokines and chemokines in ALI is in fact substantiated by clinical evidence that elevated lavage and

plasma levels of IL-6, TNF-α, IL-1β, and IL-8 correlate with disease gravity and poor outcomes in these patients [6, 12, 80–82]. Importantly, the concentration of these pro-inflammatory cytokines was found persistently elevated in the lung of non-survivors, while ALI survivors observed lower IL-6, TNF-α, IL-1β, and IL-8 levels at the onset of ALI, and over the course of the disease [6]. Overall, this evidence indicates the necessity to modulate pulmonary and systemic cytokine/ chemokine levels in order to improve ALI outcomes.

1.5.2.2. Lipid mediators in ALI

The complex inflammatory scenario associated with ALI is not limited to the above-mentioned small, protein mediators, but also includes lipid mediators of inflammation (Table 1.1). Lipid mediators, or eicosanoids, are derived from the metabolism of arachidonic acid by different enzymes: the cyclooxygenases pathway leads to the production of mediators such as isoprostanes, prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2), while the activity of lipoxygenases generates, among others, leukotriene B_4 (LTB_4) [83]. The contribution of these mediators to ALI pathogenesis can be inferred by both clinical and experimental data.

Elevated concentrations of leukotrienes have been detected in lavage samples from ALI/ARDS patients [84]; moreover, LTB_4 levels have been shown to correlate with the occurrence of lung injury in trauma patients [85]. Eicosanoids play a role in vascular tone, activation and permeability, and serve as potent chemoattractants and activators for neutrophils. In this regard, Zarbock et al. have demonstrated that TXA_2 is responsible for the recruitment and accumulation of neutrophils to the injured lung in a mouse model of acid-induced ALI [67]. Moreover, experimental work performed by Jaecklin et al. has shown the pathogenic role of circulating lipid mediators in a model of ventilation-induced lung injury. The group observed that, when perfused in the pulmonary circulation of recipient mice, lung-derived mediators isolated from mice with ventilation-induced lung injury could worsen permeability and compliance of recipient mice ventilated with non-injurious modes of MV [86]. Subsequent analysis of these soluble mediators revealed their protein and lipid nature, thereby strengthening the role of cytokines, chemokines and eicosanoids in ALI pathogenesis.

1.5.3. Targeting inflammation as a therapy for ALI

Despite the convincing evidence that overwhelming inflammation is an important pathophysiological feature contributing to the progression of ALI, interfering with this process has not been successful to date. For example, strategies involving the depletion of inflammatory cell types (i.e. alveolar macrophages or neutrophils) not only have shown conflicting experimental results [87–90], but would translate poorly into clinical practice due to issues of safety and feasibility, given the undeniable importance of innate immunity in host defense and tissue repair.

The rather intuitive approach of targeting individual cytokines or chemokines to down-modulate the inflammatory response has also proven ineffective in both experimental and clinical studies. The work of Nakamura et al. showed that mice lacking the expression of the cytokine IL-6 had similar physiological impairments than wild type mice, following exposure to three different models of ALI [91]. A more recent study by Markovic et al. demonstrated that, while solutions containing lung-derived inflammatory mediators caused liver endothelial cell dysfunction, the neutralization of IL-6 or TNF-α found in such solutions was ineffective in rescuing the alterations in endothelial cells [64]. Clinically, an antibody against TNF-α or administration of IL-1β Receptor Antagonist have been tested for the treatment of severe sepsis, a known cause of ALI (Fig. 1.1), but failed at reducing mortality in this patient population [92, 93].

Attempts at modulating the inflammatory response associated with ALI have also been made with the clinical evaluation of corticosteroids. As emerges from a recent meta-analysis of several clinical studies, corticosteroid therapy in ALI showed no effect on long term mortality, and even appeared to significantly harm patients with influenza-related lung injury [94].

Taken together, this evidence underscores the necessity for further understanding of the inflammatory response and the need for new therapeutic strategies. In this regard, we believe that lung **surfactant** and the protease **matrix metalloproteinase-3** might be such potential strategies. An overview dedicated to their role in inflammation and ALI is given in the following sections of this introductory chapter.

1.6. The pulmonary surfactant system

Being an important contributor in the pathogenesis of ALI and a marker of disease progression, the pulmonary surfactant system has been the focus of intense *in vitro*, *in vivo*, and clinical research over the last five decades.

As mentioned earlier, the biophysical role of pulmonary surfactant is to reduce the surface tension at the air-liquid interface, thereby ensuring optimal lung compliance [1]. The decrease in pulmonary compliance typical of ALI generally results from the impairment of surfactant activity [95].

In addition to its surface tension reducing properties, lung surfactant has also an important role in immune modulation and host defense within the alveolar environment [9]. Interestingly, less is known about this function in the context of ALI.

1.6.1. Surfactant composition

The composition of surfactant is conserved across mammalian species and consists of approximately 90% lipids, primarily phospholipids, and 10% surfactant associated proteins [8]. The most abundant phospholipid component is phosphatidylcholine (PC), half of which is dipalmitoylphosphatidycholine (DPPC), a disaturated species important for achieving low surface tension values at the end of expiration [8].

The protein components include four surfactant associated proteins: surfactant protein-A (SP-A), surfactant protein-B (SP-B), surfactant protein-C (SP-C) and surfactant protein-D (SP-D) [96, 97]. The large, hydrophilic SP-A and SP-D proteins are members of the collectin family and participate in the innate immune response [98]. SP-B and SP-C are small, highly hydrophobic proteins very intimately associated with the lipids. SP-B and SP-C proteins are important for promoting the formation of the surface film and supporting its biophysical function [99].

1.6.2. Surfactant metabolism

All of the surfactant components are synthesized and secreted by the alveolar type II cells (Fig. 1.2) [100]. Surfactant is exocytosed into the alveolar space from storage organelles, the lamellar bodies, found within type II cells [101]. SP-B and SP-C are assembled and secreted together with the lipids, while synthesis and release of SP-A and SP-D is mainly independent from lipid metabolism [102, 103]. There is some evidence, however, that SP-A may be secreted in association with the lamellar bodies as well (Fig. 1.2) [104, 105].

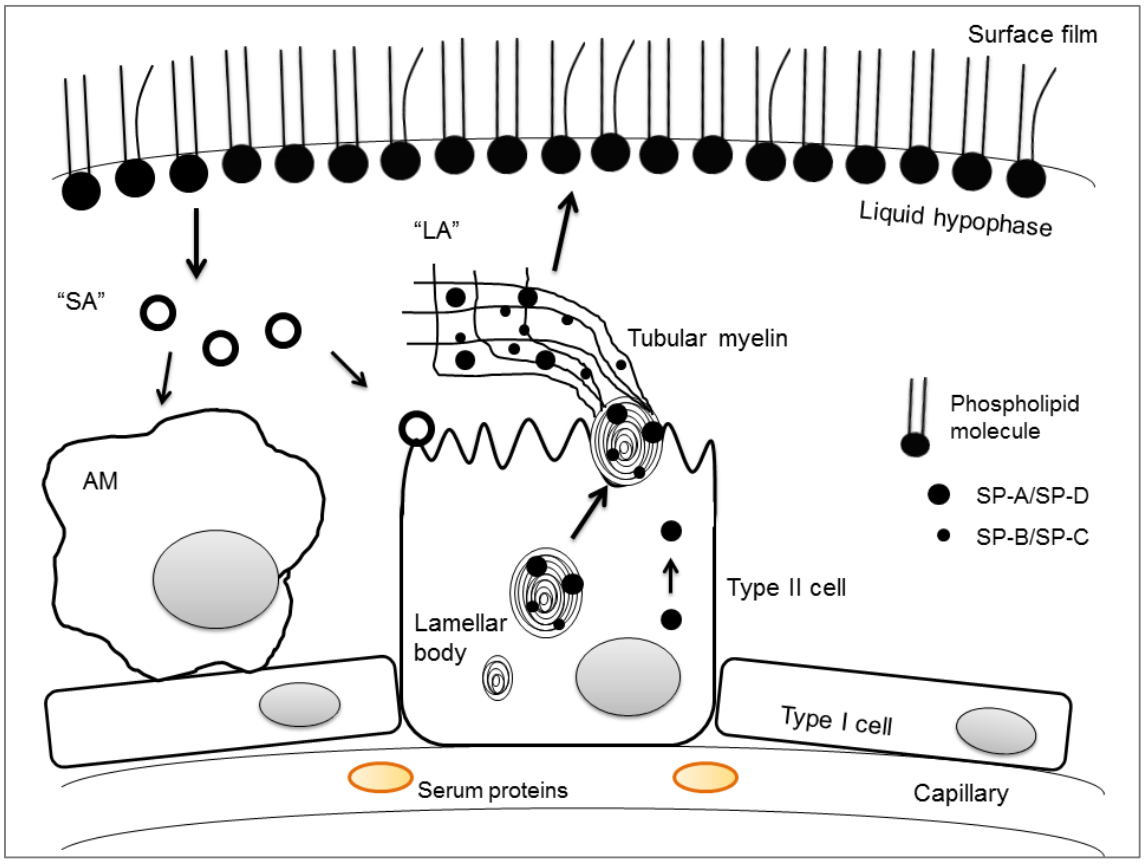

Figure 1.2: Surfactant metabolism. Surfactant is a protein-lipid mixture synthesized by type II alveolar cells, stored in lamellar bodies and secreted into the liquid hypophase. Tubular myelin is then generated and adsorption of phospholipids to the interface creates a surface film enriched in DPPC. Breathing motion causes formation of small vesicles, which are up-taken by type II cells or cleared by alveolar macrophages (AM). LA, large aggregates; SA, small aggregates.

Following secretion, the surfactant from the lamellar bodies undergoes reorganization into tubular myelin, a lattice-like structure that is then adsorbed rapidly to the air-liquid interface where it forms a monolayer film [102]. Upon ventilation, the changes in the alveolar surface area favor the conversion of surfactant into small vesicles with poor biophysical activity. Pulmonary surfactant can be collected through lung lavage, and the vesicular forms can be subsequently isolated via differential centrifugation of lung lavage samples. The process leads to the separation of a larger, heavier sub-fraction named large aggregates (LA) and small, lighter vesicles called small aggregates (SA) [106, 107]. The LA component consists of structures from the lamellar bodies, tubular myelin, surfactant proteins SP-A, SP-B, and SP-C and has excellent surface tension reducing properties [108, 109]. As mentioned, changes in the pulmonary surface area determine the conversion of large aggregates into SA, the latter being the biophysically inactive sub-fraction with lower content of surfactant-associated proteins [108]. Lastly, clearance of inactive surfactant occurs via reuptake and recycling from alveolar type II cells, or through phagocytosis and degradation within alveolar macrophages (Fig. 1.2) [102, 103].

1.6.3. Surfactant function

As previously mentioned, the biophysical function of surfactant consists in lowering the surface tension at the air-liquid interface [8]. Without surfactant, the water molecules at the very surface of the liquid hypophase would experience a net inward force, attracting them to the bulk of the liquid. In this situation, as the surface area decreases (ie. during exhalation), the high surface tension would resist lung expansion and a relatively high pressure would be necessary to re-open the lung. This would decrease alveolar stability and promote alveolar collapse. The presence of the surfactant film at the air-liquid interface assures a reduction of the surface tension to values near zero mN/m, with DPPC being the primary component contributing to alveoli stabilization at low lung volume [110, 111]. Importantly, the hydrophobic proteins SP-B and SP-C also participate in this process by promoting lipid adsorption at the interface and facilitating the re-spreading of surfactant during inspiration (corresponding to an expansion in surface area) [22, 99, 112]. The roles of SP-B and SP-C in surfactant function become particularly evident when analyzing the phenotype of mice in which the expression of either protein had been knocked out. Mice

lacking SP-B are not viable and die shortly after birth due to respiratory distress. Lack of SP-B impairs the generation of lamellar bodies and tubular myelin, highlighting the essential role of this surfactant protein in lipid organization [99, 113]. Conversely, the phenotype of mice with SP-C deficiency is not as dramatically altered, with pulmonary surfactant from these mice exhibiting minor biophysical changes at low lung volume, thereby supporting the importance of SP-C in film stabilization at the end of expiration [114].

The collectin SP-A contributes as well to the biophysical function of surfactant. SP-A aids in lipid adsorption and in the structural organization of the surfactant film undergoing cycles of compression and expansion [115, 116]. This surfactant-associated protein is also important for limiting the impairment in biophysical function consequent to intra-pulmonary leakage of serum albumin [117].

SP-A, however, together with SP-D, is primarily involved in the immuno-modulatory functions of surfactant [9]. SP-A and SP-D can opsonise viruses and bacteria and promote their clearance via phagocytosis by inflammatory cells within the lung; in line with this evidence, mice genetically modified to lack SP-A or SP-D expression succumb more easily to bacterial infection [118–120]. In addition to such activities, SP-A can influence the secretion of inflammatory mediators by peripheral immune cells and alveolar macrophages, the production of reactive oxygen species, and can inhibit lymphocyte proliferation [76, 121].

Furthermore, SP-B, SP-C, and some of the surfactant phospholipids have been shown to contribute to the immuno-modulatory properties of surfactant, as they can regulate the inflammatory response elicited by a variety of stimuli both *in vitro* and *in vivo* [122–125]. Of note, the aforementioned SP-C deficient mice appear to mount a more robust inflammatory response, and to be more severely impacted by bacterial or viral infections than wild type mice, thereby unraveling the anti-microbial and anti-inflammatory properties of SP-C [126, 127].

Overall, both the immuno-modulatory and biophysical properties of surfactant are essential for lung homeostasis, and the importance of a functional surfactant system is particularly relevant in the pathogenesis of ALI.

1.6.4. Surfactant alterations in ALI

Surfactant impairment can be considered one of the hallmarks of ALI. Analyses of lung lavage samples from numerous animal models and patients with ALI have shown changes in phospholipid composition with decreased DPPC, decreased levels of SP-A, SP-B, SP-C, and higher conversion of large aggregates into functionally inactive SA (Fig 1.3) [95, 128–131]. These alterations are likely dependent on a number of factors.

Firstly, injury to the alveolar type II cells during ALI can hinder any of the steps in surfactant metabolism, affecting phospholipid species, surfactant protein levels, and availability of functional large aggregates [128, 132].

Secondly, mechanical ventilation and the broad milieu of inflammatory mediators within the alveolar space contribute to the aforementioned alterations. Specifically, it has been observed that the changes in alveolar surface area associated with MV increase the conversion of LA into SA pools, and this is particularly relevant when ventilation with high tidal volumes is utilized [133–135]. The inflammatory mediators in the injured lung can degrade the different surfactant components via phospholipases and proteases released by immune cells or invading pathogens. For example, enzymes secreted by *Pseudomonas Aeruginosa* have been shown to degrade surfactant lipids as well as SP-A, SP-B and SP-D, and increase LA to SA conversion *in vitro* [136–138].

Lastly, leakage of serum proteins into the lung due to the more permeable alveolo-capillary barrier considerably contributes to surfactant alterations in ALI. Albumin, hemoglobin, and the accumulation of fibrin rich material favored by reduced fibrinolytic activity largely contribute to surface film impairment (Fig. 1.3) [48, 139].

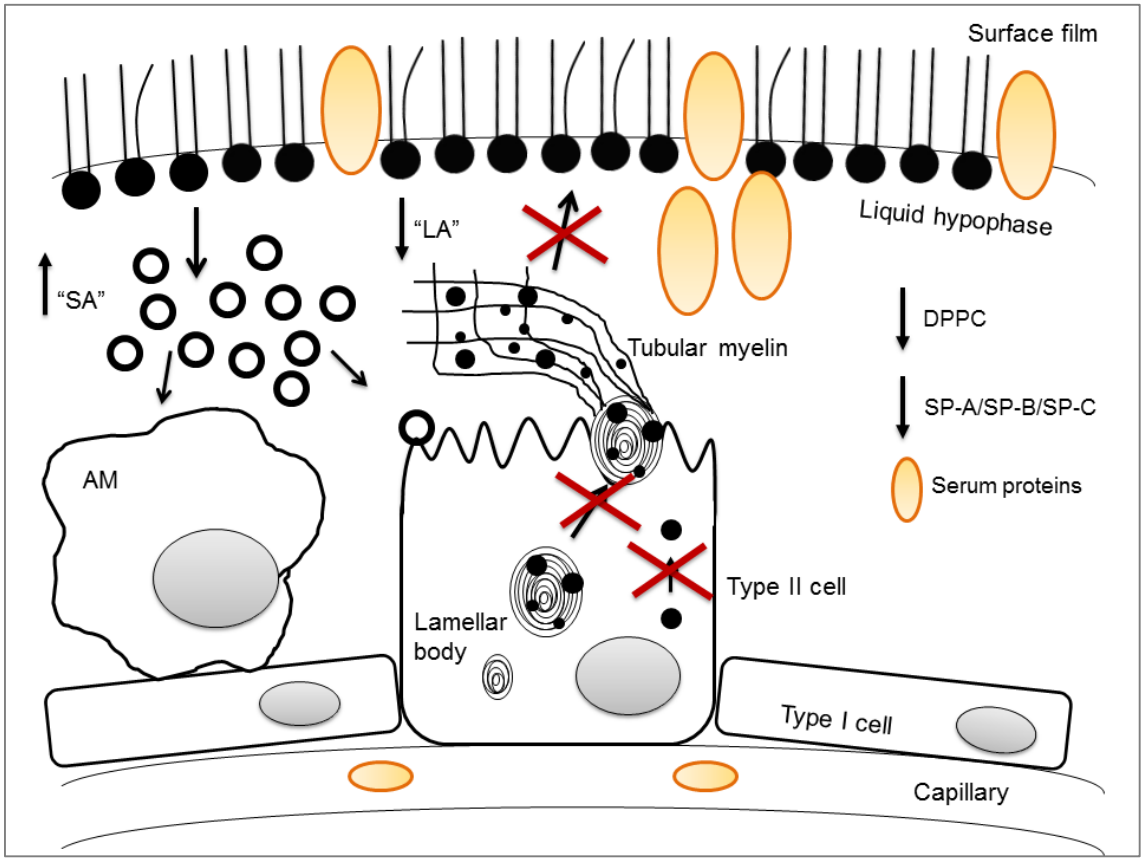

Figure 1.3: Surfactant alterations in ALI. The figure represents the increased conversion of LA into SA, dysfunction of type 2 cells, and inhibition of surfactant due to leakage of serum proteins.

The functional implications of surfactant alterations in ALI result in higher surface tension, lower lung compliance, hypoxemia, and possible loss of surfactant immuno-modulatory and host defense properties. It is important to note that the alterations of surfactant are not just the consequence of the disease process, but they also directly contribute to injury progression. Surfactant changes occur, in fact, relatively early in ALI pathophysiology, as demonstrated by the work of Maruscak and colleagues [10]. In this study, surfactant dysfunction was assessed in rats exposed to one hour or two hours of high Vt MV. Changes such as increase in SA content, decrease in percent LA, and impaired ability of isolated

surfactant to reduce surface tension occurred within the first hour of injurious MV, in the early stages of injury and before any physiological dysfunction (such as hypoxemia or low compliance) was detectable [10]. This experimental evidence is also supported by clinical observations, in which lung lavage collected from ALI patients within 24 hours from intubation showed already significantly lower PC and DPPC levels, lower SP-A/SP-B/SP-C, and higher minimum surface tension compared to healthy, spontaneously breathing controls [131].

Overall, this evidence suggests that alterations and impairment of the endogenous surfactant system contributes to lung dysfunction in ALI resulting in decreased compliance and hypoxemia. These findings constitute the main rationale for exogenous surfactant administration as a potential treatment of this disorder.

1.6.5. Exogenous surfactant treatment in ALI

The concept of exogenous surfactant treatment in ALI stems from the necessity to overcome endogenous surfactant alterations impairing the function of the lung. Throughout the years, different surfactant preparations have been investigated in both experimental and clinical settings. Such preparations differ in terms of source of surfactant (natural versus synthetic, or different animal origin) and content of surfactant associated proteins; however, none of them contains SP-A or SP-D, for reasons related to the purification process of natural surfactants or commercial considerations. Natural surfactant preparations are derived from either porcine (Curosurf, HL-10) or bovine (bLES, Alveofact, Infasurf, Survanta) sources, and contain natural lipids as well as SP-B and SP-C, while synthetic surfactants are protein-free (Exosurf and ALEC) or contain recombinant SP-C protein or a SP-B like peptide (Venticute and KL4, respectively) [40].

Extensive animal studies have been performed to evaluate the efficacy of exogenous surfactant administration in models of ALI, leading to exciting results with improvements in oxygenation and compliance in the treated animals [11, 140–142]. In line with the experimental findings, initial case reports and small clinical trials evidenced a beneficial effect of exogenous surfactant on oxygenation in patients with ALI [40, 143]. The subsequent controlled, multi-center, prospective, randomized trials focused on clinical

outcomes such as ventilator-free days (VFD) and mortality. Even though surfactant treatment proved to be safe and led to acute improvements in oxygenation, these trials showed no change in VFD or mortality in the surfactant treated patients compared to patients exposed to standard care [40, 144–146].

The factors that may have affected the outcomes of exogenous surfactant treatment are multiple, and include: the dose and method of surfactant delivery, the type of surfactant preparation used, and the severity of the underlying injury.

An additional factor that may have influenced the efficacy of surfactant treatment is the timing of administration [40]. It is in fact possible that exogenous surfactant was administered too late into ALI progression, when systemic inflammation and distal organ failure may have already developed (Fig. 1.1); therefore, administration at earlier time points may prove more beneficial.

Lastly, it is important to note that improvements in oxygenation following surfactant treatment did not correlate with a mortality benefit. Remarkably, the only approach (low Vt MV) achieving a decrease in mortality was associated with lower systemic inflammation [12, 37]. It would be, therefore, of interest to investigate the relationship between exogenous surfactant treatment and inflammation during ALI. In this respect, recent data from our lab suggests that elevated *endogenous* surfactant pool sizes can mitigate the inflammation associated with ALI, in mice exposed to injurious MV only or to a combination of lipopolysaccharide instillation and MV [147, 148]. The intriguing question of whether *exogenous* surfactant treatment can mirror those findings and have an impact on the inflammatory response during ALI will be addressed in chapter 2 of this thesis.

1.7. Overview on matrix metalloproteinases (MMPs)

As previously discussed, mitigation of the inflammation in ALI is central to hindering disease progression. As mediators of inflammation and potential therapeutic targets, matrix metalloproteinases are of particular interest, with one of these proteases, namely MMP-3, being the experimental variable under investigation in this thesis (chapters 3 and 4).

The matrix metalloproteinase family consists of 25 zinc-dependent endopeptidases produced by immune cells, epithelial cells, and fibroblasts, and characterized by similar structural features [17, 149–151]. The initial view on the function of MMPs was that of proteases exclusively dedicated to matrix remodeling. A growing body of research, however, has now shown that a large part of the MMP substrates are non-matrix molecules, and that MMPs play important roles in many different physiologic and pathologic processes. The functions of MMPs span, in fact, from organogenesis to wound repair, fibrosis, and, above all, inflammation [152–154]. Importantly, and contrary to the original view, advancements in the biology of MMPs suggest distinct and non-overlapping functions for these proteases *in vivo*. The original misconception mainly originated from *in vitro* observations, where different MMPs were shown to cleave the same substrates. The case against such redundant/overlapping roles for MMPs is supported by differences in MMPs cell expression and their diverse pericellular localization, which affects substrate availability *in vivo*. Additionally, it should be noted that there are emerging non-enzymatic activities for several MMPs that further undermine the concept of functional overlap for these enzymes [155–157].

Due to the potential effects of MMPs on multiple molecules and biological events, their activity undergoes different levels of regulation. For instance, MMPs are released as latent pro-enzymes and, once activated, their activity is limited by alpha 2 macroglobulin or specific endogenous inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs). MMPs are also regulated at the transcriptional level, with their expression being typically low in healthy tissue, and up-regulated in pathological conditions such as infection, injury and, most importantly, inflammation [17, 158].

Elevated levels and activity of various MMPs have been observed in inflammatory conditions such as atherosclerosis, asthma, sepsis, and acute lung injury [159–162]. Far from being simple by-standers, these enzymes actively participate in the inflammatory process, through regulation of the activation and availability of several cytokines and chemokines [152].

Several clinical studies have in fact observed elevated levels of some MMPs, including MMP-1, -2, -3, -8, and -9, in lavage fluids of patients with ALI [163–165]. Importantly, one of this studies showed that MMP-3 levels correlated with disease severity, incidence of multi-organ failure, and mortality [163]. In light of this evidence, it is of interest to specifically investigate MMP-3 contribution to the pathophysiology of ALI.

1.8. Matrix metalloproteinase-3 (MMP-3)

1.8.1. Characteristics of MMP-3

Matrix metalloproteinase-3, also called stromelysin-1, is an extracellular, secreted MMP whose levels are low in healthy tissue, but increase following injury or inflammation. Within the lung, MMP-3 is expressed by epithelial cells, alveolar macrophages, and fibroblasts [151, 166].

MMP-3 structure consists of a pro-domain, a catalytic domain, a hinge region and a hemopexin domain. The pro-domain contains a conserved cysteine residue, whose thiol group interacts with a zinc (Zn^{2+}) ion coordinated by the catalytic domain [167, 168]. The interaction between the thiol group of the conserved cysteine and the Zn^{2+} maintains MMP-3 in a zymogen state, and activation occurs through the proteolytic removal of the pro-domain or the disruption of the Zn^{2+}-cysteine interaction via a chaotropic agent [169]. As mentioned, MMP-3 also has a flexible hinge region, and a C-terminal hemopexin-like domain that is important in substrate recognition [168].

Since its first description in the ‘70s and isolation in the mid ‘80s, the knowledge about MMP-3 in physiology and disease has substantially increased, and new emerging roles for this protease are still being discovered. Like other members of this family, MMP-3 has

multiple and diverse substrates. It can degrade components of the extracellular matrix (ECM), such as cross-linked fibrin, fibrinogen, fibronectin, elastin, laminin, collagens and proteoglycans [162, 170–172]. Consistent with a role in ECM remodeling, MMP-3 participates in wound healing where it regulates wound contraction and speed of wound closure [173, 174]. Beside the ECM, MMP-3 has also been shown to cleave non-matrix substrates. For example, it degrades protease inhibitors, and takes part in the proteolytic activation of other MMPs such as MMP-1, -8 and MMP-9 [162, 175–178].

Cell-to-cell contacts are also affected by MMP-3 through cleavage of E-cadherin, protein situated at the junctions between epithelial cells and important for maintenance of the integrity of the alveolo-capillary barrier [151, 170]. While shedding of E-cadherin could contribute to the development of lung injury, MMP-3 role in the regulation of inflammation is of greater importance in the pathogenesis of ALI.

1.8.2. MMP-3 in the inflammatory process

MMP-3 has a widespread effect on the inflammatory signaling. MMP-3 can proteolytically activate the latent forms of TGF-β and TNF-α, and is responsible for both activation and subsequent degradation of IL-1β [17, 179, 180]. Moreover, MMP-3 cleaves several CC and CXC chemokines generating receptor antagonists (RANTES, MCP-1, MCP-2, MCP-3, and MCP-4), leading to inactivation (SDF-1), or generating chemotactic gradients [152]. It has in fact been shown that MMP-3 is essential to the generation of an unknown macrophage chemo-attractant in a model of herniated disc resorption and may participate, among other MMPs, to the activation of NAP-2, a potent neutrophils chemo-attractant [181, 182]. This considerable involvement in the inflammatory response is possibly the reason for a role of MMP-3 in various inflammatory diseases, such as atherosclerosis, endometriosis, sepsis and, importantly, ALI.

1.8.3. MMP-3 in ALI

The involvement of MMP-3 in ALI is suggested not only by the aforementioned clinical evidence, but also by some experimental data. These studies have utilized Mmp3 wild type ($Mmp3^{+/+}$) and knock out mice ($Mmp3^{-/-}$) to specifically assess the role of such protease in injury development. In the study by Warner et al, $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice were exposed

to a model of acute alveolitis; subsequently, histological analysis and measurements of protein content and cellularity in lavage were performed [183]. The authors observed reduced protein levels and neutrophils number in the lavage of injured $Mmp3^{-/-}$ mice compared to $Mmp3^{+/+}$ controls, thereby suggesting a role for MMP-3 in the development of injury [183]. These findings are supported by a later study in which lung injury was induced through the intra-tracheal instillation of the chemokine CXCL2/MIP-2 [184]. In this model as well, $Mmp3^{-/-}$ mice appeared to develop milder injury, indicating a role for MMP-3 in lung permeability and neutrophil recruitment following an inflammatory injury [184].

Even though these experimental findings are of great interest, they are characterized by some important limitations. First, these models of lung injury lack clinical relevance, since the primary insults utilized to initiate ALI do not closely resemble common ALI etiologies [2]. Second, both models are essentially based on neutrophilic infiltration, while models reflecting more of the hallmarks of ALI (see section 1.9) would be desirable. Lastly, in-depth analyses of pulmonary inflammation and ALI outcomes such as surfactant alterations and changes in lung mechanics have not yet been performed and are necessary to the understanding of MMP-3 role in such complex disease.

1.8.4. Study tool: Mmp3 knock-out mouse

As pointed out previously, the use of genetically modified mice lacking the expression of Mmp3 is a powerful tool to investigate, specifically, MMP-3 contribution to injury and inflammation.

The first MMP-knock out to be developed, $Mmp3^{-/-}$ mice are viable, fertile, and have normal lung structure and function under unchallenged conditions (chapter 4 of this thesis and [151]). Following injury, however, $Mmp3^{-/-}$ mice present different phenotype from wild type mice, and examples of the inflammatory phenotype of $Mmp3^{-/-}$ mice in several injury models are listed in Table 1.2.

Overall, the $Mmp3^{-/-}$ mouse appears to be an adequate and reliable tool to expand the understanding of MMP-3 role in the pathogenesis of ALI and, for such reasons, has been the model of choice in our studies (chapters 3 and 4).

Mouse	Inflammatory phenotypes	Ref.
Mmp3$^{-/-}$	Reduced neutrophils count in immune complex induced lung injury	[183]
	Decreased pulmonary fibrosis following bleomycin instillation	[151]
	Increased susceptibility to collagen induced arthritis	[185]
	Inhibited macrophage infiltration in an *in vitro* model of herniated disc resorption	[181]
	Impaired contact hypersensitivity and T cell response to intestinal bacterial infection	[186]

Table 1.2: Examples of inflammatory phenotypes described in Mmp3$^{-/-}$ mice.

1.9. Animal models of ALI

To effectively interfere with lung injury development and progression, the use of animal models reproducing the biological and physiological characteristics of ALI is essential. Animal models allow the use of specific research tools, for example the utilization of genetically modified animals, to elucidate specific mechanistic pathways involved in the disease, which would be otherwise impossible to directly study in human patients. A sole animal model, however, cannot resemble all of the disease features due to the variety of initiating insults and the complex pathophysiology of ALI. The optimal solution for the study of this disease resides, then, in the use of different experimental models, exposed to different initiating lung insults, and resembling the multiple hit paradigm of ALI development (Fig. 1.1). Most importantly, any animal model utilized should mirror the hallmarks of ALI (described below) to adequately reflect the disease pathophysiology.

1.9.1. Hallmarks of ALI in animal models

The hallmarks of ALI, ideally addressed by experimental models to mimic human lung injury, stem from the definition and clinical evidence for such disorder. These hallmarks include: i) *acute onset*, ii) *physiological dysfunction*, iii) *pulmonary (and systemic)*

inflammation, iv) *increased pulmonary permeability*, and v) *histological evidence of lung injury* [187, 188].

Specifically, in valid models of ALI lung injury should develop shortly after the exposure to the insults (*acute onset*), and lead to the manifestation of *physiological dysfunction*. The latter implies the presence of pulmonary surfactant alterations, impaired gas exchange, and changes in lung mechanics [2]. Analyses of pulmonary surfactant can be readily performed on isolated samples of lung lavage undergoing differential centrifugation. Following isolation of total surfactant, large, and small aggregates, the abundance of each sub-fraction can be determined through well-established biochemical assays [189, 190]. Moreover, surfactant biophysical properties can be evaluated *in vitro*, by monitoring the minimum surface tension achieved by LA samples exposed to repeated cycles of compression and expansion via captive bubble surfactometer [191] (chapter 2) or constrained sessile drop surfactometer [192] (chapter 4). With the exception of surfactant analyses, assessment of physiological dysfunction can pose a technical challenge, especially in mice, and may not be always feasible. Measurements of oxygenation often require the use of invasive arterial catheters, and are limited by a small total blood volume. Physiological parameters are also hard to measure in spontaneously breathing animals, but can be assessed in mechanically ventilated mice. MV allows *in vivo* and *ex vivo* monitoring of changes to peak pressure and/or lung compliance [193]; furthermore, as previously mentioned, MV augments the *pulmonary inflammation* associated with ALI, and contributes to the *systemic inflammatory response* [13, 58].

Pulmonary inflammation, a definite hallmark of ALI, comprises increased lavage inflammatory mediators and neutrophil numbers, and can be effectively measured in lung lavage samples through commercially available assays and analysis of total and differential cell counts. Lung lavage samples can also be utilized to evaluate increases in total protein content, thereby illustrating, with some limitations, increases in *pulmonary permeability*, another characterizing aspect of lung injury [188].

Lastly, a good animal model of ALI should provide *histological evidence of lung injury*, since histological analyses can give both qualitative and quantitative information on the

extent and uniformity of the injury, cellularity, sites of edema accumulation, and gross structural abnormalities [188].

In conclusion, it should be noted that most animal models of ALI present at least a few of the hallmarks described above. While a description of all the models available for the study of this disease goes beyond the scope of this chapter, the main characteristics of the lung injury models relevant to this thesis are described in the following paragraphs.

1.9.2. Hydrochloric acid-induced lung injury

Gastric acid aspiration refers to the unintentional inhalation of the gastric content, and is one of the direct pulmonary insults predisposing the lung to ALI (Fig. 1.1). It accounts for about 30% of all the deaths due to anesthesia, and can also occur in subjects experiencing drug overdose or seizures [194]. Even though gastric aspiration exposes the lung to both particulate matter and acidic juices from the stomach, this injury has been widely modelled through the intra-tracheal instillation of hydrochloric acid (HCl) solutions with low pH [58, 195, 196].

Animal models of acid-induced ALI are characterized by acute pulmonary changes, detectable as early as four hours post HCl instillation (chapters 2 and 3) [58]. Lung injury results from the chemical burn of the pulmonary parenchyma, subsequently leading to elevated lavage concentrations of inflammatory cytokines and chemokines involved in neutrophils recruitment. Acid-induced ALI, in fact, appears to be neutrophil dependent, as demonstrated by marked pulmonary infiltrates and, conversely, by the development of milder injury when neutrophils accumulation in the lung is inhibited or decreased [67, 197, 198]. While it is not entirely clear how acid injury activates the immune system, some recent study suggests that the Toll-like receptor 4 (TLR4) pathway may be involved [199]. Being a direct lung insult, the alveolar epithelium is especially affected, with loss of epithelial cells, alterations of the alveolo-capillary barrier and consequent surfactant impairment [187]. Additional signs of physiological dysfunction, such as increased peak pressure and decreased compliance, have also been observed in this model of ALI [58, 196].

1.9.3. Lipopolysaccharide-induced lung injury

Bacterial pneumonia is one of the most common causes of ALI [2]. This injury has been extensively reproduced in experimental models through the intra-nasal or intra-tracheal administration of bacteria or lipopolysaccharide (LPS), an immunogenic molecule found in the outer membrane of Gram negative bacteria. While administration of live bacteria constitutes a valid alternative in modeling pneumonia, LPS has the advantage of being easy to prepare and administer, and ensures a good degree of reproducibility [187].

LPS instilled intra-tracheally directly affects both the alveolar macrophages and epithelium by binding to its receptor, TLR4, on these cells and activating intracellular pathways leading to synthesis and release of multiple cytokines and chemokines [200]. LPS-induced lung injury then develops presenting most of the hallmarks of ALI described above, as highlighted by the analyses of pulmonary permeability and inflammation, physiological dysfunction and histological damage described throughout chapters 3 and 4 of this thesis. For such reasons, LPS-induced ALI is a very useful and informative model in the study of this disorder.

1.9.4. Ventilation associated lung injury and *ex vivo* ventilation

As previously mentioned, mechanical ventilation is the main supportive treatment in ALI necessary to improve oxygenation, and a crucial component of ALI pathophysiology as a potential secondary insult. Experimentally, the role of MV in ALI has been investigated in isolation, overstretching the lung by applying high tidal volume MV to healthy lungs. This injury model is referred to as ventilator-induced lung injury (VILI) and allows the study of specific alterations in pulmonary function, inflammation and cell signaling resulting from excessive mechanical stretch and strain implemented on otherwise normal lungs [201, 202].

While VILI is an experimental tool of great analytical value, in a clinical setting ventilation is utilized to support lung function, which is already impaired due to a pre-existing injury. The study of ventilation in clinically relevant, *in vivo* or *ex vivo* models is therefore performed by implementing MV on lungs that have been pre-exposed to a clinically significant insult, such as aspiration or pneumonia. Such models of ventilation-associated

lung injury (VALI) directly examine the role of MV as a secondary insult in propagating lung injury and in contributing to the development of systemic inflammation and associated distal organ failure [14, 52, 58]. Overall, models of VILI and VALI have been essential in demonstrating that MV can lead to damage via over-distention and repeated opening/closing of alveolar units, and have convincingly pointed out that the extent of the injury is proportional to the degree of mechanical stress applied to the lung.

The mechanisms through which ventilation can cause and exacerbate injury are barotrauma (gross lung injury due to excessive airway pressure), volutrauma (excessive volume applied to the lungs) and, particularly relevant for this thesis, biotrauma [55, 203]. The concept of biotrauma refers to the release of biologically active mediators from lungs exposed to injurious modalities of MV, mediators that can potentially translocate into the circulation and damage distal organs [56]. Evidence for the biotrauma hypothesis stems from a vast body of *in vitro, in vivo* and *ex vivo* research. Numerous studies have in fact shown release of inflammatory molecules and changes in gene expression in alveolar epithelial cells and macrophages exposed to cycles of mechanical stretch [56, 204, 205]. These *in vitro* findings have been mirrored by *in vivo* experimental observations, in which high pressure or high volume ventilation caused elevated pulmonary and systemic levels of cytokines and chemokines, often in combination with changes in peripheral organs [14, 58, 79]. Notably, Imai and colleagues have demonstrated that, in a model of acid-induced ALI followed by injurious MV, lung derived mediators in the systemic circulation led to increased apoptosis in the kidneys and small intestine of the experimental animals, thereby strongly supporting the biotrauma concept [61].

A particularly useful tool to study biotrauma and the effects of MV on the lung in isolation is a system for *ex vivo* ventilation, namely the isolated and perfused mouse lung (IPML) set up [59]. The IPML set up allows for *ex vivo* MV of mouse lungs and simultaneous re-circulation of a cell free solution through the pulmonary blood vessels. One of the main advantages of this system resides in the possibility to collect and analyze inflammatory mediators specifically released from the lung into the circulation throughout MV [59, 86]. Importantly, the IPML system allows the monitoring of potential changes in the levels of

these circulating, lung-derived mediators resulting from lung specific treatments, as illustrated in chapter 2 of this thesis.

1.9.4.1. Measurements of lung mechanics in small rodents

An undoubtable advantage associated with the use of MV in small rodents is the possibility to evaluate lung mechanics. Measurement of physiological parameters in spontaneously breathing animals is, in fact, quite difficult and usually not very precise [206]. For example, whole-body plethysmography can be used in unrestrained, conscious animals; this technique, however, provides only general information about the pattern of ventilation, and it can generate errors and artifacts. On the other hand, MV is a more invasive procedure, requiring restrain, surgery and anesthesia management for *in vivo* models, but providing the valuable advantage of reliable, reproducible, and accurate measurements [207]. While *in vivo* MV was not utilized in this thesis work, models of *ex vivo* ventilation and *ex vivo* measurements of lung function were applied to terminally sacrificed mice [59, 208, 209]. These strategies allowed the monitoring of peak inspiratory pressure changes throughout MV (chapter 2) and the specific assessment of several parameters, such as: compliance, resistance, and elastance for the whole respiratory system; quasi-static compliance and elastance; central airway resistance (R_N); tissue resistance or tissue damping (G); and tissue elastance (H) (chapter 4); thereby providing complete and novel information on physiological dysfunction in our models of ALI.

1.10. Summary and overall objective

The lung is the organ responsible for gas exchange, a vital function that is severely impaired during acute lung injury. This life threatening condition can be initiated by multiple and diverse insults to the lung, resulting in profound alterations of the alveolar microenvironment. Among such alterations, the impairment of the surfactant system and the development of an extensive pulmonary inflammatory response are key events in the progression of the disease. Despite extensive research, the highly complex pathophysiology of ALI has hindered the development of an effective therapeutic treatment capable of reducing the high mortality associated with this disorder. Further investigation

is, therefore, necessary to improve applicable interventions and identify key mediators of lung injury that could serve as potential therapeutic targets.

The **overarching hypothesis** of this thesis work is that interventions targeting the inflammatory response associated with ALI can improve outcomes. The **overall objective** is then to expand our understanding of the ALI pathophysiology, in order to better interfere with disease progression and provide potential future alternatives in the treatment of ALI.

1.11. References

1. West JB: *Respiratory Physiology: The Essentials*. 5th edition. Lippincott Williams & Wilkins; .

2. Ware LB, Matthay MA: **The Acute Respiratory Distress Syndrome**. *N Engl J Med* 2000, **342**:1334–1349.

3. Wheeler AP, Bernard GR: **Acute lung injury and the acute respiratory distress syndrome: a clinical review.** *Lancet* 2007, **369**:1553–1564.

4. Walkey AJ, Summer R, Ho V, Alkana P: **Acute respiratory distress syndrome: epidemiology and management approaches.** *Clin Epidemiol* 2012, **4**:159–169.

5. Parsons PE, Eisner MD, Thompson BT, Matthay MA, Ancukiewicz M, Bernard GR, Wheeler AP, and the NHLBI Acute Respiratory Distress Syndrome Clinical Trials Network: **Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury**. *Crit Care Med* 2005, **33**:1–6.

6. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A: **Inflammatory Cytokines in the BAL of Patients with ARDS. Persistent Elevation Over Time Predicts Poor Outcome**. *Chest* 1995, **108**:1303–1314.

7. Veldhuizen RAW, Welk B, Harbottle R, Hearn S, Nag K, Petersen N, Possmayer F: **Mechanical ventilation of isolated rat lungs changes the structure and biophysical properties of surfactant**. *J Appl Physiol* 2002, **92**:1169–1175.

8. Goerke J: **Pulmonary surfactant: functions and molecular composition**. *Biochim Biophys Acta* 1998, **1408**:79–89.

9. Wright JR: **Immunomodulatory functions of surfactant.** *Physiol Rev* 1997, **77**:931–962.

10. Maruscak AA, Vockeroth DW, Girardi B, Sheikh T, Possmayer F, Lewis JF, Veldhuizen RAW: **Alterations to surfactant precede physiological deterioration during high tidal volume ventilation.** *Am J Physiol Lung Cell Mol Physiol* 2008, **294**:L974–L983.

11. Rasaiah VP, Malloy JL, Lewis JF, Veldhuizen RAW: **Early surfactant administration protects against lung dysfunction in a mouse model of ARDS**. *Am J Physiol Cell Mol Physiol* 2003, **284**:L783–L790.

12. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, Slutsky AS: **Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial**. *JAMA* 1999, **282**:54–61.

13. Chiumello D, Pristine G, Slutsky AS: **Mechanical ventilation affects local and systemic cytokines in an animal model of acute respiratory distress syndrome**. *Am J Respir Crit Care Med* 1999, **160**:109–116.

14. Dhanireddy S, Altemeier WA, Matute-Bello G, O'Mahony DS, Glenny RW, Martin TR, Liles WC: **Mechanical ventilation induces inflammation, lung injury, and extra-pulmonary organ dysfunction in experimental pneumonia**. *Lab Invest* 2006, **86**:790–799.

15. Montgomery AB, Stager MA, Carrico CJ, Hudson LD: **Causes of mortality in patients with the adult respiratory distress syndrome**. *Am Rev Respir Dis* 1985, **132**:485–489.

16. Stapleton RD, Wang BM, Hudson LD, Rubenfeld GD, Caldwell ES, Steinberg KP: **Causes and timing of death in patients with ARDS**. *Chest* 2005, **128**:525–532.

17. Parks WC, Wilson CL, López-Boado YS: **Matrix metalloproteinases as modulators of inflammation and innate immunity**. *Nat Rev Immunol* 2004, **4**:617–629.

18. Mason RJ: **Biology of alveolar type II cells.** *Respirology* 2006, **11 Suppl**:S12–S15.

19. Du Bois RM: **The alveolar macrophage.** *Thorax* 1985, **40**:321–7.

20. Wright JR: **Clearance and recycling of pulmonary surfactant**. *Am J Physiol* 1990, **259**:L1–L12.

21. West JB: *Pulmonary Pathophysiology: The Essentials*. 5th edition. Lippincott Williams & Wilkins.

22. Veldhuizen EJ, Haagsman HP: **Role of pulmonary surfactant components in surface film formation and dynamics.** *Biochim Biophys Acta* 2000, **1467**:255–270.

23. Avery ME, Mead J: **Surface properties in relation to atelectasis and hyaline membrane disease.** *AMA J Dis Child* 1959, **97**:517–523.

24. Ashbaugh DG, Bigelow DB, Petty TL, Levine BE: **Acute respiratory distress in adults**. *Lancet* 1967, **2**:319–323.

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

26. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS: **Acute respiratory distress syndrome: the Berlin Definition.** *JAMA* 2012, **307**:2526–2533.

27. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD: **Incidence and outcomes of acute lung injury**. *N Engl J Med* 2005, **353**:1685–1693.

28. Phua J, Badia JR, Adhikari NKJ, Friedrich JO, Fowler R a, Singh JM, Scales DC, Stather DR, Li A, Jones A, Gattas DJ, Hallett D, Tomlinson G, Stewart TE, Ferguson ND: **Has mortality from acute respiratory distress syndrome decreased over time?: A systematic review.** *Am J Respir Crit Care Med* 2009, **179**:220–227.

29. Matthay MA, Ware LB, Zimmerman GA: **The acute respiratory distress syndrome**. *J Clin Invest* 2012, **122**:2731–2740.

30. Bellingan G.J.: **The pulmonary physician in critical care * 6 : The pathogenesis of ALI/ARDS.** *Thorax* 2002, **57**:540–546.

31. Johnson ER, Matthay MA: **Acute lung injury: Epidemiology, Pathogenesis, and Treatment**. *J Aerosol Med Pulm Drug Deliv* 2010, **23**:243–252.

32. Del Sorbo L, Slutsky AS: **Acute respiratory distress syndrome and multiple organ failure.** *Curr Opin Crit Care* 2011, **17**:1–6.

33. Pelosi P, D'Onofrio D, Chiumello D, Paolo S, Chiara G, Capelozzi V, Barbas C, Chiaranda M, Gattinoni L: **Pulmonary and extrapulmonary acute respiratory distress syndrome are different**. *Eur Respir J* 2003, **22**:48s–56s.

34. Rocco PRM, Pelosi P: **Pulmonary and extrapulmonary acute respiratory distress syndrome: myth or reality?**. *Curr Opin Crit Care* 2008, **14**:50–55.

35. Castro CY: **ARDS and diffuse alveolar damage: a pathologist's perspective.** *Semin Thorac Cardiovasc Surg* 2006, **18**:13–19.

36. Petty TL, Silvers GW, Paul GW, Stanford RE: **Abnormalities in lung elastic properties and surfactant function in adult respiratory distress syndrome.** *Chest* 1979, **75**:571–574.

37. The Acute Respiratory Distress Syndrome Network: **Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network**. *N Engl J Med* 2000, **342**:1301–1308.

38. Brower RG: **Treatment of ARDS**. *Chest* 2001, **120**:1347–1367.

39. Levitt JE, Matthay MA: **Treatment of acute lung injury: historical perspective and potential future therapies.** *Semin Respir Crit Care Med* 2006, **27**:426–437.

40. Lewis JF, Veldhuizen R: **The role of exogenous surfactant in the treatment of acute lung injury**. *Annu Rev Physiol* 2003, **65**:613–642.

41. Davidson WJ, Dorscheid D, Spragg R, Schulzer M, Mak E, Ayas NT: **Exogenous pulmonary surfactant for the treatment of adult patients with acute respiratory distress syndrome: results of a meta-analysis**. *Crit Care* 2006, **10**:R41.

42. Goodman RB, Pugin J, Lee JS, Matthay MA: **Cytokine-mediated inflammation in acute lung injury**. *Cytokine Growth Factor Rev* 2003, **14**:523–535.

43. Bhatia M, Moochhala S: **Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome.** *J Pathol* 2004, **202**:145–56.

44. Bosma KJ, Taneja R, Lewis JF: **Pharmacotherapy for prevention and treatment of acute respiratory distress syndrome: current and experimental approaches.** *Drugs* 2010, **70**:1255–1282.

45. Yamashita CM, Lewis JF: **Emerging therapies for treatment of acute lung injury and acute respiratory distress syndrome**. *Expert Opin Emerg Drugs* 2012, **17**:1–4.

46. Pelosi P, Caironi P, Gattinoni L: **Pulmonary and extrapulmonary forms of acute respiratory distress syndrome.** *Semin Respir Crit Care Med* 2001, **22**:259–268.

47. Bhattacharya J, Matthay MA: **Regulation and repair of the alveolar-capillary barrier in acute lung injury.** *Annu Rev Physiol* 2013, **75**:593–615.

48. Seeger W, Grube C, Gunther A, Schmidt R: **Surfactant inhibition by plasma proteins: differential sensitivity of various surfactant preparations**. *Eur Respir J* 1993, **6**:971–977.

49. Da Silva K, McCaig L, Veldhuizen RAW, Possmayer F: **Protein inhibition of surfactant during mechanical ventilation of isolated rat lungs**. *Exp Lung Res* 2005, **31**:745–758.

50. Pittet JF, Mackersie RC, Martin TR, Matthay MA: **Biological markers of acute lung injury: prognostic and pathogenetic significance**. *Am J Respir Crit Care Med* 1997, **155**:1187–1205.

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

52. O'Mahony DS, Liles WC, Altemeier WA, Dhanireddy S, Frevert CW, Liggitt D, Martin TR, Matute-Bello G: **Mechanical ventilation interacts with endotoxemia to induce extrapulmonary organ dysfunction.** *Crit Care* 2006, **10**:R136.

53. Altemeier WA, Matute-Bello G, Gharib SA, Glenny RW, Martin TR, Liles WC: **Modulation of lipopolysaccharide-induced gene transcription and promotion of lung injury by mechanical ventilation.** *J Immunol* 2005, **175**:3369–3376.

54. Nakamura T, Malloy J, McCaig L, Yao L, Joseph M, Lewis J, Veldhuizen R: **Mechanical ventilation of isolated septic rat lungs : effects on surfactant and inflammatory cytokines**. *J Appl Physiol* 2001, **91**:811–820.

55. Tremblay LN, Slutsky AS: **Ventilator-induced injury: from barotrauma to biotrauma**. *Proc Assoc Am Physicians* 1998, **110**:482–488.

56. Dos Santos CC, Zhang H, Liu M, Slutsky AS: **Bench-to-bedside review: Biotrauma and modulation of the innate immune response.** *Crit Care* 2005, **9**:280–286.

57. Wilson MR, Choudhury S, Goddard ME, O'Dea KP, Nicholson AG, Takata M: **High tidal volume upregulates intrapulmonary cytokines in an in vivo mouse model of ventilator-induced lung injury**. *J Appl Physiol* 2003, **95**:1385–1393.

58. Walker MG, Yao LJ, Patterson EK, Joseph MG, Cepinskas G, Veldhuizen RA, Lewis JF, Yamashita CM: **The effect of tidal volume on systemic inflammation in Acid-induced lung injury**. *Respiration* 2011, **81**:333–342.

59. Von Bethmann AN, Brasch F, Nusing R, Vogt K, Volk HD, Muller KM, Wendel A, Uhlig S: **Hyperventilation induces release of cytokines from perfused mouse lung**. *Am J Respir Crit Care Med* 1998, **157**:263–272.

60. Haitsma JJ, Uhlig S, Goggel R, Verbrugge SJ, Lachmann U, Lachmann B: **Ventilator-induced lung injury leads to loss of alveolar and systemic compartmentalization of tumor necrosis factor-alpha**. *Intensive Care Med* 2000, **26**:1515–1522.

61. Imai Y, Parodo J, Kajikawa O, De Perrot M, Fischer S, Edwards V, Cutz E, Liu M, Keshavjee S, Martin TR, Marshall JC, Ranieri VM, Slutsky AS: **Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of acute respiratory distress syndrome**. *JAMA* 2003, **289**:2104–2112.

62. Plötz FB, Slutsky AS, van Vught AJ, Heijnen CJ: **Ventilator-induced lung injury and multiple system organ failure: a critical review of facts and hypotheses.** *Intensive Care Med* 2004, **30**:1865–1872.

63. Slutsky AS, Tremblay LN: **Multiple system organ failure. Is mechanical ventilation a contributing factor?**. *Am J Respir Crit Care Med* 1998, **157**(6 Pt 1):1721–1725.

64. Markovic N, McCaig LA, Stephen J, Mizuguchi S, Veldhuizen RA, Lewis JF, Cepinskas G: **Mediators released from LPS-challenged lungs induce inflammatory responses in liver vascular endothelial cells and neutrophilic leukocytes**. *Am J Physiol liver Physiol* 2009, **297**:G1066–1076.

65. Medzhitov R: **Origin and physiological roles of inflammation**. *Nature* 2008, **454**:428–435.

66. Grommes J, Soehnlein O: **Contribution of neutrophils to acute lung injury.** *Mol Med* 2011, **17**:293–307.

67. Zarbock A, Singbartl K, Ley K: **Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation**. *J Clin Invest* 2006, **116**:3211–3219.

68. Riva CM, Morganroth ML, Ljungman AG, Schoeneich SO, Marks RM, Todd RF 3rd, Ward PA, Boxer LA: **Iloprost inhibits neutrophil-induced lung injury and neutrophil adherence to endothelial monolayers**. *Am J Respir Cell Mol Biol* 1990, **3**:301–309.

69. Lee CT, Fein AM, Lippmann M, Holtzman H, Kimbel P, Weinbaum G: **Elastolytic activity in pulmonary lavage fluid from patients with adult respiratory-distress syndrome**. *N Engl J Med* 1981, **304**:192–196.

70. Braude S, Apperley J, Krausz T, Goldman JM, Royston D: **Adult respiratory distress syndrome after allogeneic bone-marrow transplantation: evidence for a neutrophil-independent mechanism**. *Lancet* 1985, **1**:1239–1242.

71. Aggarwal NR, King LS, D'Alessio FR: **Diverse macrophage populations mediate acute lung inflammation and resolution.** *Am J Physiol Lung Cell Mol Physiol* 2014, **306**:L709–L725.

72. Kuebler WM: **Inflammatory pathways and microvascular responses in the lung**. *Pharmacol reports* 2005, **57 Suppl**:196–205.

73. Dos Santos CC, Han B, Andrade CF, Bai X, Uhlig S, Hubmayr R, Tsang M, Lodyga M, Keshavjee S, Slutsky AS, Liu M: **DNA microarray analysis of gene expression in alveolar epithelial cells in response to TNFalpha, LPS, and cyclic stretch.** *Physiol Genomics* 2004, **19**:331–342.

74. Vlahakis NE, Schroeder MA, Limper AH, Hubmayr RD: **Stretch induces cytokine release by alveolar epithelial cells in vitro**. *Am J Physiol* 1999, **277**(1 Pt 1):L167–L173.

75. Manicone AM: **Role of the pulmonary epithelium and inflammatory signals in acute lung injury**. *Expert Rev Clin Immunol* 2009, **5**:63–75.

76. Wright JR: **Immunoregulatory functions of surfactant proteins**. *Nat Rev* 2005, **5**:58–68.

77. Puneet P, Moochhala S, Bhatia M: **Chemokines in acute respiratory distress syndrome**. *Am J Physiol Lung Cell Mol Physiol* 2005, **288**:L3–L15.

78. Braunwald E, Fauci AS, Kasper DL, Hauser SL, Longo DL, Jameson JL: *Harrison's Principles of Internal Medicine*. 15th edition. McGraw Hill.

79. Gurkan OU, O'Donnell C, Brower R, Ruckdeschel E, Becker PM: **Differential effects of mechanical ventilatory strategy on lung injury and systemic organ inflammation in mice**. *Am J Physiol Cell Mol Physiol* 2003, **285**:L710–L718.

80. Meduri G, Headley S, Kohler G, Stentz F, Tolley E, Umberger R, Leeper K: **Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time.** *CHEST J* 1995, **107**:1062–1073.

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

82. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC, Grant IS, Pollok AJ, Haslett C: **Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups.** *Lancet* 1993, **341**:643–647.

83. McCarthy MK, Weinberg JB: **Eicosanoids and respiratory viral infection: coordinators of inflammation and potential therapeutic targets.** *Mediators Inflamm* 2012, **2012**:1–13.

84. Stephenson AH, Lonigro AJ, Hyers TM, Webster RO, Fowler AA: **Increased concentrations of leukotrienes in bronchoalveolar lavage fluid of patients with ARDS or at risk for ARDS**. *Am Rev Respir Dis* 1988, **138**:714–719.

85. Auner B, Geiger E V, Henrich D, Lehnert M, Marzi I, Relja B: **Circulating leukotriene B4 identifies respiratory complications after trauma**. *Mediators Inflamm* 2012, **2012**:1–8.

86. Jaecklin T, Engelberts D, Otulakowski G, O'Brodovich H, Post M, Kavanagh BP: **Lung-derived soluble mediators are pathogenic in ventilator-induced lung injury**. *Am J Physiol Cell Mol Physiol* 2011, **300**:L648–L658.

87. Traeger T, Kessler W, Hilpert A, Mikulcak M, Entleutner M, Koerner P, Westerholt A, Cziupka K, van Rooijen N, Heidecke C-D, Maier S: **Selective depletion of alveolar macrophages in polymicrobial sepsis increases lung injury, bacterial load and mortality but does not affect cytokine release.** *Respiration* 2009, **77**:203–213.

88. Eyal FG, Hamm CR, Parker JC: **Reduction in alveolar macrophages attenuates acute ventilator induced lung injury in rats.** *Intensive Care Med* 2007, **33**:1212–1218.

89. Kotani M, Kotani T, Ishizaka A, Fujishima S, Koh H, Tasaka S, Sawafuji M, Ikeda E, Moriyama K, Kotake Y, Morisaki H, Aikawa N, Ohashi A, Matsushima K, Huang Y-CT, Takeda J: **Neutrophil depletion attenuates interleukin-8 production in mild-overstretch ventilated normal rabbit lung.** *Crit Care Med* 2004, **32**:514–519.

90. Gavett SH, Carakostas MC, Belcher LA, Warheit DB: **Effect of circulating neutrophil depletion on lung injury induced by inhaled silica particles.** *J Leukoc Biol* 1992, **51**:455–461.

91. Nakamura T, Moyer BZ, Veldhuizen RA, Lewis JF: **Interleukin-6 has no effect on surfactant or lung function in different lung insults.** *Exp Lung Res* 2006, **32**:27–42.

92. Abraham E, Anzueto A, Gutierrez G, Tessler S, San Pedro G, Wunderink R, Dal Nogare A, Nasraway S, Berman S, Cooney R, Levy H, Baughman R, Rumbak M, Light RB, Poole L, Allred R, Constant J, Pennington J, Porter S: **Double-blind randomised controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock. NORASEPT II Study Group.** *Lancet* 1998, **351**:929–933.

93. Opal SM, Fisher CJ, 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: **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* 1997, **25**:1115–1124.

94. Ruan S-Y, Lin H-H, Huang C-T, Kuo P-H, Wu H-D, Yu C-J: **Exploring the heterogeneity of effects of corticosteroids on acute respiratory distress syndrome: a systematic review and meta-analysis.** *Crit Care* 2014, **18**:R63.

95. Veldhuizen RA, McCaig LA, Akino T, Lewis JF: **Pulmonary surfactant subfractions in patients with the acute respiratory distress syndrome**. *Am J Respir Crit Care Med* 1995, **152**(6 Pt 1):1867–1871.

96. Possmayer F: **A proposed nomenclature for pulmonary surfactant-associated proteins**. *Am Rev Respir Dis* 1988, **138**:990–998.

97. Kuroki Y, Voelker DR: **Pulmonary surfactant proteins**. *J Biol Chem* 1994, **269**:25943–25946.

98. Sano H, Kuroki Y: **The lung collectins, SP-A and SP-D, modulate pulmonary innate immunity.** *Mol Immunol* 2005, **42**:279–287.

99. Weaver TE, Conkright JJ: **Function of surfactant proteins B and C**. *Annu Rev Physiol* 2001, **63**:555–578.

100. Wright JR, Hawgood S: **Pulmonary surfactant metabolism**. *Clin Chest Med* 1989, **10**:83–93.

101. Wright JR, Dobbs LG: **Regulation of pulmonary surfactant secretion and clearance**. *Annu Rev Physiol* 1991, **53**:395–414.

102. Perez-Gil J, Weaver TE: **Pulmonary surfactant pathophysiology: current models and open questions.** *Physiology (Bethesda)* 2010, **25**:132–141.

103. Hawgood S, Poulain FR: **The pulmonary collectins and surfactant metabolism**. *Annu Rev Physiol* 2001, **63**:495–519.

104. Froh D, Gonzales LW, Ballard PL: **Secretion of surfactant protein A and phosphatidylcholine from type II cells of human fetal lung**. *Am J Respir Cell Mol Biol* 1993, **8**:556–561.

105. Fisher AB, Dodia C, Ruckert P, Tao JQ, Bates SR: **Pathway to lamellar bodies for surfactant protein A**. *Am J Physiol Cell Mol Physiol* 2010, **299**:L51–L58.

106. Gross NJ, Narine KR: **Surfactant subtypes in mice: characterization and quantitation**. *J Appl Physiol* 1989, **66**:342–349.

107. Gross NJ, Narine KR: **Surfactant subtypes of mice: metabolic relationships and conversion in vitro**. *J Appl Physiol* 1989, **67**:414–421.

108. Brackenbury AM, Malloy JL, McCaig LA, Yao LJ, Veldhuizen RA, Lewis JF: **Evaluation of alveolar surfactant aggregates in vitro and in vivo**. *Eur Respir J* 2002, **19**:41–46.

109. Putz G, Goerke J, Clements JA: **Surface activity of rabbit pulmonary surfactant subfractions at different concentrations in a captive bubble**. *J Appl Physiol* 1994, **77**:597–605.

110. Possmayer F, Yu SH, Weber JM, Harding PG: **Pulmonary surfactant.** *Can J Biochem Cell Biol* 1984, **62**:1121–1133.

111. Veldhuizen R, Nag K, Orgeig S, Possmayer F: **The role of lipids in pulmonary surfactant**. *Biochim Biophys Acta* 1998, **1408**:90–108.

112. Veldhuizen EJ, Batenburg JJ, van Golde LM, Haagsman HP: **The role of surfactant proteins in DPPC enrichment of surface films**. *Biophys J* 2000, **79**:3164–3171.

113. Clark JC, Wert SE, Bachurski CJ, Stahlmant MT, Stripp BR, Weaver TE, Whitsett JA: **Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis , causing respiratory failure in newborn mice**. *Proc Natl Acad Sci U S A* 1995, **92**(August):7794–7798.

114. Glasser SW, Burhans MS, Korfhagen TR, Na CL, Sly PD, Ross GF, Ikegami M, Whitsett JA: **Altered stability of pulmonary surfactant in SP-C-deficient mice**. *Proc Natl Acad Sci U S A* 2001, **98**:6366–6371.

115. McCormack FX: **Structure, processing and properties of surfactant protein A**. *Biochim Biophys Acta* 1998, **1408**:109–131.

116. Veldhuizen RAW, Yao L, Hearn SA, Possmayer F, Lewis JF: **Surfactant-associated Protein A is important for maintaining surfactant large-aggregate forms during surface-area cycling**. *Biochem J* 1996, **313**:835–840.

117. Cockshutt AM, Weitz J, Possmayer F: **Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro.** *Biochemistry* 1990, **29**:8424–8429.

118. LeVine AM, Kurak KE, Wright JR, Watford WT, Bruno MD, Ross GF, Whitsett JA, Korfhagen TR: **Surfactant protein-A binds group B streptococcus enhancing phagocytosis and clearance from lungs of surfactant protein-A-deficient mice**. *Am J Respir Cell Mol Biol* 1999, **20**:279–286.

119. Crouch E, Wright JR: **Surfactant proteins A and D and pulmonary host defense**. *Annu Rev Physiol* 2001, **63**:521–554.

120. McCormack FX, Whitsett JA: **The pulmonary collectins, SP-A and SP-D, orchestrate innate immunity in the lung**. *J Clin Invest* 2002, **109**:707–712.

121. Borron PJ, Mostaghel EA, Doyle C, Walsh ES, McHeyzer-Williams MG, Wright JR: **Pulmonary surfactant proteins A and D directly suppress CD3+/CD4+ cell function: evidence for two shared mechanisms.** *J Immunol* 2002, **169**:5844–5850.

122. Wolfson MR, Wu J, Hubert TL, Gregory TJ, Mazela J, Shaffer TH: **Lucinactant attenuates pulmonary inflammatory response, preserves lung structure, and improves physiologic outcomes in a preterm lamb model of RDS.** *Pediatr Res* 2012, **72**:375–383.

123. Van Iwaarden JF, Claassen E, Jeurissen SH, Haagsman HP, Kraal G: **Alveolar macrophages, surfactant lipids, and surfactant protein B regulate the induction of immune responses via the airways.** *Am J Respir Cell Mol Biol* 2001, **24**:452–458.

124. Numata M, Chu HW, Dakhama A, Voelker DR: **Pulmonary surfactant phosphatidylglycerol inhibits respiratory syncytial virus-induced inflammation and infection.** *Proc Natl Acad Sci U S A* 2010, **107**:320–325.

125. Abate W, Alghaithy AA, Parton J, Jones KP, Jackson SK: **Surfactant lipids regulate LPS-induced interleukin-8 production in A549 lung epithelial cells by inhibiting translocation of TLR4 into lipid raft domains.** *J Lipid Res* 2010, **51**:334–344.

126. Glasser SW, Senft AP, Whitsett JA, Maxfield MD, Ross GF, Richardson TR, Prows DR, Xu Y, Korfhagen TR: **Macrophage dysfunction and susceptibility to pulmonary Pseudomonas aeruginosa infection in surfactant protein C-deficient mice**. *J Immunol* 2008, **181**:621–628.

127. Glasser SW, Witt TL, Senft AP, Baatz JE, Folger D, Maxfield MD, Akinbi HT, Newton DA, Prows DR, Korfhagen TR: **Surfactant protein C-deficient mice are susceptible to respiratory syncytial virus infection**. *Am J Physiol Cell Mol Physiol* 2009, **297**:L64–L72.

128. Lewis JF, Ikegami M, Jobe AH: **Altered surfactant function and metabolism in rabbits with acute lung injury**. *J Appl Physiol* 1990, **69**:2303–2310.

129. Lewis JF, Veldhuizen R, Possmayer F, Sibbald W, Whitsett J, Qanbar R, McCaig L: **Altered alveolar surfactant is an early marker of acute lung injury in septic adult sheep.** *Am J Respir Crit Care Med* 1994, **150**:123–130.

130. Gregory TJ, Longmore WJ, Moxley MA, Whitsett JA, Reed CR, Fowler a a, Hudson LD, Maunder RJ, Crim C, Hyers TM: **Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome.** *J Clin Invest* 1991, **88**:1976–1981.

131. Schmidt R, Markart P, Ruppert C, Wygrecka M, Kuchenbuch T, Walmrath D, Seeger W, Guenther A: **Time-dependent changes in pulmonary surfactant function and composition in acute respiratory distress syndrome due to pneumonia or aspiration.** *Respir Res* 2007, **8**:55.

132. Holm BA, Matalon S, Finkelstein JN, Notter RH: **Type II pneumocyte changes during hyperoxic lung injury and recovery.** *J Appl Physiol* 1988, **65**:2672–2678.

133. Ito Y, Veldhuizen RA, Yao LJ, McCaig LA, Bartlett AJ, Lewis JF: **Ventilation strategies affect surfactant aggregate conversion in acute lung injury**. *Am J Respir Crit Care Med* 1997, **155**:493–499.

134. Veldhuizen RA, Yao LJ, Lewis JF: **An examination of the different variables affecting surfactant aggregate conversion in vitro**. *Exp Lung Res* 1999, **25**:127–141.

135. Veldhuizen RA, Ito Y, Marcou J, Yao LJ, McCaig L, Lewis JF: **Effects of lung injury on pulmonary surfactant aggregate conversion in vivo and in vitro**. *Am J Physiol* 1997, **272**(5 Pt 1):L872–L878.

136. Malloy JL, Veldhuizen RAW, Thibodeaux BA, Callaghan RJO, Wright JR: **Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions**. *Am J Physiol Lung Cell Mol Physiol* 2005, **288**:L409–L418.

137. Beatty AL, Malloy JL, Wright JR: **Pseudomonas aeruginosa Degrades Pulmonary Surfactant and Increases Conversion In Vitro**. *Am J Respir Cell Mol Biol* 2005, **32**:128–134.

138. Mariencheck WI, Alcorn JF, Palmer SM, Wright JR: **Pseudomonas aeruginosa Elastase Degrades Surfactant Proteins A and D**. *Am J Respir Cell Mol Biol* 2003, **28**:528–537.

139. Seeger W, Elssner A, Günther A, Krämer HJ, Kalinowski HO: **Lung surfactant phospholipids associate with polymerizing fibrin: loss of surface activity.** *Am J Respir Cell Mol Biol* 1993, **9**:213–220.

140. Welk B, Malloy JL, Joseph M, Yao LJ, Veldhuizen A: **Surfactant treatment for ventilation-induced lung injury in rats: effects on lung compliance and cytokines.** *Exp Lung Res* 2001, **27**:505–520.

141. Häfner D, Germann PG, Hauschke D, Kilian U: **Effects of early treatment with rSP-C surfactant on oxygenation and histology in rats with acute lung injury.** *Pulm Pharmacol Ther* 1999, **12**:193–201.

142. Vreugdenhil HA, Lachmann B, Haitsma JJ, Zijlstra J, Heijnen CJ, Jansen NJ, van Vught AJ: **Exogenous surfactant restores lung function but not peripheral immunosuppression in ventilated surfactant-deficient rats.** *Exp Lung Res* 2006, **32**:1–14.

143. Lewis JF, Brackenbury A: **Role of exogenous surfactant in acute lung injury**. *Crit Care Med* 2003, **31**(4 Suppl):S324–S328.

144. Anzueto A, Baughman RP, Guntupalli KK, Weg JG, Wiedemann HP, Raventos AA, Lemaire F, Long W, Zaccardelli DS, Pattishall EN: **Aerosolized surfactant in adults with sepsis-induced acute respiratory distress syndrome. Exosurf Acute Respiratory Distress Syndrome Sepsis Study Group**. *N Engl J Med* 1996, **334**:1417–1421.

145. Gregory TJ, Steinberg KP, Spragg R, Gadek JE, Hyers TM, Longmore WJ, Moxley MA, Cai GZ, Hite RD, Smith RM, Hudson LD, Crim C, Newton P, Mitchell BR, Gold AJ: **Bovine surfactant therapy for patients with acute respiratory distress syndrome**. *Am J Respir Crit Care Med* 1997, **155**:1309–1315.

146. Spragg RG, Lewis JF, Walmrath HD, Johannigman J, Bellingan G, Laterre PF, Witte MC, Richards GA, Rippin G, Rathgeb F, Hafner D, Taut FJ, Seeger W: **Effect of recombinant surfactant protein C-based surfactant on the acute respiratory distress syndrome**. *N Engl J Med* 2004, **351**:884–892.

147. Walker MG, Tessolini JM, McCaig L, Yao LJ, Lewis JF, Veldhuizen RA: **Elevated endogenous surfactant reduces inflammation in an acute lung injury model**. *Exp Lung Res* 2009, **35**:591–604.

148. Yamashita C, Forbes A, Tessolini JM, Yao LJ, Lewis JF, Veldhuizen RA: **Protective effects of elevated endogenous surfactant pools to injurious mechanical ventilation**. *Am J Physiol Cell Mol Physiol* 2008, **294**:L724–L732.

149. Brinckerhoff CE, Matrisian LM: **Matrix metalloproteinases: a tail of a frog that became a prince.** *Nat Rev Mol Cell Biol* 2002, **3**:207–214.

150. Webster NL, Crowe SM: **Matrix metalloproteinases, their production by monocytes and macrophages and their potential role in HIV-related diseases**. *J Leukoc Biol* 2006, **80**:1052–1066.

151. Yamashita CM, Dolgonos L, Zemans RL, Young SK, Robertson J, Briones N, Suzuki T, Campbell MN, Gauldie J, Radisky DC, Riches DW, Yu G, Kaminski N, McCulloch CA, Downey GP: **Matrix metalloproteinase 3 is a mediator of pulmonary fibrosis**. *Am J Pathol* 2011, **179**:1733–1745.

152. Gill SE, Parks WC: **Metalloproteinases and their inhibitors: regulators of wound healing.** *Int J Biochem Cell Biol* 2008, **40**:1334–1347.

153. Manicone AM, McGuire JK: **Matrix metalloproteinases as modulators of inflammation**. *Semin Cell Dev Biol* 2008, **19**:34–41.

154. Page-McCaw A, Ewald AJ, Werb Z: **Matrix metalloproteinases and the regulation of tissue remodelling**. *Nat Rev cell Biol* 2007, **8**:221–233.

155. Iyer RP, Patterson NL, Fields GB, Lindsey ML: **The history of matrix metalloproteinases: milestones, myths, and misperceptions.** *Am J Physiol Heart Circ Physiol* 2012, **303**:H919–H930.

156. Kessenbrock K, Dijkgraaf GJP, Lawson DA, Littlepage LE, Shahi P, Pieper U, Werb Z: **A role for matrix metalloproteinases in regulating mammary stem cell function via the Wnt signaling pathway.** *Cell Stem Cell* 2013, **13**:300–313.

157. Correia AL, Mori H, Chen EI, Schmitt FC, Bissell MJ: **The hemopexin domain of MMP3 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with HSP90β.** *Genes Dev* 2013, **27**:805–817.

158. Khokha R, Murthy A, Weiss A: **Metalloproteinases and their natural inhibitors in inflammation and immunity.** *Nat Rev Immunol* 2013, **13**:649–665.

159. Silence J, Lupu F, Collen D, Lijnen HR: **Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation**. *Arterioscler Thromb Vasc Biol* 2001, **21**:1440–1445.

160. Gueders MM, Foidart J-M, Noel A, Cataldo DD: **Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the respiratory tract: potential implications in asthma and other lung diseases.** *Eur J Pharmacol* 2006, **533**:133–144.

161. Yazdan-Ashoori P, Liaw P, Toltl L, Webb B, Kilmer G, Carter DE, Fraser DD: **Elevated plasma matrix metalloproteinases and their tissue inhibitors in patients with severe sepsis**. *J Crit Care* 2011, **26**:556–565.

162. Davey A, McAuley DF, O'Kane CM: **Matrix metalloproteinases in acute lung injury: mediators of injury and drivers of repair**. *Eur Respir J* 2011, **38**:959–970.

163. Fligiel SE, Standiford T, Fligiel HM, Tashkin D, Strieter RM, Warner RL, Johnson KJ, Varani J: **Matrix metalloproteinases and matrix metalloproteinase inhibitors in acute lung injury**. *Hum Pathol* 2006, **37**:422–430.

164. Kong MYF, Gaggar A, Li Y, Winkler M, Blalock JE, Clancy JP: **Matrix metalloproteinase activity in pediatric acute lung injury.** *Int J Med Sci* 2009, **6**:9–17.

165. O'Kane CM, McKeown SW, Perkins GD, Bassford CR, Gao F, Thickett DR, McAuley DF: **Salbutamol up-regulates matrix metalloproteinase-9 in the alveolar space in the acute respiratory distress syndrome**. *Crit Care Med* 2009, **37**:2242–2249.

166. Warner RL, Bhagavathula N, Nerusu KC, Lateef H, Younkin E, Johnson KJ, Varani J: **Matrix metalloproteinases in acute inflammation: induction of MMP-3 and MMP-9 in fibroblasts and epithelial cells following exposure to pro-inflammatory mediators in vitro**. *Exp Mol Pathol* 2004, **76**:189–195.

167. Nagase H, Woessner JF: **Matrix metalloproteinases.** *J Biol Chem* 1999, **274**:21491–21494.

168. Massova I, Kotra LP, Fridman R, Mobashery S: **Matrix metalloproteinases: structures, evolution, and diversification.** *FASEB J* 1998, **12**:1075–1095.

169. Van Wart HE, Birkedal-Hansen H: **The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family.** *Proc Natl Acad Sci U S A* 1990, **87**:5578–5582.

170. Lochter A, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ: **Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells.** *J Cell Biol* 1997, **139**:1861–1872.

171. Sympson CJ, Talhouk RS, Alexander CM, Chin JR, Clift SM, Bissell MJ, Werb Z: **Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression.** *J Cell Biol* 1994, **125**:681–693.

172. Bini A, Itoh Y, Kudryk BJ, Nagase H: **Degradation of cross-linked fibrin by matrix metalloproteinase 3 (stromelysin 1): hydrolysis of the gamma Gly 404-Ala 405 peptide bond.** *Biochemistry* 1996, **35**:13056–13063.

173. Bullard KM, Lund L, Mudgett JS, Mellin TN, Hunt TK, Murphy B, Ronan J, Werb Z, Banda MJ: **Impaired wound contraction in stromelysin-1-deficient mice.** *Ann Surg* 1999, **230**:260–265.

174. Bullard KM, Mudgett J, Scheuenstuhl H, Hunt TK, Banda MJ: **Stromelysin-1-Deficient Fibroblasts Display Impaired Contraction in Vitro**. *J Surg Res* 1999, **84**:31–34.

175. Lijnen HR, Arza B, Van Hoef B, Collen D, Declerck PJ: **Inactivation of plasminogen activator inhibitor-1 by specific proteolysis with stromelysin-1 (MMP-3).** *J Biol Chem* 2000, **275**:37645–50.

176. Knäuper V, Wilhelm SM, Seperack PK, DeClerck YA, Langley KE, Osthues A, Tschesche H: **Direct activation of human neutrophil procollagenase by recombinant stromelysin.** *Biochem J* 1993, **295**:581–586.

177. Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H: **Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin).** *Biochemistry* 1990, **29**:10261–10270.

178. Ogata Y, Enghild JJ, Nagase H: **Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9.** *J Biol Chem* 1992, **267**:3581–3584.

179. Maeda S, Dean DD, Gomez R, Schwartz Z, Boyan BD: **The first stage of transforming growth factor beta1 activation is release of the large latent complex from the extracellular matrix of growth plate chondrocytes by matrix vesicle stromelysin-1 (MMP-3).** *Calcif Tissue Int* 2002, **70**:54–65.

180. Schönbeck U, Mach F, Libby P: **Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing.** *J Immunol* 1998, **161**:3340–3346.

181. Haro H, Crawford HC, Fingleton B, MacDougall JR, Shinomiya K, Spengler DM, Matrisian LM: **Matrix metalloproteinase-3-dependent generation of a macrophage chemoattractant in a model of herniated disc resorption.** *J Clin Invest* 2000, **105**:133–141.

182. Kruidenier L, MacDonald TT, Collins JE, Pender SLF, Sanderson IR: **Myofibroblast matrix metalloproteinases activate the neutrophil chemoattractant CXCL7 from intestinal epithelial cells.** *Gastroenterology* 2006, **130**:127–136.

183. Warner RL, Beltran L, Younkin EM, Lewis CS, Weiss SJ, Varani J, Johnson KJ: **Role of stromelysin 1 and gelatinase B in experimental acute lung injury**. *Am J Respir Cell Mol Biol* 2001, **24**:537–544.

184. Nerusu KC, Warner RL, Bhagavathula N, McClintock SD, Johnson KJ, Varani J: **Matrix metalloproteinase-3 (stromelysin-1) in acute inflammatory tissue injury**. *Exp Mol Pathol* 2007, **83**:169–176.

185. Mudgett JS, Hutchinson NI, Chartrain NA, Forsyth AJ, Mcdonnell J, Singer II, Bayne EK, Flanagan J, Kawka D, Shen CF, Stevens K, Chen H, Trumbauer M, Visco DM: **Susceptibility of stromelysin 1-deficient mice to collagen-induced arthritis and cartilage destruction**. *Arthritis Rheum* 1998, **41**:110–121.

186. Li CKF, Pender SLF, Pickard KM, Chance V, Holloway J a, Huett A, Goncalves NS, Mudgett JS, Dougan G, Frankel G, MacDonald TT, Gonçalves NS: **Impaired immunity to intestinal bacterial infection in stromelysin-1 (matrix metalloproteinase-3)-deficient mice**. *J Immunol* 2004, **173**:5171–5179.

187. Matute-Bello G, Frevert CW, Martin TR: **Animal models of acute lung injury**. *Am J Physiol Cell Mol Physiol* 2008, **295**:L379–L399.

188. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, Kuebler WM, Group ALI in AS: **An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals.** *Am J Respir Cell Mol Biol* 2011, **44**:725–738.

189. Duck-Chong CG: **A rapid sensitive method for determining phospholipid phosphorous involving digestion with magnesium nitrate.** *Lipids* 1979, **14**:492–497.

190. Bligh EG, Dyer WJ: **A rapid method of total lipid extraction and purification**. *Can J Biochem Physiol* 1959, **37**:911–917.

191. Schurch S, Bachofen H, Possmayer F: **Surface activity in situ, in vivo, and in the captive bubble surfactometer**. *Comp Biochem Physiol A, Mol Integr Physiol* 2001, **129**:195–207.

192. Yu LMY, Lu JJ, Chan YW, Ng A, Zhang L, Hoorfar M, Policova Z, Grundke K, Neumann AW: **Constrained sessile drop as a new configuration to measure low surface tension in lung surfactant systems.** *J Appl Physiol* 2004, **97**:704–715.

193. Schwarte LA, Zuurbier CJ, Ince C: **Mechanical ventilation of mice.** *Basic Res Cardiol* 2000, **95**:510–520.

194. Marik PE: **Aspiration Pneumonitis and Aspiration Pneumonia**. *N Engl J Med* 2001, **344**:665.

195. Brackenbury AM, Puligandla PS, McCaig LA, Nikore V, Yao LJ, Veldhuizen RA, Lewis JF: **Evaluation of exogenous surfactant in HCL-induced lung injury**. *Am J Respir Crit Care Med* 2001, **163**:1135–1142.

196. Aspros AJ, Coto CG, Lewis JF, Veldhuizen RA: **High-frequency oscillation and surfactant treatment in an acid aspiration model**. *Can J Physiol Pharmacol* 2010, **88**:14–20.

197. Modelska K, Pittet JF, Folkesson HG, Courtney Broaddus V, Matthay MA: **Acid-induced lung injury. Protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits**. *Am J Respir Crit Care Med* 1999, **160**(5 Pt 1):1450–1456.

198. St John RC, Mizer LA, Kindt GC, Weisbrode SE, Moore SA, Dorinsky PM: **Acid aspiration-induced acute lung injury causes leukocyte-dependent systemic organ injury.** *J Appl Physiol* 1993, **74**:1994–2003.

199. Imai Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, van Loo G, Ermolaeva M, Veldhuizen R, Leung YHC, Wang H, Liu H, Sun Y, Pasparakis M, Kopf M, Mech C, Bavari S, Peiris JSM, Slutsky AS, Akira S, Hultqvist M, Holmdahl R, Nicholls J, Jiang C, Binder CJ, Penninger JM: **Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury.** *Cell* 2008, **133**:235–249.

200. Sender V, Stamme C: **Lung cell-specific modulation of LPS-induced TLR4 receptor and adaptor localization.** *Commun Integr Biol* 2014, **7**:1–9.

201. Vadász I, Brochard L: **Update in acute lung injury and mechanical ventilation 2011.** *Am J Respir Crit Care Med* 2012, **186**:17–23.

202. Herold S, Gabrielli NM, Vadász I: **Novel concepts of acute lung injury and alveolar-capillary barrier dysfunction.** *Am J Physiol Lung Cell Mol Physiol* 2013, **305**:L665–L681.

203. Slutsky AS: **Lung injury caused by mechanical ventilation.** *Chest* 1999, **116**(1 Suppl):9S–15S.

204. Vlahakis NE, Schroeder MA, Limper AH, Hubmayr RD: **Stretch induces cytokine release by alveolar epithelial cells in vitro**. *Am J Physiol Lung Cell Mol Physiol* 2011, **227**:L167–L173.

205. Frank JA, Wray CM, McAuley DF, Schwendener R, Matthay MA: **Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury.** *Am J Physiol Lung Cell Mol Physiol* 2006, **291**:L1191–L1198.

206. Irvin CG, Bates JHT: **Measuring the lung function in the mouse: the challenge of size.** *Respir Res* 2003, **4**:4.

207. Glaab T, Taube C, Braun A, Mitzner W: **Invasive and noninvasive methods for studying pulmonary function in mice.** *Respir Res* 2007, **8**:63.

208. Manali ED, Moschos C, Triantafillidou C, Kotanidou A, Psallidas I, Karabela SP, Roussos C, Papiris S, Armaganidis A, Stathopoulos GT, Maniatis NA: **Static and dynamic mechanics of the murine lung after intratracheal bleomycin.** *BMC Pulm Med* 2011, **11**:33.

209. Martin EL, Truscott EA, Bailey TC, Leco KJ, McCaig LA, Lewis JF, Veldhuizen RA: **Lung mechanics in the TIMP3 null mouse and its response to mechanical ventilation**. *Exp Lung Res* 2007, **33**:99–113.

CHAPTER 2:

The effects of exogenous surfactant administration on ventilation-induced inflammation in mouse models of lung injury

A version of this chapter has been published:

Puntorieri V, Hiansen JQ, McCaig LA, Yao LJ, Veldhuizen RAW, Lewis JF. **The effects of exogenous surfactant administration on ventilation-induced inflammation in mouse models of lung injury.** *BMC Pulm Med.* 2013 Nov 20; **13**:67.

2.1. Introduction

Pulmonary surfactant is a mixture of phospholipids, surfactant-associated proteins and neutral lipids which has an important role in the lung in both host defence mechanisms such as modulating pulmonary inflammation and in stabilizing the alveoli by reducing surface tension [1, 2]. Both biophysical and immuno-modulatory properties of endogenous surfactant are essential for normal lung function. Importantly, both properties are severely impaired during the course of acute lung injury (ALI) [3, 4].

ALI is a life threatening condition characterized by bilateral pulmonary infiltrates on chest radiograph, alveolar edema and hypoxemia [5]. Mortality is approximately 30-40%, with the main cause of death resulting from multiple organ failure (MOF) rather than respiratory failure. The former is thought to develop in large part due to the release of inflammatory mediators from the lung into the circulation thereby contributing to excessive systemic inflammation. This, in turn, causes MOF and death [6-8].

The main supportive therapy required to maintain adequate oxygenation for patients with ALI is mechanical ventilation (MV). Unfortunately, this intervention is also an important component of the complex pathophysiology of ALI, since it can increase pulmonary inflammation and contribute to the development of the associated systemic inflammation leading to MOF [9-13]. A pharmacological therapy capable of mitigating the specific inflammatory effects of MV thereby reducing the contribution of the lung to the systemic inflammation is needed. Based on the known properties of surfactant within the lung, the current study investigated on such potential therapy namely exogenous surfactant administration.

Exogenous surfactant has been investigated as a possible therapy for ALI in many experimental and clinical studies [14-17]. Traditionally surfactant treatment has been administered to improve the biophysical function of this material within the lung. Although extensive research has shown improvements in physiological and biophysical outcomes following surfactant treatment, there was no effect on mortality [18]. Contrasting this extensively investigated approach, only a limited number of studies have evaluated surfactant with the aim to down-regulate the systemic inflammation associated

with ALI and MV. Previous studies in our laboratory demonstrated that elevated endogenous surfactant pool sizes *prior to MV* attenuated the development of pulmonary and systemic inflammation in animal models where injurious MV was applied to normal lungs [19] or conventional ventilation was applied to lungs with a pre-existing injury (lipopolysaccharide-induced ALI) [20]. Whether exogenous surfactant can mirror these observations obtained with elevated endogenous surfactant is not known. It was therefore hypothesized that administration of exogenous surfactant *prior* to MV would reduce the *systemic inflammation* associated with lung injury.

To test this hypothesis, two separate mouse models were utilized: i) a model of mechanical ventilation in animals with otherwise normal lungs and ii) a model of acid-induced lung injury followed by MV. For both experiments, exogenous surfactant was administered *prior to* MV, and the ventilation was performed *ex vivo* using an isolated and perfused mouse lung (IPML) setup. The inflammatory mediators released by the lungs into the circulation were collected (via left ventricle) in perfusate and re-circulated (via pulmonary artery) throughout MV. This *ex vivo* circulatory system in the IPML setup allowed us to isolate the contribution of mechanically ventilated lungs to the systemic system, with perfusate representing a surrogate of systemic inflammation.

2.2. Materials and methods

2.2.1. Experimental design and ethics statement

A total of 36 male 129X1/SVJ mice (Jackson Laboratories, Bar Harbor, Me., USA) were utilized for two separate animal experiments. All procedures were approved by the Animal Use Subcommittee at the University of Western Ontario (Permit Number: 2010-272) and, whenever necessary, adequate anesthetic regimen was used to minimize suffering. For both experiments, mice were allowed to acclimatize for a minimum period of 72 hours in an animal facility, during which time they were allowed free access to water and standard chow.

In order to test our hypothesis of an anti-inflammatory role of surfactant toward the effects of MV, administration of exogenous surfactant was performed in two separate models of lung injury: *experiment 1* involved the use of MV only and *experiment 2* involved the use of intra-tracheal (i.t.) instillation of hydrochloric acid (HCl) followed by conventional MV.

In *experiment 1*, mice were anaesthetized and subsequently randomized to either exogenous surfactant administration or no treatment. After the completion of the i.t. surfactant instillation, mice were connected to the IPML setup and exposed immediately following re-perfusion to MV with a tidal volume (Vt) of 20ml/kg, a positive end expiratory pressure (PEEP) of 3 cmH_2O, and a respiratory rate (RR) of 30 breaths/min. This resulted in the randomization of a total of 12 mice to one of the two experimental conditions: i) No Treatment group or ii) bLES group.

In *experiment 2,* a total of 24 male 129X1/SVJ mice were anaesthetized and then randomized to receive an intra-tracheal instillation of HCl or air. Four hours after the development of acid-induced lung injury, mice were randomized to receive an intra-tracheal exogenous surfactant administration (or no treatment) before *ex vivo, in situ* MV. The IPML setup was used to ventilate these animals with the following ventilation parameters: Vt=5ml/kg, PEEP=3 cmH_2O, RR=60 breaths/min. This resulted in the following experimental conditions: i) air + no treatment; ii) air + bLES; iii) acid + no treatment; iv) acid + bLES.

2.2.2. Intra-tracheal hydrochloric acid instillation

Mice were randomized to receive either an intra-tracheal (i.t.) administration of HCl or air as a control, as previously described [9]. Briefly, mice were anesthetised with an intra-peritoneal injection of ketamine (130 mg/kg; Sandoz, Quebec, Que., Canada) and xylazine (6 mg/kg; Bayer, Toronto, Ont., Canada). Once the proper depth of anesthesia was reached, mice were positioned dorsally on a vertical stand and their trachea was intubated with a 20-gauge catheter coupled with a fiber-optic stylet (BioLite intubation system for small rodents, BioTex, Inc., Houston, Tex., USA). Animals randomized to the acid instillation group were given 50 μl of 0.05 N HCl in a drop-wise fashion through the endotracheal tube. Animals randomized to the control group were intubated as described and allowed to breathe spontaneously through the tube. The total procedure took approximately 5 minutes. Mice were then extubated, positioned on a horizontal inclined stand and administered sub-cutaneous injections of buprenorphine (0.05-0.1 mg/kg) and 1ml of sterile normal saline. Subsequently, mice were returned to the cage and allowed to recover for 4 hours with free access to water and food. Mice were carefully monitored during the 4 hours recovery period.

2.2.3. Intra-tracheal surfactant instillation

Mice were anesthetised with an intra-peritoneal (i.p.) injection of ketamine (130 mg/kg) and xylazine (6 mg/kg). Animals were then positioned dorsally on a vertical rodent stand and the trachea was intubated trans-orally with a 20-gauge catheter coupled with a fiber-optic stylet (BioLite intubation system for small rodents, BioTex, Inc., Houston, Tex., USA). Mice randomized to the surfactant administration group were given 50 mg/kg bLES (BLES Biochemicals, London, Ont., Canada) in a drop wise fashion through the endotracheal tube. This natural, bovine lipid extracted surfactant is composed of approximately 97% phospholipids, 3% neutral lipids, and about 1% by weight proteins [21]. After the surfactant was spontaneously inhaled by the animals, mice were extubated, positioned on a horizontal inclined stand. To allow for peripheral surfactant distribution, based on preliminary experiments, mice were allowed to spontaneously breathe for 12-15 minutes before MV. Animals randomized to the no treatment group were intubated as described and allowed to breathe spontaneously.

2.2.4. Isolated and Perfused Mouse Lung setup

Mice were ventilated for a total of 2 hours using the IPML setup. Following exogenous surfactant administration (or no treatment), the anesthetised mice were sacrificed with an additional i.p. injection of ketamine (200 mg/kg) and xylazine (10 mg/kg). A tracheostomy tube was then inserted and secured in the trachea, and the animals were subsequently connected to the IPML apparatus as described by Von Bethmann et al. [22]. Briefly, the heart and lungs were surgically exposed and the lungs were ventilated with a volume cycled, positive pressure ventilator (Flexivent, Scireq, Montreal, Que., Canada) with different ventilation strategies as described in detail under the *experimental design* section. Perfusate (RPMI lacking phenol red + 2% w/v low endotoxin grade Bovine Serum Albumin; Sigma, St. Louis, Mo., USA) was circulated into the pulmonary vasculature through a catheter inserted in the pulmonary artery and collected by a second catheter in the left ventricle. Once the lungs were cleared of all the blood, perfusate was delivered in a re-circulating fashion (rate 1ml/min) during the 2 hours of MV. One milliliter of perfusate was collected at baseline (time 0, immediately after vascular clearing and before perfusate re-circulation) and every 30 minutes of MV thereafter. Samples were frozen and stored at -80ºC for subsequent measurement of inflammatory mediators. Physiological parameters such as peak inspiratory pressure (PIP) and perfusion pressure were monitored throughout ventilation utilizing Chart v.4.12 software (AD Instruments, Castle Hill, Australia).

2.2.5. Surfactant and total lung lavage protein measurements

Immediately after MV using the IPML setup, lungs were lavaged with 3 x 1ml aliquots of 0.9% NaCl solution with each aliquot instilled and withdrawn 3 times. The total lavage volume was recorded and average recoveries of lavage fluid were 2.7 mL and 2.8 mL for *experiment 1* and *experiment 2*, respectively. Total lavage was then immediately centrifuged at 380 g for 10 min. at 4ºC to remove the cellular component, and the collected supernatant was termed total surfactant (TS). A 1 ml aliquot of TS was stored at -80ºC for cytokine and protein analysis. In order to separate the small aggregate sub-fraction (SA) from the large aggregate (LA) sub-fraction, 1 ml of TS was centrifuged at 40,000 g for 15 min at 4ºC. The LA pellet was then re-suspended in 0.3 ml of 0.9% NaCl,

while the supernatant represented the SA fraction. The leftover volume of TS was used for analysis of total surfactant pool size. TS, LA and SA were frozen and stored at -80ºC.

Measurement of the phospholipid content in TS, LA and SA was performed by phosphorous assay on chloroform-methanol extracted samples, as previously described [23, 24]. Total protein content in lavage was assessed using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA) according to manufacturer's instructions.

2.2.6. Biophysical functional analysis of surfactant

LA sub-fractions from animals within each experimental group were pooled together for functional analysis. An aliquot from each pooled sample was utilized to measure the total phospholipid content by phosphorous assay, while the remaining pooled LA was centrifuged at 40,000g for 15 min at 4ºC. The supernatant was then discarded and the purified LA pellet re-suspended in a buffer solution (1.5 mM CaCl2, 5 mM TRIS) to a final phospholipid concentration of 5 mg/ml. The surface activity of the LA samples was assessed using a computer-controlled captive bubble surfactometer (CBS, 3 runs for each pooled sample) as previously described [25, 26].

2.2.7. Measurement of inflammatory mediators

Interleukin-6 (IL-6) levels were measured in aliquots of lung lavage and in perfusate aliquots obtained at different time points using an enzyme-linked immunosorbent assay (ELISA) kit following manufacturer's instructions (BD Biosciences, San Diego, CA., USA). A broader array of inflammatory mediators was measured in perfusate samples collected at the end of MV using a Milliplex Map mouse cytokine/chemokine panel (MPXMCYTO-70K-12; Millipore Corporation, Billerica, MA, USA) for the following 12 analytes: eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-6, IL-13, interferon-γ-induced protein 10 (IP-10), keratinocyte chemoattractant (KC), lipopolysaccharide-induced CXC chemochine (LIX), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein 2 (MIP-2) and tumor necrosis factor-alpha (TNF-α). Samples were analyzed utilizing the Luminex® xMAP® detection system on the Luminex^{100} (Linco Research, St. Charles, Mo., USA) according to the manufacturer's instructions. Perfusate

samples collected at the end of MV in *experiment 2* were further analyzed for eicosanoids levels (8-isoprostane, prostaglandin E_2, leukotriene B_4, thromboxane B_2) using colorimetric competitive enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA) according to manufacturer's instructions.

2.2.8. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using the GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA., USA). Data were analysed with a t-test or one way ANOVA with a Tukey's post hoc test when appropriate (*experiment 1)*. For *experiment 2*, a two-way ANOVA (variables: presence of primary insult and treatment effects) followed by a one-way ANOVA with a Tukey's post hoc test was used to analyse the data. A repeated measures two-way ANOVA was performed when appropriate with a Bonferroni post hoc test. $P < 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Experiment 1.

In experiment 1 the effects of exogenous surfactant administration on lung and systemic inflammation during MV of otherwise normal lungs were determined. Peak inspiratory pressure (PIP) was recorded throughout MV. PIP ranged between 20.62±1.6 cmH_2O and 22.6±2.7 cmH_2O for the No Treatment group (time 0 and time 120min, respectively) and varied between 22.6±2.7 cmH_2O and 26.3±2.7 cmH_2O for the bLES group (time 0 and time 120min, respectively). Exogenous surfactant administration did not reduce PIP values in the surfactant treated group compared to No Treatment. Perfusion pressure was also monitored throughout MV and maintained between 4 and 6 mmHg for both groups (Fig. 2.1).

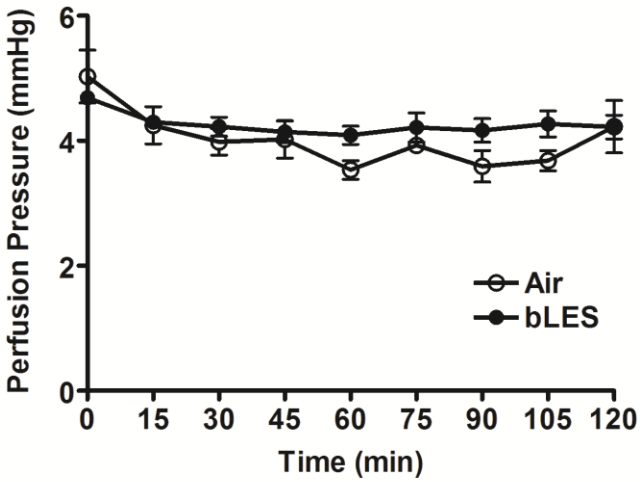

Figure 2.1 ***Experiment 1.*** Perfusion pressure measured throughout MV. Values are expressed as mean ± SEM; n=6 per group.

Lavage Analysis

Results reflecting local inflammation, as assessed by pulmonary permeability changes and inflammatory markers are shown in Table 2.1. The total protein content and IL-6 levels in lung lavage collected at the end of MV were not affected by surfactant treatment, with no statistically significant differences noted in these values between bLES treated and non-treated groups. Recoveries of lung lavage fluid were not statistically significant between groups (data not shown).

	Mechanical Ventilation	
	No treatment	**bLES**
Total lavage protein (mg/kg body weight)	13.4 ± 1.2	34.6 ± 18.9
Lavage IL-6 (pg/mL)	136.8 ± 31.4	474.1 ± 233.8

Table 2.1 ***Experiment 1.*** Total protein levels and IL-6 concentrations in lung lavage at the end of MV. Values are expressed as mean ± SEM; n=6 per group.

Surfactant pool sizes of TS, LA and SA sub-fractions isolated from lung lavage for the two groups are shown in Figure 2.2A. As expected, TS pools were significantly higher in the bLES treated group compared to No Treatment mice. Similarly to TS values, LA and SA pools were significantly higher in the bLES group compared to the No Treatment group (Fig. 2.2A). The functional activity of the LA samples measured during four different dynamic compression-expansion cycles is shown in Figure 2.2B for each experimental group. No significant differences in surface tension were found between bLES treated and No Treatment mice for any of the cycles. Within each group, the minimum achievable surface tension was significantly higher during cycle 10 compared with cycles 1 and 2 (Fig. 2.2B).

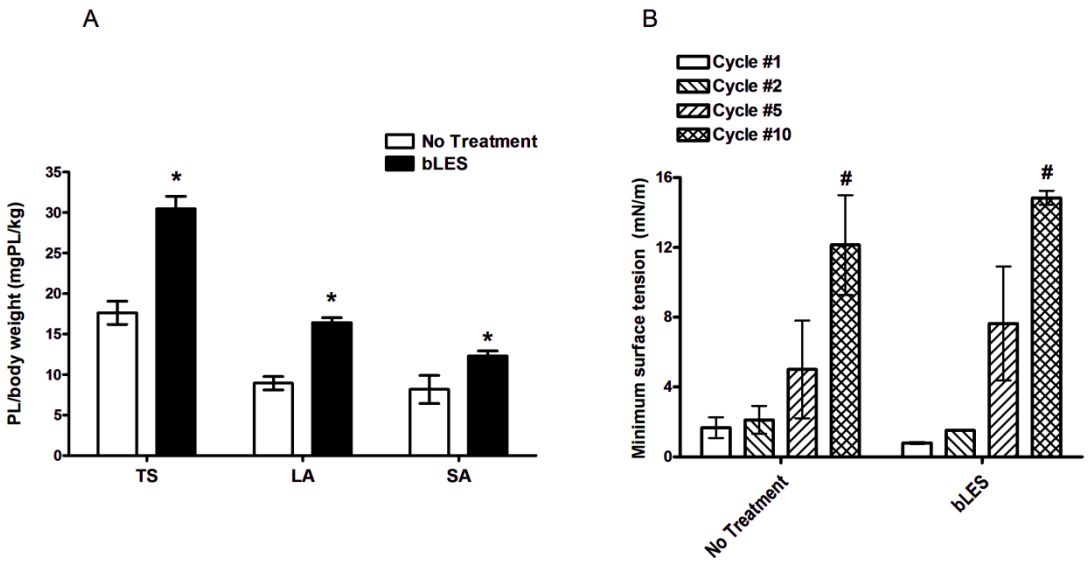

Figure 2.2 ***Experiment 1.*** Surfactant recovery in lung lavage and surface activity of LA. **A**: surfactant pool size of TS, LA and SA sub-fractions measured by phosphorous assay. Data are expressed as amount of phospholipids/kg body weight. Within each sub-fraction, *p<0.05 vs the No Treatment condition. **B**: minimum surface tension of pooled LA samples during different dynamic compression-expansion cycles. #p<0.05 versus cycle 1 and 2 within each experimental conditions. Values are expressed as mean ± SEM.; n=6 per group.

Perfusate Analysis

The concentration of IL-6 was measured in perfusate samples in order to assess the effects of exogenous surfactant on the development of systemic inflammation (Figure 2.3). IL-6 levels were not detectable within the first 30 minutes of MV (time 0 and 30 min; data not shown). A gradual increase in perfusate IL-6 was measured at 60 and 90 minutes in both groups; however, there was no statistically significant difference in this cytokine level between bLES treated and No Treatment mice at any time point throughout MV.

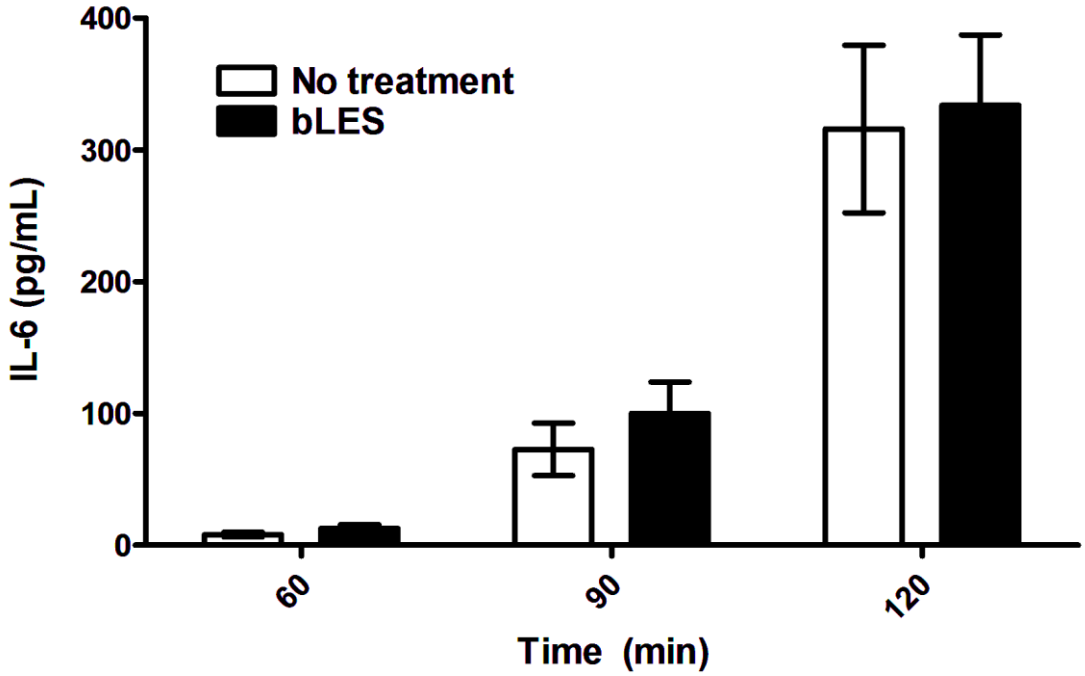

Figure 2.3 ***Experiment 1.*** IL-6 levels measured in lung perfusate at 60, 90 and 120 min. Values are expressed as mean ± SEM.; n=6 per group.

Perfusate concentrations of 11 cytokines/chemokines measured at the end of MV by multiplex assay are shown in Table 2.2. Perfusate IL-13 levels were not detectable (data not shown). There was no statistically significant effect of exogenous surfactant administration on cytokines/chemokines concentrations in perfusate, with no differences between No treatment and bLES groups.

	Mechanical Ventilation	
Mediator (pg/ml)	**No treatment**	**bLES**
Eotaxin	42.8±3.6	42.7±6.5
G-CSF	9.5±2.0	11.7±2.8
GM-CSF	1.6±1.6	5.1±2.4
IL-6	520.6±117.2	463.0±75.2
IL-1 β	0.8±0.4	0.6±0.3
KC	868.6±254.3	853.8±222.6
LIX	71.3±14.8	66.1±11.4
MCP-1	12.2±2.7	7.5±1.8
MIP-2	753.9±193.8	658.5±167.8
TNF-α	23.4±7.8	17.4±7.0
IP-10	39.9±5.4	37.8±6.2

Table 2.2 ***Experiment 1*****.** Cytokine and chemokine analysis in lung perfusate at the end of MV. Data are expressed as mean ± SEM; n=6 per group.
G-CSF=granulocyte colony-stimulating factor, GM-CSF= granulocyte-macrophage CSF, IL-6=interleukin-6, IP-10=interferon-γ-induced protein 10, KC=keratinocyte chemoattractant, LIX=lipopolysaccharide-induced CXC chemokine, MCP-1=monocyte chemotactic protein-1, MIP-2=macrophage inflammatory protein 2 and TNF-α=tumor necrosis factor-alpha.

2.3.2. Experiment 2.

In experiment 2, the effect of exogenous surfactant on systemic inflammation during MV was assessed in the presence of a pre-existing acid-induced lung injury/inflammation. Physiological parameters such as peak inspiratory pressure and perfusion pressure were monitored throughout ventilation as in experiment 1, and PIP values are shown in Figure 2.4. Although all experimental groups were exposed to the same ventilation strategy, the peak inspiratory pressure was significantly higher in Acid injured mice compared to the

respective Air groups (Acid No Treatment vs Air No Treatment; Acid bLES vs Air bLES). Exogenous surfactant administration led to a significant increase in PIP values during the first hour of MV (10 to 75 min) in the Air bLES group compared to Air No Treatment group and, importantly, did not reduce PIP values in the Acid bLES group compared to Acid No Treatment group at any time point. Perfusion pressure was monitored during MV and maintained between 5 and 7 mmHg for all groups (Fig. 2.5).

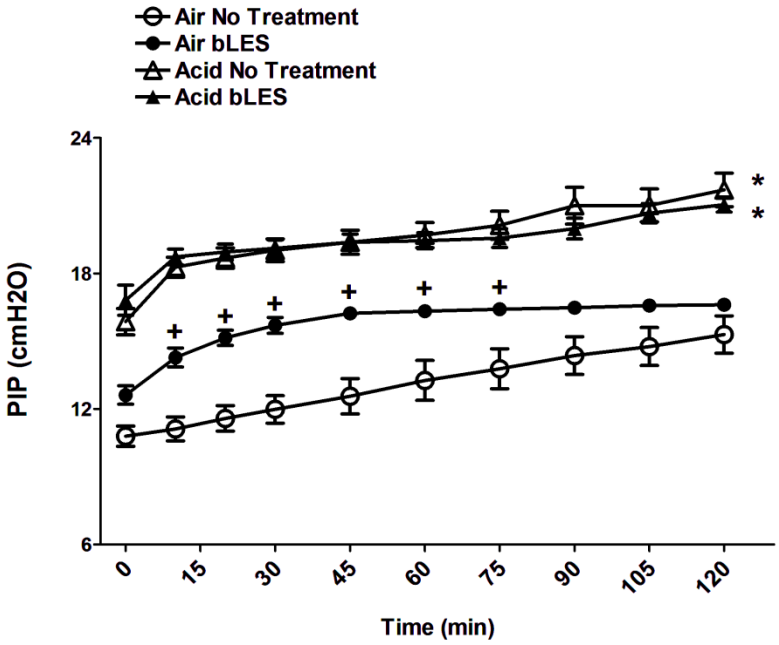

Figure 2.4 ***Experiment 2.*** Peak Inspiratory Pressure (PIP) was measured over the course of MV. Values are expressed as mean ± SEM. +$p<0.05$ versus Air No Treatment at the specific time point indicated, *$p<0.05$ versus the respective Air control at each time point; n=6 per group.

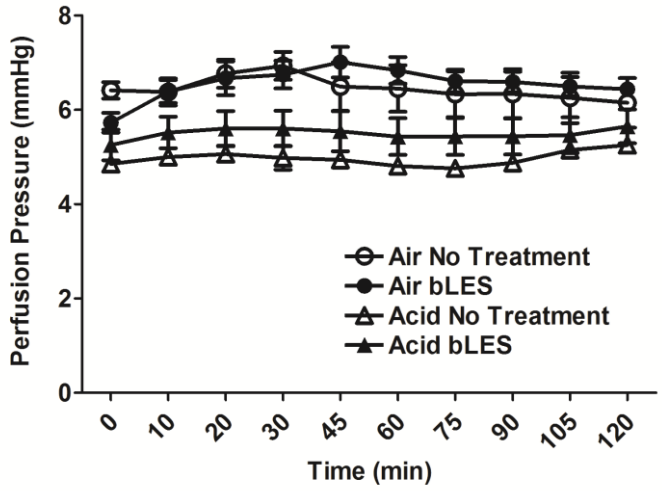

Figure 2.5 ***Experiment 2*****.** Perfusion pressure measured throughout MV. Values are expressed as mean ± SEM; n=6 per group.

Lavage Analysis

Lung permeability, as reflected by total protein content in lung lavage (Table 2.3), was significantly higher in the acid injured animals versus the air control groups, whether they were given surfactant or not (Acid No Treatment vs Air No Treatment; Acid bLES vs Air bLES). No significant difference was noted between Air bLES versus Air No Treatment and Acid bLES versus Acid No Treatment. Similar results were observed for IL-6 concentration in lung lavage (Table 2.3). Acid–instilled animals showed greater IL-6 levels in lavage compared to the respective air-instilled controls. Exogenous surfactant did not affect lavage IL-6 levels in both air groups (Air bLES vs Air No Treatment); however, there was a significantly higher cytokine concentration in the lavage of Acid bLES mice compared to the Acid No Treatment group. Recoveries of lung lavage fluid were not statistically significant between groups (data not shown).

	Air		Acid	
	No treatment	**bLES**	**No treatment**	**bLES**
Total lavage protein (mg/kg body weight)	46.3 ± 8.1	32.8 ± 5.6	215.4 ± 21.1*	194.3 ± 26.9*
Lavage IL-6 (pg/mL)	237.8 ± 72.9	635.1 ± 120.2	5034.9 ± 653.4*	6775.5 ± 1476.1*,#

Table 2.3 ***Experiment 2.*** Total protein levels and IL-6 concentrations were measured in lung lavage at the end of MV. Data are expressed as mean ± SEM; n=6 per group. *p<0.05 versus the respective Air control, #p<0.05 versus Acid no Treatment.

Surfactant sub-fractions and the surface activity of isolated LA are shown in Figures 2.6A and B respectively. Acid instillation did not change TS, LA and SA pool sizes compared to their respective Air control groups (Fig. 2.6A). This was similar for both not treated and surfactant treated groups. As expected and observed in experiment 1, total surfactant and LA values were significantly higher in surfactant treated groups than non-surfactant treated controls (Air bLES vs Air No Treatment; Acid bLES vs Acid No Treatment). There was no difference in SA values among the various experimental groups.

There were no statistically significant differences noted between any of the experimental groups in the biophysical activity of the LA samples (Fig. 2.6B). Within some of the groups, however, significant differences in surface tension were measured between the different dynamic cycles. In particular, surface tension was significantly higher during compression-expansion of cycles 5 and 10 when compared to cycle 1 within the acid instilled groups (in both Acid No Treatment and Acid bLES). LA from the Air No Treatment and Air bLES groups maintained low surface tension values throughout the 10 dynamic compression-expansion cycles.

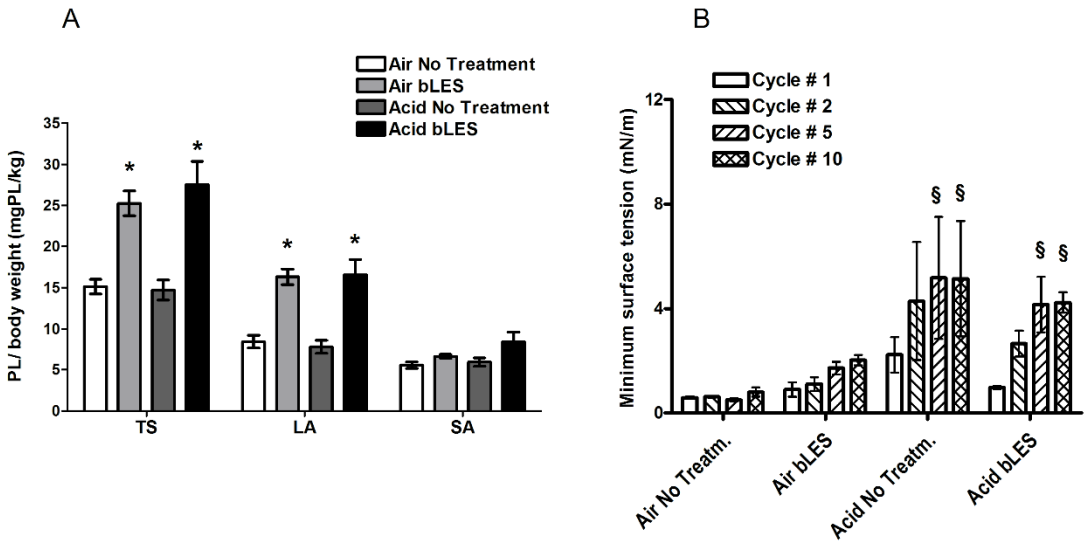

Figure 2.6 ***Experiment 2.*** Surfactant recovery in lung lavage and surface activity of crude LA. **A**: surfactant pool size of TS, LA and SA sub-fractions measured by phosphorous assay. Data are expressed as amount of phospholipids/kg body weight. Within each sub-fraction,*$p<0.05$ versus the respective No Treatment condition. **B**: surface tension of pooled LA samples during different dynamic compression-expansion cycles. §$p<0.05$ versus cycle 1 within each experimental condition. Values are expressed as mean ± SEM; n=6 per group.

Perfusate Analysis

To test the hypothesis of a role for exogenous surfactant in down-modulating systemic inflammation in ALI, sequential lung perfusate samples, as a surrogate for systemic inflammation, were analyzed for IL-6 concentrations. As shown in Figure 2.7, there were significantly higher levels of IL-6 in the perfusate of acid-instilled mice compared to the respective air-instilled controls at every time point (0, 30, 60, 90, 120 min; Acid No Treatment vs Air No Treatment; Acid bLES vs Air bLES). Perfusate IL-6 levels were not significantly affected by exogenous surfactant administration, with no differences between Air bLES and Air No Treatment and no change between Acid bLES and Acid No treatment.

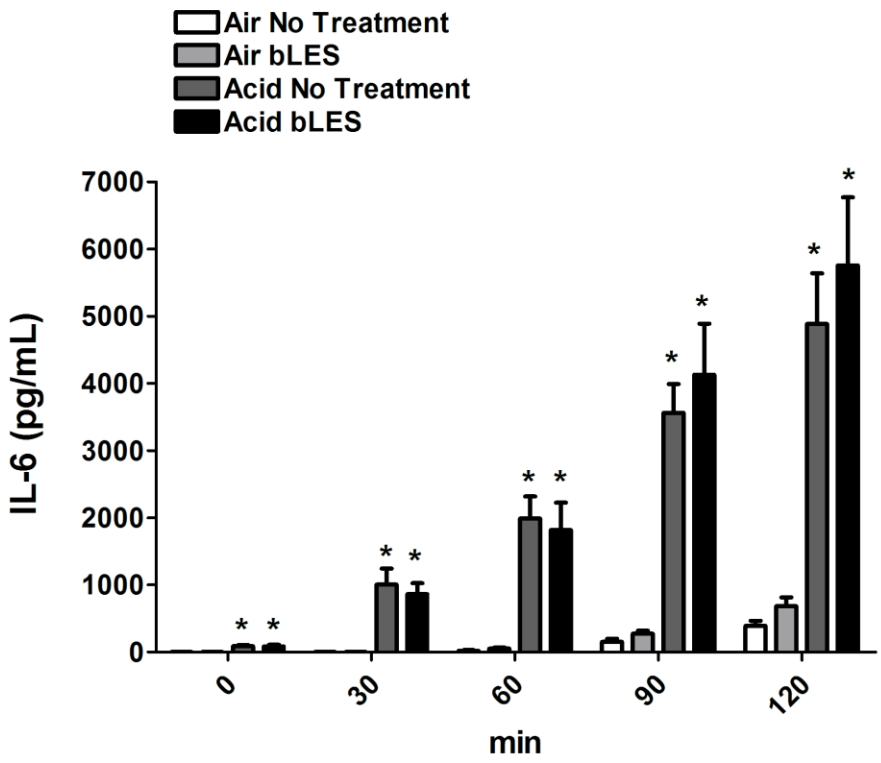

Figure 2.7 ***Experiment 2.*** IL-6 levels measured in lung perfusate at 0, 30, 60, 90, 120 min. Data are expressed as mean ± SEM. *$p<0.05$ versus respective Air control at each time point; n=6 per group.

Lung perfusate samples collected at 120 min were further analyzed for a wider array of cytokines/chemokines. Among the 12 mediators measured (Table 2.4), IL-13 levels were not detectable (data not shown), while there were significantly greater levels of eotaxin, IL-6, KC, MIP-2 in acid-instilled animals compared to the respective air instilled control. Overall, exogenous surfactant administration did not affect eotaxin, GM-CSF, IL-6, IL-1β, KC, TNF-α and IP-10 levels, with no statistical difference between the bLES and No Treatment group in both Air and Acid instilled mice.

A statistically significant increase of MIP-2 levels in the perfusate of Acid bLES mice was determined compared to Acid No Treatment, as well as significantly higher perfusate levels of G-CSF, LIX and MCP-1 in acid injured mice treated with surfactant compared to the Air bLES.

	Air		Acid	
Mediator (pg/ml)	**No treatment**	**bLES**	**No treatment**	**bLES**
Eotaxin	17.4±1.3	25.7±0.9	135.2±13.6*	142.8±15.9*
G-CSF	45.9±10.5	59.4±3.4	890.8±75.8	1270.3±187.8*
GM-CSF	0	0	13.4±2.8	16.3±2.2
IL-6	567.7±62.7	1119.0±99.1	8303.2±323.6*	10720.1±764.2*
IL-1 β	1.0±0.40	0.2±0.1	0.3±0.1	3.2±0.6
KC	579.2±65.8	907.0±75.4	3617.2±174.3*	6212.7±504.8*
LIX	113.7±11.1	98.3±13.0	284.6±17.9	447.4±31.9*
MCP-1	12.9±1.8	30.9±4.9	337.4±26.2	558.0±71.5*
MIP-2	584.4±68.2	725.7±41.9	1840.2±76.7*	3113.4±204.8*,#
TNF-α	63.4±11.6	72.2±7.0	119.7±3.1	143.3±8.1
IP-10	32.9±3.6	40.8±1.7	391.3±80.8	283.3±27.7

Table 2.4 ***Experiment 2*****.** Cytokine and chemokine measured in lung perfusate at the end of MV. Data are expressed as mean ± SEM; n=6 per group. *p<0.05 versus respective Air control, #p<0.05 versus Acid No Treatment.

G-CSF=granulocyte colony-stimulating factor, GM-CSF= granulocyte-macrophage CSF, IL-6=interleukin-6, IP-10=interferon-γ-induced protein 10, KC=keratinocyte chemoattractant, LIX=lipopolysaccharide-induced CXC chemokine, MCP-1=monocyte chemotactic protein-1, MIP-2=macrophage inflammatory protein 2 and TNF-α=tumor necrosis factor-alpha.

Finally, in order to further characterize the effect of exogenous surfactant administration on lung-derived mediators in perfusate, eicosanoids levels were also measured at the 120 min. time point (Table 2.5). Although increased levels of thromboxane B_2 and prostaglandin E_2 were recorded in the perfusate of acid-instilled animals compared to their respective Air controls, these changes did not reach statistical significance. Perfusate concentrations of 8-isoprostane were significantly higher in the acid injured groups compared to air controls. Surfactant treatment did not affect thromboxane B_2 and 8-isoprostane concentrations. Prostaglandin E_2 levels were significantly elevated only in the perfusate of Acid bLES mice compared to Air bLES controls. Leukotriene B_4 levels were increased in the perfusate of Acid bLES mice but this difference failed to be statistically significant.

	Air		**Acid**	
Mediator (pg/ml)	**No treatment**	**bLES**	**No treatment**	**bLES**
Prostaglandin E_2	14.3±2.0	26.8±3.9	147.4±40.3	221.7±89.2*
Leukotriene B_4	14.4±5.2	10.6±5.3	13.1±7.3	41.9±12.3
Thromboxane B_2	52.7±10.1	67.8±12.9	109.9±24.8	134.9±43.6
8-Isoprostane	11.3±1.6	19.7±3.0	47.2±8.1*	70.4±12.5*

Table 2.5 ***Experiment 2.*** Concentrations of prostaglandin E2, leukotriene B4, thromboxane B2 and 8-isoprostane measured in lung perfusate samples collected at the end of MV. Data are expressed as mean ± SEM; n=6 per group. *$p<0.05$ versus the respective Air control.

2.4. Discussion

The overall objective of this study was to evaluate the anti-inflammatory effects of exogenous surfactant when administered prior to mechanical ventilation, either in the absence (experiment 1) or in the presence (experiment 2) of an initiating pulmonary insult. For both lung injury models, the IPML setup was utilized to specifically evaluate the contribution of ventilation to the development of systemic inflammation. MV of normal lungs resulted in the release of IL-6 (locally) into the airspace and several mediators (systemically) in the perfusate. Surfactant administration, however, was not effective in reducing the systemic inflammation associated with MV. Conventional ventilation of HCl instilled mice led to higher levels of both IL-6 and total protein in lavage, and significantly higher levels of pro-inflammatory mediators in perfusate without any beneficial effect of bLES instillation. Notably, significantly higher lavage IL-6 and perfusate MIP-2 concentrations were observed in acid-injured mice receiving bLES, compared to Acid-No Treatment controls. Based on these results, it was concluded that administration of exogenous surfactant prior to MV does not reduce the systemic inflammation associated with lung injury in these models.

An important feature of the current study was to examine the effects of surfactant therapy in two different models. Analysis of the data showed important differences between the models, such as the degree of lung edema. Mechanical stretch of uninjured lungs did not affect lung permeability, whereas acid injured mice had increased total lavage proteins after two hours of MV. Another aspect that distinguishes the two models is represented by different levels of pulmonary and perfusate inflammation, which becomes particularly evident when comparing cytokine levels measured in the perfusate of the MV only, No Treatment group to the cytokine levels of the Acid No Treatment group. For example, MV of normal lungs caused a moderate increase in circulating Eotaxin, IL-6, KC and MIP-2, while acid instilled animals subjected to conventional MV had perfusate concentrations of these mediators that were at least two times greater. Given the greater inflammation characterizing the acid-injury model and the important role of lipid mediators in the development and progression of lung injury [27-32], eicosanoids levels were analyzed only on samples from experiment 2. Unambiguous conclusions about the

effects of exogenous surfactant on systemic inflammation were therefore inferred from two experimental models with very different characteristics. This allowed us to rule out possible causes for the lack of efficacy of our treatment (such as presence/lack of pre-existing injury, specific effects of ventilation), and strengthened the understanding of the biological response.

Exogenous surfactant administration has been extensively investigated as a potential adjunctive therapy in acute lung injury [33-36]. The traditional approach with surfactant treatment has been to evaluate its efficacy in terms of physiological and biophysical improvements. Many experimental studies have in fact demonstrated that exogenous surfactant instilled *after* the onset of ventilation improved oxygenation, lung volume and compliance; moreover, it improved the surface tension reducing properties of the surfactant recovered from lung lavage subsequent to administration [15, 37, 38]. Nevertheless, despite this encouraging experimental evidence, clinical trials showed no improvement in mortality in surfactant treated patients even in the presence of an initial improvement in oxygenation [16, 18]. It is possible that surfactant treatment in the previous studies was administered too late into ALI development; therefore earlier surfactant administration *prior to* or *at the onset* of MV could be more effective at mitigating disease progression. Since mortality can be improved by ameliorating ventilation – induced systemic inflammation [39], it was our interest to investigate whether exogenous surfactant could mitigate the effects of MV thereby down-modulating inflammation.

To our knowledge, the effect of surfactant on ventilation-induced release of inflammatory mediators in perfusate of an IPML model has been specifically addressed in two previous studies. Stamme and colleagues [40] showed elevated TNFα and IL-6 concentrations in the perfusate of surfactant treated animals compared to controls, in their mouse model of high pressure ventilation. In contrast, our group has shown a reduced level of inflammatory cytokines in perfusate due to elevated endogenous surfactant in an LPS model of injury [20]. Together with the current study in which surfactant did not impact inflammation in two models of injury, these data illustrate the complexity of surfactant treatment in which specific details of the experimental model may impact outcome.

Furthermore, such details are obviously important to understand in the context of a potential clinical utilization of surfactant treatment to down-regulate systemic inflammation as well as to understand the mechanisms by which surfactant may affect inflammation.

Despite the lack of effect of surfactant treatment in our study, we speculate that mitigation of MV induced inflammation is still the best approach for an early intervention. Our data support earlier studies which showed that cytokines can be detected in perfusate rapidly after the onset of ventilation [22, 41]. This loss of alveolar and systemic cytokine compartmentalization can lead to peripheral organ dysfunction, a condition of difficult clinical management. Therefore, targeting the lung with anti-inflammatory agents prior to MV may be a successful treatment option leading to improved outcomes. In this respect, surfactant could be utilized as a carrier for delivering lung specific anti-inflammatory agents prior to MV in future studies.

Along with the strengths of the present study, some limitations need to be addressed. Due to the lack of blood perfusion in the IPML setup, the lungs were not exposed during *ex vivo* MV to circulating soluble factors and immune cells which could have affected the progression of the injury. Moreover, *ex vivo* ventilation of perfused lungs did not favor the use of severe lung injury models, due to potential technical failure of the preparation. Consequently, the injury from ventilation was mild to moderate, thereby explaining the lack of change in surface tension or surfactant pool sizes. It is believed, however, that these limitations of the IPML setup were counter balanced by the advantage of specifically isolating lung-derived mediators released into the circulation, without the confounding contribution of systemic factors to the development of inflammation. Intra-tracheal instillation was also used for administering surfactant, ensuring the presence of large amounts of active material in the airspace before ventilation, as shown by higher levels of TS and LA in the lung lavage of treated animals. It should be acknowledged, however, that some inadequate distribution of surfactant might have occurred following instillation. Obstruction of smaller airways, with consequent heterogeneous lung inflation and regional over-distension might have been responsible for the increase in PIP (experiment 2, Air treated groups), and for the non-significant trend towards higher

lavage IL-6 levels in the surfactant treated groups. Nevertheless, the instilled surfactant retained excellent biophysical properties as shown by the very low minimum surface tension achieved during dynamic compression-expansion of the crude LA. Overall, we believe that instillation did not account for the lack of efficacy of our treatment.

In conclusion, this study expands the knowledge about exogenous surfactant treatment. It specifically focuses on the anti-inflammatory effects of a lung targeted therapy administered prior to MV on the development of systemic inflammation using two different mouse models. Although our data suggest a lack of efficacy for exogenous surfactant in down-modulating inflammation, future studies might focus on surfactant as a carrier for anti-inflammatory drugs or antibiotics in order to better interfere with ALI progression.

2.5. References

1. Goerke J: **Pulmonary surfactant: functions and molecular composition.** *Biochim Biophys Acta* 1998, **1408**:79-89.

2. Wright JR: **Immunomodulatory functions of surfactant.** *Physiol Rev* 1997, **77**(4):931-962.

3. Lewis JF, Jobe AH: **Surfactant and the adult respiratory distress syndrome.** *Am Rev Respir Dis* 1993, **147**(1):218-233.

4. Veldhuizen RA, McCaig LA, Akino T, Lewis JF: **Pulmonary surfactant subfractions in patients with the acute respiratory distress syndrome.** *Am J Respir Crit Care Med* 1995, **152**(6 Pt 1):1867-1871.

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

6. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD: **Incidence and outcomes of acute lung injury.** *N Engl J Med* 2005, **353**(16):1685-1693.

7. Stapleton RD, Wang BM, Hudson LD, Rubenfeld GD, Caldwell ES, Steinberg KP: **Causes and timing of death in patients with ARDS.** *Chest* 2005, **128**(2):525-532.

8. Montgomery AB, Stager MA, Carrico CJ, Hudson LD: **Causes of mortality in patients with the adult respiratory distress syndrome.** *Am Rev Respir Dis* 1985, **132**(3):485-489.

9. Walker MG, Yao LJ, Patterson EK, Joseph MG, Cepinskas G, Veldhuizen RA, Lewis JF, Yamashita CM: **The effect of tidal volume on systemic inflammation in Acid-induced lung injury.** *Respiration* 2011, **81**(4):333-342.

10. Gurkan OU, O'Donnell C, Brower R, Ruckdeschel E, Becker PM: **Differential effects of mechanical ventilatory strategy on lung injury and systemic organ inflammation in mice.** *Am J Physiol Lung Cell Mol Physiol* 2003, **285**(3):L710-8.

11. Dhanireddy S, Altemeier WA, Matute-Bello G, O'Mahony DS, Glenny RW, Martin TR, Liles WC: **Mechanical ventilation induces inflammation, lung injury, and extra-pulmonary organ dysfunction in experimental pneumonia.** *Lab Invest* 2006, **86**(8):790-799.

12. O'Mahony DS, Liles WC, Altemeier WA, Dhanireddy S, Frevert CW, Liggitt D, Martin TR, Matute-Bello G: **Mechanical ventilation interacts with endotoxemia to induce extrapulmonary organ dysfunction.** *Crit Care* 2006, **10**(5):R136.

13. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, Slutsky AS: **Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial.** *JAMA* 1999, **282**(1):54-61.

14. Aspros AJ, Coto CG, Lewis JF, Veldhuizen RA: **High-frequency oscillation and surfactant treatment in an acid aspiration model.** *Can J Physiol Pharmacol* 2010, **88**(1):14-20.

15. Vazquez de Anda GF, Lachmann RA, Gommers D, Verbrugge SJ, Haitsma J, Lachmann B: **Treatment of ventilation-induced lung injury with exogenous surfactant.** *Intensive Care Med* 2001, **27**(3):559-565.

16. Spragg RG, Lewis JF, Walmrath HD, Johannigman J, Bellingan G, Laterre PF, Witte MC, Richards GA, Rippin G, Rathgeb F, Hafner D, Taut FJ, Seeger W: **Effect of recombinant surfactant protein C-based surfactant on the acute respiratory distress syndrome.** *N Engl J Med* 2004, **351**(9):884-892.

17. Spragg RG, Taut FJ, Lewis JF, Schenk P, Ruppert C, Dean N, Krell K, Karabinis A, Gunther A: **Recombinant surfactant protein C-based surfactant for patients with severe direct lung injury.** *Am J Respir Crit Care Med* 2011, **183**(8):1055-1061.

18. Davidson WJ, Dorscheid D, Spragg R, Schulzer M, Mak E, Ayas NT: **Exogenous pulmonary surfactant for the treatment of adult patients with acute respiratory distress syndrome: results of a meta-analysis.** *Crit Care* 2006, **10**(2):R41.

19. Yamashita C, Forbes A, Tessolini JM, Yao LJ, Lewis JF, Veldhuizen RA: **Protective effects of elevated endogenous surfactant pools to injurious mechanical ventilation.** *Am J Physiol Lung Cell Mol Physiol* 2008, **294**(4):L724-L732.

20. Walker MG, Tessolini JM, McCaig L, Yao LJ, Lewis JF, Veldhuizen RA: **Elevated endogenous surfactant reduces inflammation in an acute lung injury model.** *Exp Lung Res* 2009, **35**(7):591-604.

21. Yu S, Harding PG, Smith N, Possmayer F: **Bovine pulmonary surfactant: chemical composition and physical properties.** *Lipids* 1983, **18**(8):522-529.

22. von Bethmann AN, Brasch F, Nusing R, Vogt K, Volk HD, Muller KM, Wendel A, Uhlig S: **Hyperventilation induces release of cytokines from perfused mouse lung.** *Am J Respir Crit Care Med* 1998, **157**(1):263-272.

23. Bligh EG, Dyer WJ: **A rapid method of total lipid extraction and purification.** *Can J Biochem Physiol* 1959, **37**(8):911-917.

24. Duck-Chong CG: **A rapid sensitive method for determining phospholipid phosphorous involving digestion with magnesium nitrate.** *Lipids* 1979, **14**(5):492-497.

25. Vockeroth D, Gunasekara L, Amrein M, Possmayer F, Lewis JF, Veldhuizen RA: **Role of cholesterol in the biophysical dysfunction of surfactant in ventilator-induced lung injury.** *Am J Physiol Lung Cell Mol Physiol* 2010, **298**(1):L117-L125.

26. Gunasekara L, Schoel WM, Schurch S, Amrein MW: **A comparative study of mechanisms of surfactant inhibition.** *Biochim Biophys Acta* 2008, **1778**(2):433-444.

27. Ngiam N, Peltekova V, Engelberts D, Otulakowski G, Post M, Kavanagh BP: **Early growth response-1 worsens ventilator-induced lung injury by up-regulating prostanoid synthesis.** *Am J Respir Crit Care Med* 2010, **181**(9):947-956.

28. Jaecklin T, Engelberts D, Otulakowski G, O'Brodovich H, Post M, Kavanagh BP: **Lung-derived soluble mediators are pathogenic in ventilator-induced lung injury.** *Am J Physiol Lung Cell Mol Physiol* 2011, **300**(4):L648-L658.

29. Zarbock A, Singbartl K, Ley K: **Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation.** *J Clin Invest* 2006, **116**(12):3211-3219.

30. Stephenson AH, Lonigro AJ, Hyers TM, Webster RO, Fowler AA: **Increased concentrations of leukotrienes in bronchoalveolar lavage fluid of patients with ARDS or at risk for ARDS.** *Am Rev Respir Dis* 1988, **138**(3):714-719.

31. Auner B, Geiger EV, Henrich D, Lehnert M, Marzi I, Relja B: **Circulating leukotriene B4 identifies respiratory complications after trauma.** *Mediators Inflamm* 2012, **2012**:536156.

32. Eun JC, Moore EE, Banerjee A, Kelher MR, Khan SY, Elzi DJ, McLaughlin NJ, Silliman CC: **Leukotriene b4 and its metabolites prime the neutrophil oxidase and induce proinflammatory activation of human pulmonary microvascular endothelial cells.** *Shock* 2011, **35**(3):240-244.

33. Lewis JF, Veldhuizen R: **The role of exogenous surfactant in the treatment of acute lung injury.** *Annu Rev Physiol* 2003, **65**:613-642.

34. Ito Y, Manwell SE, Kerr CL, Veldhuizen RA, Yao LJ, Bjarneson D, McCaig LA, Bartlett AJ, Lewis JF: **Effects of ventilation strategies on the efficacy of exogenous surfactant therapy in a rabbit model of acute lung injury.** *Am J Respir Crit Care Med* 1998, **157**(1):149-155.

35. Brackenbury AM, Puligandla PS, McCaig LA, Nikore V, Yao LJ, Veldhuizen RA, Lewis JF: **Evaluation of exogenous surfactant in HCL-induced lung injury.** *Am J Respir Crit Care Med* 2001, **163**(5):1135-1142.

36. Lewis JF, Goffin J, Yue P, McCaig LA, Bjarneson D, Veldhuizen RA: **Evaluation of exogenous surfactant treatment strategies in an adult model of acute lung injury.** *J Appl Physiol* 1996, **80**(4):1156-1164.

37. Welk B, Malloy JL, Joseph M, Yao LJ, Veldhuizen AW: **Surfactant treatment for ventilation-induced lung injury in rats: effects on lung compliance and cytokines.** *Exp Lung Res* 2001, **27**(6):505-520.

38. Rasaiah VP, Malloy JL, Lewis JF, Veldhuizen RA: **Early surfactant administration protects against lung dysfunction in a mouse model of ARDS.** *Am J Physiol Lung Cell Mol Physiol* 2003, **284**(5):L783-L790.

39. The Acute Respiratory Distress Syndrome Network: **Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome.** *N Engl J Med* 2000, **342**(18):1301-1308.

40. Stamme C, Brasch F, von Bethmann A, Uhlig S: **Effect of surfactant on ventilation-induced mediator release in isolated perfused mouse lungs.** *Pulm Pharmacol Ther* 2002, **15**(5):455-461.

41. Truscott EA, McCaig LA, Yao LJ, Veldhuizen RA, Lewis JF: **Surfactant protein-A reduces translocation of mediators from the lung into the circulation.** *Exp Lung Res* 2010, **36**(7):431-439.

CHAPTER 3:

Lack of matrix metalloproteinase-3 in mouse models of lung injury ameliorates the pulmonary inflammatory response in female but not in male mice.

Valeria Puntorieri, Lynda A. McCaig, Li-Juan Yao, James F. Lewis, Cory M. Yamashita, and Ruud A.W. Veldhuizen.

3.1. Introduction

Acute lung injury (ALI) is a pulmonary inflammatory disorder with a mortality of approximately 40% [1, 2]. Clinically, ALI is defined by the presence of bilateral infiltrates on chest radiograph, alveolar edema in the absence of cardiac failure, and hypoxemia [3, 4]. Despite a relatively simplistic clinical definition, ALI pathophysiology is complex, with a variable disease progression, and many different insults, such as bacterial pneumonia or gastric acid aspiration, capable of initiating lung injury [5, 6]. Within this complex disease process, pulmonary inflammation represents a key aspect of ALI pathophysiology that is common to all patients regardless of the initiating insult [7, 8], and which is strongly related to outcomes of ALI [9]. Numerous experimental studies have shown that strategies capable of modulating the lung inflammatory response greatly affect disease progression to systemic inflammation and consequent multi-organ failure, the main cause of death in ALI [10–15].

Clinical evidence for the importance of lung inflammation in ALI stems from clinical trials over the past decade, which consistently demonstrated that lower indices of lung inflammatory mediators were associated with diminished systemic inflammation, and subsequent lower mortality [16, 17]. In these trials, the decrease in pulmonary inflammation resulted from the use of non-injurious strategies of mechanical ventilation, the main supportive therapy in ALI [16, 17]. Since then, however, no other lung-targeted pharmacological or anti-inflammatory treatment has been identified that is capable of reducing mortality [18, 19], thereby stressing the importance of further research into key modulators of pulmonary inflammation in ALI.

One potential key mediator of pulmonary inflammation is matrix metalloproteinase-3 (MMP-3), a protease expressed in the lung by alveolar macrophages (AM), alveolar epithelial cells, and fibroblasts [20–22]. MMP-3 is a member of the matrix metalloproteinases (MMPs) family, which consists of over 20 extracellular, zinc-dependent proteolytic enzymes involved in several physiological processes such as matrix turnover, tissue repair, and inflammation [23–25]. Interestingly, metalloproteinase activity and expression levels are often elevated in injured tissues and in inflammatory conditions such as asthma, sepsis, and ALI [26–30]. Specifically, recent clinical studies have

confirmed elevated MMPs levels in lavage samples from patients with ALI; importantly, detectable lavage levels of a specific member of this family, namely MMP-3, were associated with severity of ALI, distal organ failure, and mortality [27, 31]. In addition to this clinical evidence, two experimental studies suggest a potential role for MMP-3 in ALI [32, 33]. These studies showed that, compared to wild type animals, mice lacking Mmp3 expression had lower neutrophils and total protein accumulation within the lung following intra-tracheal instillation of the chemokine MIP-2 and in a model of immunoglobulin G immune-complex-induced lung injury [32, 33]. Although supportive of a role for MMP-3 in ALI, these studies have utilized neutrophil-dependent models of ALI that lack clinical relevance, since they do not resemble any of the most common initiating insults in the pathogenesis of this disorder. It is, therefore, necessary to investigate the role of MMP-3 in clinically relevant models of ALI, with a specific focus on pulmonary inflammation, as it appears to be a key driving factor in disease progression.

It was hypothesized that MMP-3 is a key mediator in the pathogenesis of ALI by affecting pulmonary inflammation. To test this hypothesis, male and female wild type mice ($Mmp3^{+/+}$) and mice lacking Mmp3 expression ($Mmp3^{-/-}$) were exposed to two clinically relevant models of lung injury: i) a model of lipopolysaccharide-induced lung injury, resembling bacterial pneumonia, and ii) a model of acid-induced ALI, which mirrors lung injury due to gastric acid aspiration. Each model reproduces major pathophysiological events in ALI, such as the intra-pulmonary accumulation of proteinaceous edema fluid, the increased pulmonary release of cytokines and chemokines by resident alveolar macrophages and lung epithelial cells, and the recruitment of inflammatory neutrophils to the injured lung [5].

Interestingly, initial analyses showed a more prominent role of MMP-3 in female mice; therefore, data from female mice and from male mice have been analyzed separately, as distinct data sets. A sex-dependent role of MMP-3 in lung inflammation was observed in both ALI models, which led to initial *in vitro* investigations into mechanisms and inflammatory cell population (AM) potentially contributing to these findings. Experiments were performed using bone marrow-derived macrophages (BMDMs) as a surrogate for AM. Questions on possible inherent differences in the inflammatory response of

male/female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ BMDMs, and on the potential effect of 17β-estradiol (E_2), were preliminarily addressed in this work.

3.2. Methods

3.2.1. Animal procedures and experimental design

Mice lacking the expression of Mmp3 ($Mmp3^{-/-}$) were originally generated as described by Mudgett JS et al. [34].; breeding pairs of $Mmp3^{-/-}$ mice were kindly provided by Dr. G.P. Downey (National Jewish Hospital, Denver, Colorado) to initiate and re-derive a pathogen free colony of $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice. All the animals were group housed in a research animal facility, exposed daily to a 12 hours light/dark cycle and allowed unrestricted access to standard chow food and water.

A total of 109 female and male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice were utilized for three separate experiments – a model of lipopolysaccharide-induced lung injury (*experiment 1*), a model of acid-induced lung injury (*experiment 2*), and BMDMs isolation (*experiment 3*) – and all animal procedures were approved by the Animal Use Subcommittee at Western University (protocol number: 2010-272).

3.2.2. Experiment 1: Lipopolysaccharide induced lung injury

A total of 47 $Mmp3^{+/+}$ and $Mmp3^{-/-}$, female and male mice (13.9 ± 0.4 weeks old; average ± sem) were randomized to receive an intra-tracheal (i.t.) instillation of lipopolysaccharide (LPS from E.Coli, 0111:B4, 20 µg/mouse; Sigma, St. Louis, Mo., USA) or saline (0.15M NaCl solution) as a control for this model. Considering that the data were analyzed by sex, for both female and male mice the following 4 experimental groups were obtained: i) $Mmp3^{+/+}$ Saline, ii) $Mmp3^{+/+}$ LPS, iii) $Mmp3^{-/-}$ Saline, iv) $Mmp3^{-/-}$ LPS.

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg; Sandoz, Quebec, Que., Canada) and medetomidine (1 mg/kg; Orion Corporation, Espoo, Finland). Once adequate sedation was achieved, mice were placed dorsally on a vertical, slightly

inclined stand, and the oral cavity was opened to visualize the vocal cords. Subsequently, a 20-gauge catheter combined with a fiber-optic stylet (BioLite intubation system for small rodents, BioTex, Inc., Houston, Tex., USA) was inserted through the vocal cords into the trachea. Mice randomized to the LPS group received 40 µL of a 0.5 mg/mL LPS solution in sterile saline (0.15M NaCl), instilled drop-wise through the endotracheal tube. Mice randomized to the Saline group were intubated and received 40 µL of a sterile 0.15M NaCl solution. The whole procedure required approximately 15 minutes. Mice were then extubated, injected subcutaneously with 0.5 mL sterile saline and immediately returned to their cage. Preliminary studies performed during development of the model determined that mice did not require any analgesia following instillation. Within 30 minutes from the injection of the anesthetic mix, mice received an intra-peritoneal injection of atipamezole (1 mg/kg; Zoetis, Florham Park, NJ, USA), the reversal agent for medetomidine. Mice were left to recover for a total of 18 hours with free access to water and food. At the end of the 18 hours, mice were sacrificed with an intraperitoneal injection of sodium pentobarbital (110 mg/kg; Lundbeck, Valby, Denmark) followed by exsanguination, and processed for various analyses as described in sections 3.2.4 to 3.2.7.

3.2.3. Experiment 2: Acid-induced lung injury

A total of 44 $Mmp3^{+/+}$ and $Mmp3^{-/-}$, female and male mice (12.7 ± 0.5 weeks old) were randomized to receive an intra-tracheal instillation of hydrochloric acid (HCl) or air as a control. Similar to Experiment 1, the data from each sex were analyzed as different data sets. For both female and male mice, the following 4 experimental groups were analyzed: i) $Mmp3^{+/+}$ Air, ii) $Mmp3^{+/+}$ Acid, iii) $Mmp3^{-/-}$ Air, iv) $Mmp3^{-/-}$ Acid.

An intraperitoneal injection of ketamine (100 mg/kg; Sandoz, Quebec, Que., Canada) and xylazine (5 mg/kg; Bayer, Toronto, Ont., Canada) was given to anesthetize mice and subsequently proceed with the endotracheal intubation as described above (under ‘lipopolysaccharide-induced lung injury’). Mice randomized to acid instillation received 50 µL of 0.05N HCl, instilled intra-tracheally in a drop-wise fashion. Mice randomized to the air group were intubated as described and allowed to spontaneously breathe through the tube. Overall, the procedure lasted about 15 minutes. Mice were then extubated, put on an inclined stand and injected subcutaneously with buprenorphine (0.05-0.1 mg/kg

Animalgesic Labs Inc., Millersville, MD, USA) and 0.3 mL sterile saline. Mice were then returned to their cage and 30 minutes after the injection of anesthetic, they were injected intra-peritoneally with atipamezole (1 mg/kg), reversal agent for xylazine.

Mice were allowed to recover for 4 hours, with free access to food and water, and sacrificed at the end of the recovery period with an intraperitoneal injection of a ketamine (200 mg/kg)-xylazine (10 mg/kg) solution followed by exsanguination.

3.2.4. Lung lavage isolation and total protein analysis

At the end of their respective recovery periods (18 hours for LPS-induced lung injury, 4 hours for acid-induced lung injury), mice were given a euthanizing dose of anesthetic as previously described, and placed in a supine position. When deep anesthesia was attained, the abdominal content and thorax were exposed through an abdominal midline incision, and exsanguination was performed through excision of the inferior vena cava. A tracheostomy tube was subsequently inserted and secured in the trachea, the diaphragm cut and the chest wall opened via a midline incision to expose the lungs. Lungs were then lavaged with 3 × 1 mL aliquots of saline, and each aliquot was instilled and withdrawn 3 times. The total volume of lavage fluid collected from each mouse was recorded. Lung lavage was immediately centrifuged at 380 g for 10 minutes at 4˚C to isolate the cell pellet; the supernatant was collected and 4 × 0.25 mL aliquots were frozen at -80˚C for analyses of inflammatory mediators.

Measurements of total protein content in lavage were also performed on this supernatant using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA), as per manufacturer's instructions.

3.2.5. Lavage cell analysis

Following centrifugation of lavage samples, the cellular component was isolated and resuspended in Plasmalyte (300 to 500 µL depending on cell density). An aliquot of cell suspension was mixed with an equal volume of trypan blue to assess viability through trypan blue exclusion, and subsequently utilized to determine total cell counts with a hemocytometer and light microscopy. Aliquots of the resuspended cell pellet were also

spun down on cytospin slides at 1000 rpm for 6 minutes at room temperature, stained with Hemacolor® stain (Harleco, EMD Chemicals Inc., Gibbstown, NJ, USA) and utilized to perform differential cell counts under light microscopy. Specifically, 5 fields were counted and averaged for each slide, and the relative percentage of each inflammatory cell type was calculated. The absolute number of neutrophils in lavage was then obtained by multiplying percentages by the total cell number previously determined.

3.2.6. Measurements of MMP-3 and inflammatory mediators in lung lavage

Total MMP-3 levels were measured in lavage aliquots from experiment 1 and 2, using a commercially available enzyme-linked immune-sorbent assay (ELISA) according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Lavage interleukin-6 (IL-6) levels were also assessed through the use of a mouse IL-6 ELISA (BD Biosciences, San Diego, Ca., USA) according to manufacturer's instructions.

An array of inflammatory cytokines and chemokines was measured in lung lavage samples from both injury models. For both experiment 1 and 2, mouse Milliplex multi-analyte panels (Map) (MCYTOMAG-70K, Millipore Corporation, Billerica, MA, USA) were utilized to measure lavage levels of the following cytokine/chemokine: granulocyte colony stimulating factor (G-CSF), interleukin 10 (IL-10), keratinocyte chemoattractant (KC), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), and tumor necrosis factor alpha (TNF-α). Samples analysis was performed on the Luminex® xMAP® detection system on the Luminex^{100} (Linco Research, St. Charles, Mo., USA), as per manufacturer's protocol.

3.2.7. Experiment 3: Isolation of Mmp3$^{+/+}$ and Mmp3$^{-/-}$ bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were isolated from the femurs and tibias of mice as previously described [35]. Briefly, a total of 18 Mmp3$^{+/+}$ and Mmp3$^{-/-}$ mice (19.9 ± 1.7 weeks old) were sacrificed with an intraperitoneal injection of sodium pentobarbital (110 mg/kg) and exsanguinated. The femur and tibia of both hind legs were isolated, the soft tissue was removed, and the ends of each bone were cut off before placing each pair

of long bones in a 0.65 mL Eppendorf tube with a punctured bottom. Each one of these tubes was then placed into a 1.5 mL Eppendorf, thereby allowing for the collection of the bone marrow cell pellet upon centrifugation of the bones at 2000 g for 1 minute at 4˚C. After lysis of red blood cells and subsequent wash with cold, sterile 1x PBS, bone marrow samples from each mouse were pulled together and centrifuged at 400 g, for 10 minutes, 4˚C, and the resulting cell pellet was then resuspended in macrophage differentiation media (Mac media: RPMI 1640, 10% fetal bovine serum, 2 mM glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin, and 30% L-cell supernatant as a source of monocyte-colony stimulating factor). Bone marrow-derived cells were seeded and cultured in a 100 mm Petri dish at 37˚C with 5% CO_2 for 24 hours; at the end of this time, the non-adherent and loosely adherent cells were transferred to a 150 mm tissue culture dish with fresh Mac media, and cultured as previously described. Following a 7 days culture period, cells were fully differentiated into BMDMs and ready for experimental use.

All reagents used for the culture of bone marrow derived macrophages were provided by Gibco® (Life Technologies, Grand Island, NY, USA), unless otherwise specified. L-cell supernatant was a generous gift of Dr. Gill (Western University, London ON, Canada).

3.2.8. *In vitro* stimulation of Mmp3$^{+/+}$ and Mmp3$^{-/-}$ bone marrow-derived macrophages

The effects of LPS or a combination of LPS and estrogen were assessed *in vitro* on BMDMs isolated as described above. Fully differentiated BMDMs from male and female Mmp3$^{+/+}$ and Mmp3$^{-/-}$ mice were harvested and 2×10^5 cells per well were seeded in a 24 well plate. The following day, macrophage differentiation media was substituted with stimulation media (RPMI 1640, 10% fetal bovine serum, 2 mM glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin) and BMDMs were exposed to one of the following conditions: i) control – media only, ii) 0.1µg/mL LPS (E.Coli, 0111:B4, Sigma, St. Louis, Mo., USA), iii) 1µg/mL LPS, iv) 10µg/mL LPS. BMDMs were stimulated at 37˚C, 5% CO_2 for 18 hours. At the end of the stimulation period, individual cell supernatants were collected, spun at 380 g for 10 minutes at 4˚C and frozen at -80 ˚C for subsequent analysis of IL-6 levels. BMDMs from the stimulated 24 well plate were lysed on ice in lysis buffer (0.5% Sodium dodecyl sulfate, 50 mM Tris pH 7.5, 1 mM EDTA) with protease inhibitor

(cOmplete, Mini, EDTA-free, Roche Diagnostic GmbH, Mannheim, Germany) for measurements of total protein content (Micro BCA protein assay kit, Pierce, Rockford, Ill., USA). IL-6 levels of each individual cell supernatant were then normalized by the corresponding total protein content, to account for differences in total cell numbers between wells. A total of three independent experiments were performed.

In a second set of experiments, differentiated BMDMs from female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice were harvested and 2×10^5 cells per well were seeded in a 24 well plate to be stimulated in the presence of 17-β estradiol (E_2, Sigma, St. Louis, Mo., USA). On the next day, stimulation media was utilized to expose BMDMs to one of the following conditions: i) control media (with vehicle), ii) 0.1nM E_2, iii) 1nM E_2, iv) 10nM E_2, v) 1µg/mL LPS, vi) 0.1nM E_2 + 1µg/mL LPS, vii) 1nM E_2 + 1µg/mL LPS, and viii) 10nM E_2 + 1µg/mL LPS. Following 18 hours, cell supernatants were collected for IL-6 analysis and total protein content was assessed on cell lysates, as previously described. A total of three independent experiments were performed.

3.2.9. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). GraphPad Prism statistical software (La Jolla, CA, USA) was utilized to perform statistical analyses. Data were analyzed using a two-way ANOVA (variables: genotype and treatment) followed by a one-way ANOVA with a Tukey's post-hoc test. When appropriate, an unpaired two tailed Student's t-test was utilized for analysis. $P<0.05$ was considered statistically significant.

3.3. Results

3.3.1. Experiment 1: Lipopolysaccharide-induced lung injury

Lavage MMP-3

The role of MMP-3 in ALI was first assessed utilizing an 18-hour model of LPS-induced lung injury, in which $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice of both sexes received an intra-tracheal administration of LPS or saline as a control.

Total MMP-3 was measured in lavage samples from $Mmp3^{+/+}$ mice. The results in figure 3.1A show significantly higher lavage MMP-3 concentrations in LPS-injured mice compared to Saline controls. Given the use of both female and male mice for this study, the data was subsequently analyzed by sex. Notably, female $Mmp3^{+/+}$ mice showed significantly elevated total MMP-3 following LPS injury compared to Saline (Fig. 3.1B); however MMP-3 concentrations in male, LPS-instilled $Mmp3^{+/+}$ mice were not significantly higher than the respective Saline control (Fig. 3.1C). In light of this difference in results from female and male mice, the remaining of the experimental data was analyzed separately by sex.

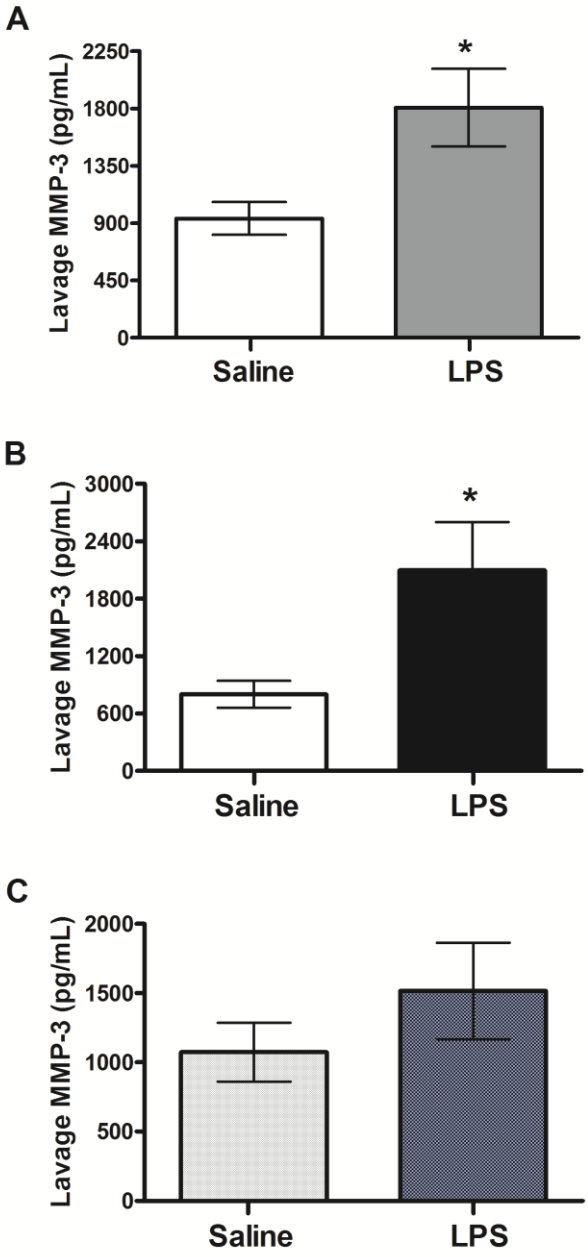

Figure 3.1 ***Experiment 1***: MMP-3 levels measured in lung lavage samples from $Mmp3^{+/+}$ mice, 18 hours after intra-tracheal saline or LPS instillation. **A**: Lavage MMP-3 levels assessed in all $Mmp3^{+/+}$ mice, n=11-12 per group; **B**: in female $Mmp3^{+/+}$ mice, n=5-6 per group; **C:** in male $Mmp3^{+/+}$ mice, n=6 per group. Data are expressed as mean ± SEM; *$p<0.05$ vs Saline control.

Lavage protein

The average lavage volume recovered from all animals was 2.9 ± 0.02 mL, with no significant differences among groups (data not shown). Total protein content in lavage is shown in Table 3.1. No differences due to genotype were observed for Saline-instilled mice of both sexes. Overall, no significant differences were observed in total lavage protein of LPS-instilled $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice compared to their respective Saline controls. Similar results were observed in both sexes, although female LPS-injured $Mmp3^{+/+}$ mice appeared to have higher lavage protein than Saline control, but this difference did not reach significance. The genotype had no significant effect on this outcome following LPS instillation.

Total lavage protein (mg/kg body weight)	$Mmp3^{+/+}$		$Mmp3^{-/-}$	
	Saline	LPS	Saline	LPS
Female mice	36.4±9.0	81.0±22.4	24.4±3.6	30.2±4.5
Male mice	24.9±9.1	28.2±7.4	19.3±3.9	25.7±3.0

Table 3.1 ***Experiment 1*****:** Total protein content assessed in lavage from $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice of either sex at the end of the 18 hours recovery period. Data are expressed as mean ± SEM; n=5-6 per group.

Lavage inflammatory cells

Pulmonary inflammation associated with this model of ALI was first investigated through the analysis of the lung lavage cellular infiltrate. Results in figure 3.2A and B showed no differences at baseline (Saline instillation) due to genotype in female mice. A higher but not significantly different total cell number was found in female, LPS-injured $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice compared to the respective Saline controls, with no effect of the genotype

(Fig. 3.2A). Similarly, determination of total PMN cells in lavage showed a non-significant trend toward higher neutrophils in female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice exposed to LPS, with no differences due to genotype (Fig. 3.2B).

In male mice, no differences due to genotype were found in the total number of cells (Fig. 3.2C) and of PMN (Fig. 3.2D) in the lavage of Saline-instilled controls. The increase in lavage cell number was statistically significant in male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice exposed to LPS-induced lung injury compared to controls, with no effect of the genotype (Fig. 3.2C). Male mice of both genotypes had also significantly higher lavage numbers of neutrophils following LPS-induced lung injury compared to Saline controls, with no effect stemming from lack of Mmp3 expression (Fig. 3.2D).

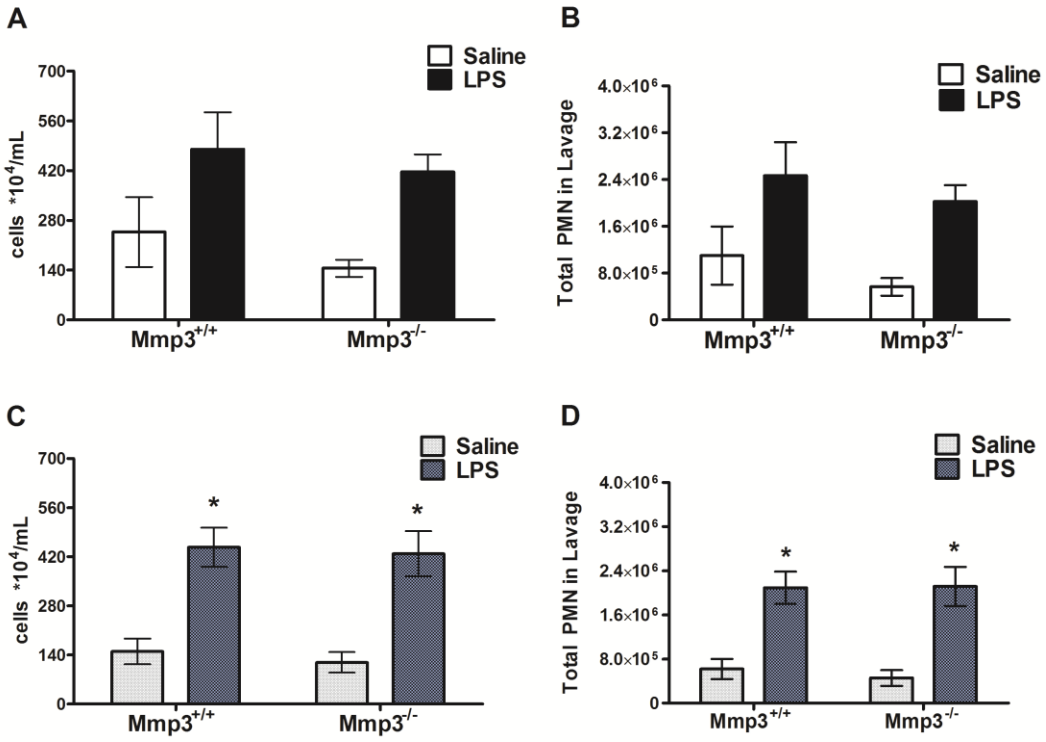

Figure 3.2 ***Experiment 1***: Total (**A, C**) and differential (**B, D**) cell counts performed in lavage samples 18 hours post Saline or LPS intra-tracheal instillation. Total lavage cell numbers were determined in $Mmp3^{+/+}$ and $Mmp3^{-/-}$ female (**A**) and male (**C**) mice. Following differential cell counts performed on cytospin slides, the total number of PMN neutrophils in lavage was obtained for female (**B**) and male (**D**) mice of both genotypes. Data are expressed as mean ± SEM; n=5-6 per group. *$p<0.05$ vs Saline control within the genotype.

Lavage inflammatory mediators

To further characterize the role of MMP-3 in the pulmonary inflammatory response associated with ALI, the concentration of several cytokines and chemokines was measured in lung lavage. Data from $Mmp3^{+/+}$ and $Mmp3^{-/-}$ female mice are shown in figure 3.3A to F, while results for male mice are shown in figure 3.4A to F. Overall, lavage concentrations of cytokines and chemokines did not differ between genotypes of Saline-instilled mice from both sexes (Figures 3.3 & 3.4).

In female mice, LPS instillation led to significantly higher lavage concentrations of MIP-2 in $Mmp3^{+/+}$ mice compared to Saline controls. Significantly elevated lavage concentrations of IL-6, G-CSF, KC, MCP-1, and TNF-α were found in female, LPS-instilled $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice compared to the respective Saline controls (Fig. 3.3A-F). Significantly lower lavage concentrations of IL-6, G-CSF, MIP-2, TNF-α, were observed in female, LPS-injured $Mmp3^{-/-}$ mice compared to the LPS-instilled $Mmp3^{+/+}$ group, while differences in these experimental groups did not reach significance for the KC concentration (Fig. 3.3A-F). No differences were found for IL-10 (data not shown).

The same array of inflammatory mediators was measured in the lavage of male mice and showed that LPS-induced injury caused significant increases in IL-6, G-CSF, KC, MIP-2, and TNF-α in $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice compared to Saline mice, with no effect of the genotype (Fig. 3.4A-F) . No differences were detected in the lavage concentrations of MCP-1 (Fig. 3.4D) and IL-10 (data not shown).

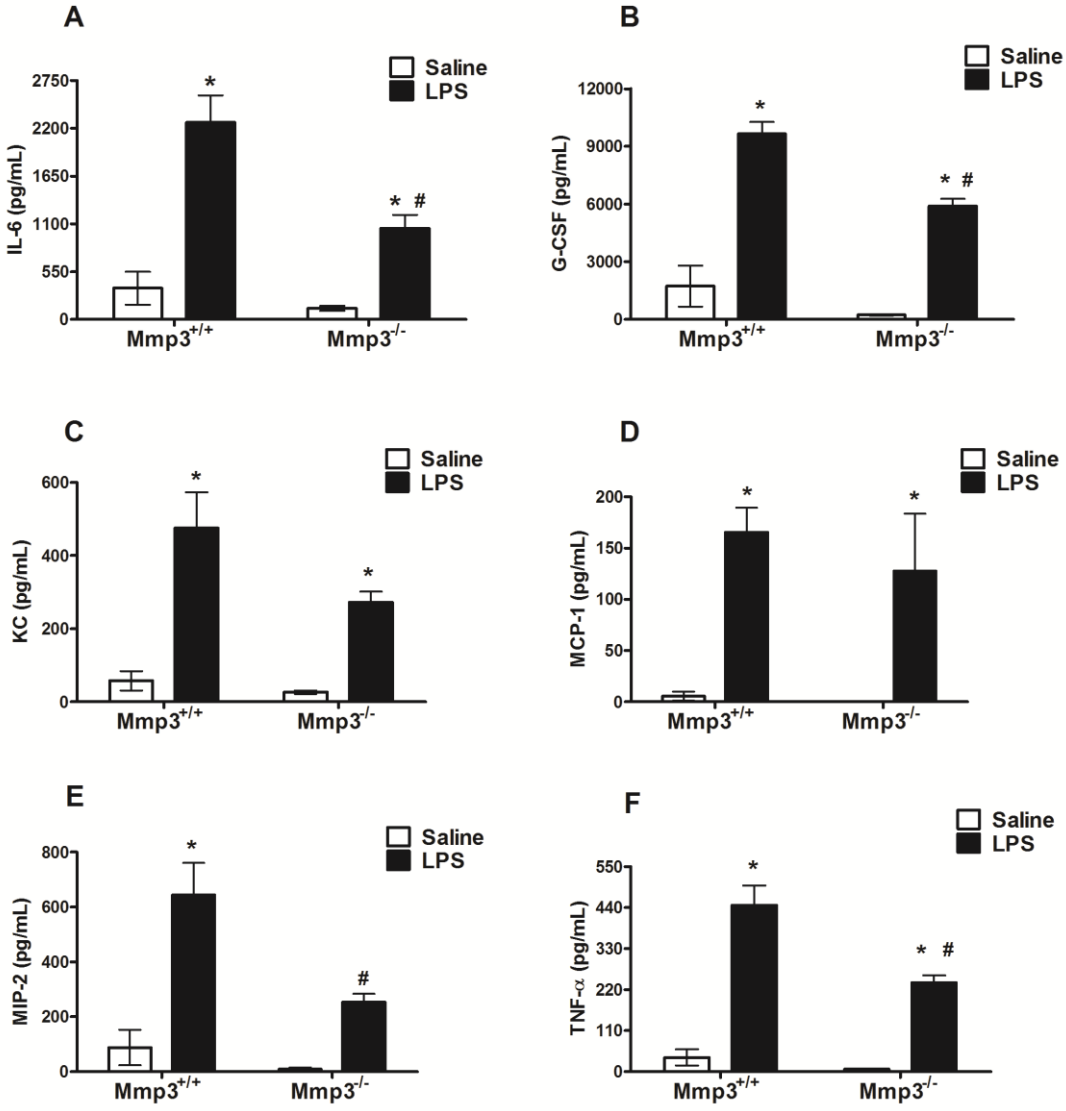

Figure 3.3 ***Experiment 1***: Cytokine and chemokine levels measured in lung lavage from $Mmp3^{+/+}$ and $Mmp3^{-/-}$ female mice (**A-F**), 18 hours after LPS or saline instillation. Data are expressed as mean ± SEM; n=5-6 per group. *$p<0.05$ vs Saline control within the genotype; #$p<0.05$ vs $Mmp3^{+/+}$ LPS group.

IL-6= interleukin-6, G-CSF= granulocyte colony stimulating factor, KC= keratinocyte chemoattractant, MCP-1= monocyte chemotactic protein-1, MIP-2= macrophage inflammatory protein 2, and TNF-α= tumor necrosis factor alpha.

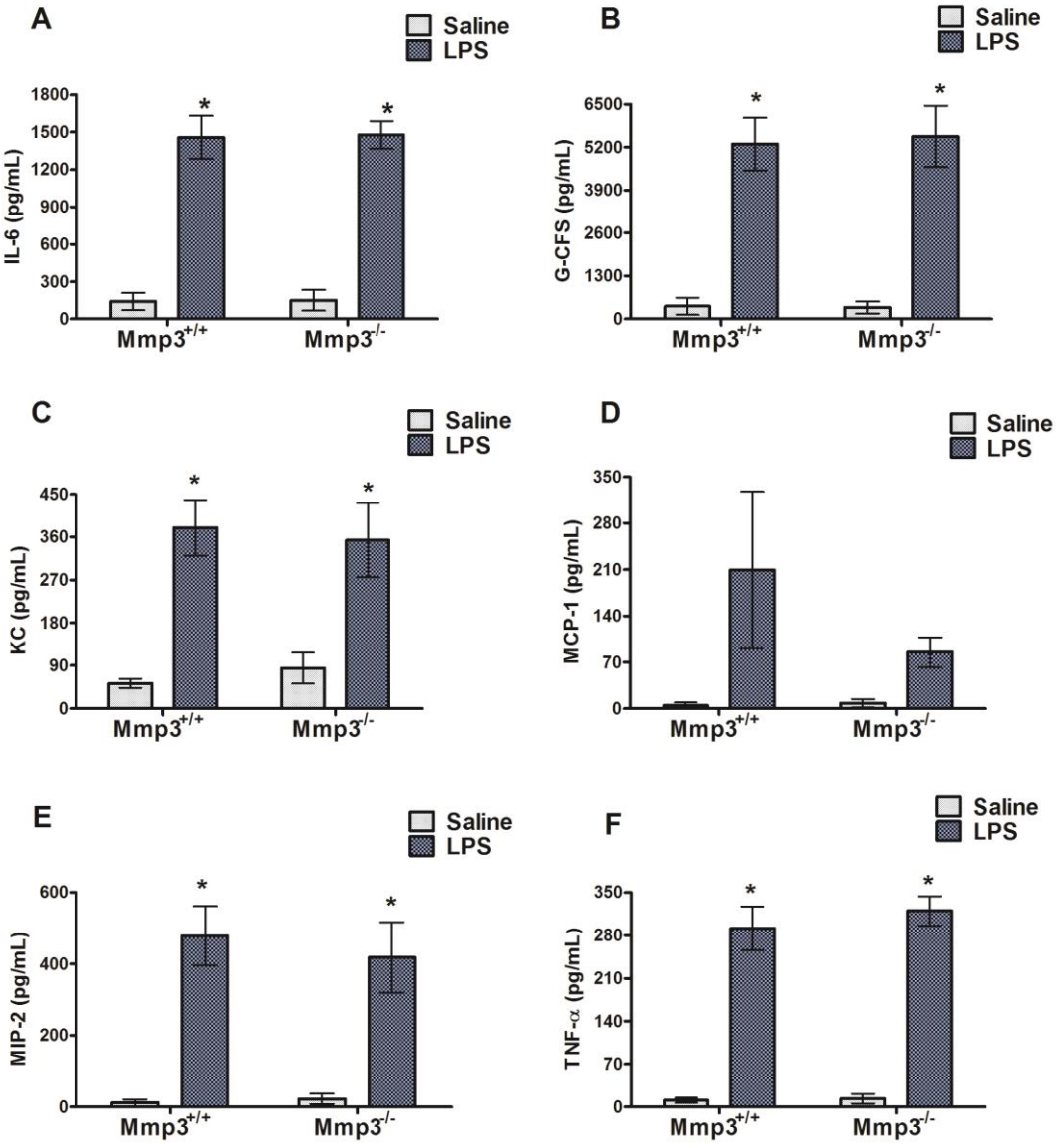

Figure 3.4 ***Experiment 1***: Cytokine and chemokine levels measured in lung lavage from $Mmp3^{+/+}$ and $Mmp3^{-/-}$ male mice (**A-F**), 18 hours after LPS or saline instillation. Data are expressed as mean ± SEM; n=5-6 per group. *$p<0.05$ vs Saline control within the genotype; #$p<0.05$ vs $Mmp3^{+/+}$ LPS group.

IL-6= interleukin-6, G-CSF= granulocyte colony stimulating factor, KC= keratinocyte chemoattractant, MCP-1= monocyte chemotactic protein-1, MIP-2= macrophage inflammatory protein 2, and TNF-α= tumor necrosis factor alpha.

3.3.2. Experiment 2: Acid-induced lung injury

Lavage MMP-3

The role of MMP-3 in ALI was also investigated in a model of acid-induced lung injury, in which female and male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice were exposed to an intra-tracheal instillation of HCl or Air, and sacrificed after a period of 4 hours. Similar to Experiment 1, the data have been separated by sex.

Analysis of total lavage MMP-3 levels (Fig. 3.5) showed that acid injury led to significantly higher MMP-3 levels in the lavage of female and male $Mmp3^{+/+}$ mice (Fig. 3.5A, B), compared to the respective Air controls.

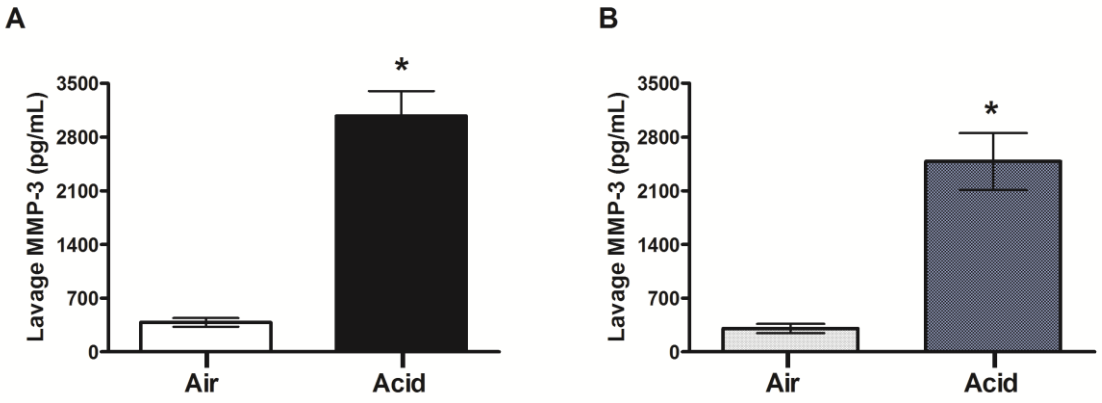

Figure 3.5 ***Experiment 2***: MMP-3 levels measured in lavage samples from female (**A**) and male (**B**) $Mmp3^{+/+}$ mice, 4 hours after intra-tracheal instillation of HCl or Air as a control. Data are expressed as mean ± SEM; n=5-6 per group. *$p<0.05$ vs Air control.

Lavage protein

The average lavage recovery volume from all animals was 2.9 ± 0.01 mL, with a significant difference only observed in male acid-instilled $Mmp3^{-/-}$ mice compared to $Mmp3^{-/-}$ Air control (2.99 ± 0.55 mL versus 2.81 ± 0.03 mL, respectively). Total protein content was measured in the lavage of female and male, $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice (Table 3.2). When comparing Air-instilled mice within each sex, no differences in total protein content were

observed due to genotype. Overall, total protein content was significantly higher in mice exposed to acid-induced ALI compared to Air controls. These results were observed in both female and male mice, with no effect of the Mmp3 genotype (Table 3.2).

Total lavage protein (mg/kg body weight)	$Mmp3^{+/+}$		$Mmp3^{-/-}$	
	Air	Acid	Air	Acid
Female mice	16.9±4.2	197.7±34.0*	13.5±1.1	251.5±13.1*
Male mice	11.8±0.3	169.4±25.2*	10.8±1.0	243.4±38.1*

Table 3.2 ***Experiment 2***: Total protein content measured in lavage samples from female and male mice of both genotypes. Data are expressed as mean ± SEM; n=5-6 per group. *$p<0.05$ vs Air control within the genotype.

Lavage inflammatory cells

The total number of neutrophils in the lavage of Air-instilled mice did not differ between genotypes in both female (Fig. 3.6A) and male mice (Fig. 3.6B). The results in figure 3.6A showed a significantly higher number of neutrophils in female, acid-injured $Mmp3^{+/+}$ mice compared to Air control. Nonetheless, the increase in lavage neutrophil numbers in female, acid-injured $Mmp3^{-/-}$ mice was not significant compared to the respective Air mice. Lower neutrophil abundance was observed in the lavage of female, acid-injured $Mmp3^{-/-}$ mice compared to $Mmp3^{+/+}$ counterpart (Fig. 3.6A); however this difference did not reach significance.

In male mice the increases in lavage neutrophils following acid injury were not statistically significant, and no effect stemmed from the difference in genotype (Fig. 3.6B).

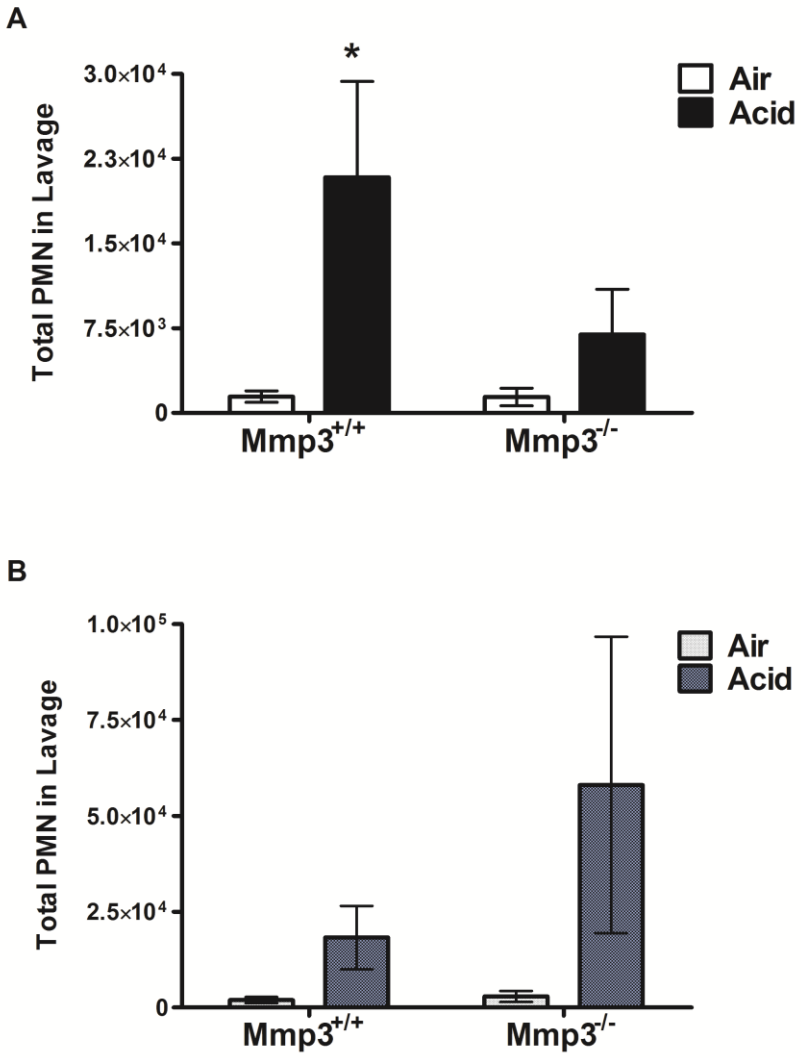

Figure 3.6 ***Experiment 2***: Total number of PMN neutrophils in lavage samples from female (**A**) and male (**B**) mice of both genotypes, 4 hours post air or acid instillation. Data are expressed as mean ± SEM; n=5-6 per group. *$p<0.05$ vs Air control.

Lavage inflammatory mediators

Lavage concentrations of inflammatory mediators from Air-instilled mice of either sex did not show any difference due to genotype (Figures 3.7 & 3.8).

In female mice (Fig. 3.7A-F), acid instillation led to significant increases in lavage IL-6, G-CSF, KC, MCP-1, MIP-2, and TNF-α in $Mmp3^{+/+}$ mice compared to $Mmp3^{+/+}$ Air mice; however in acid-injured $Mmp3^{-/-}$ mice such increases only reached significance for MIP-2 and TNF-α (Fig 3.7A-F) compared to Air controls. Although non-significant, female acid-injured $Mmp3^{-/-}$ mice appeared to have lower lavage concentrations of IL-6, G-CSF, KC, MCP-1, and had significantly lower concentrations of MIP-2 and TNF-α compred to acid-instilled $Mmp3^{+/+}$ mice (Fig 3.7A-F). IL-10 levels were increased following acid injury, with no effect of the genotype (data not shown).

Analysis of the same inflammatory mediators in the lavage of male mice showed significantly higher concentrations of IL-6, MCP-1, and TNF-α in acid-instilled $Mmp3^{+/+}$ mice compared to Air controls (Fig 3.8A-F). Significant increases in IL-6, MCP-1, G-CSF, KC, and MIP-2 were also observed in acid-injured $Mmp3^{-/-}$ mice compared to Air-instilled controls (Fig 3.8A-F). No differences were observed due to the Mmp3 genotype in male mice (Fig 3.8A-F) following acid injury; moreover, no effect of the genotype was noticed in IL-10 lavage levels (data not shown).

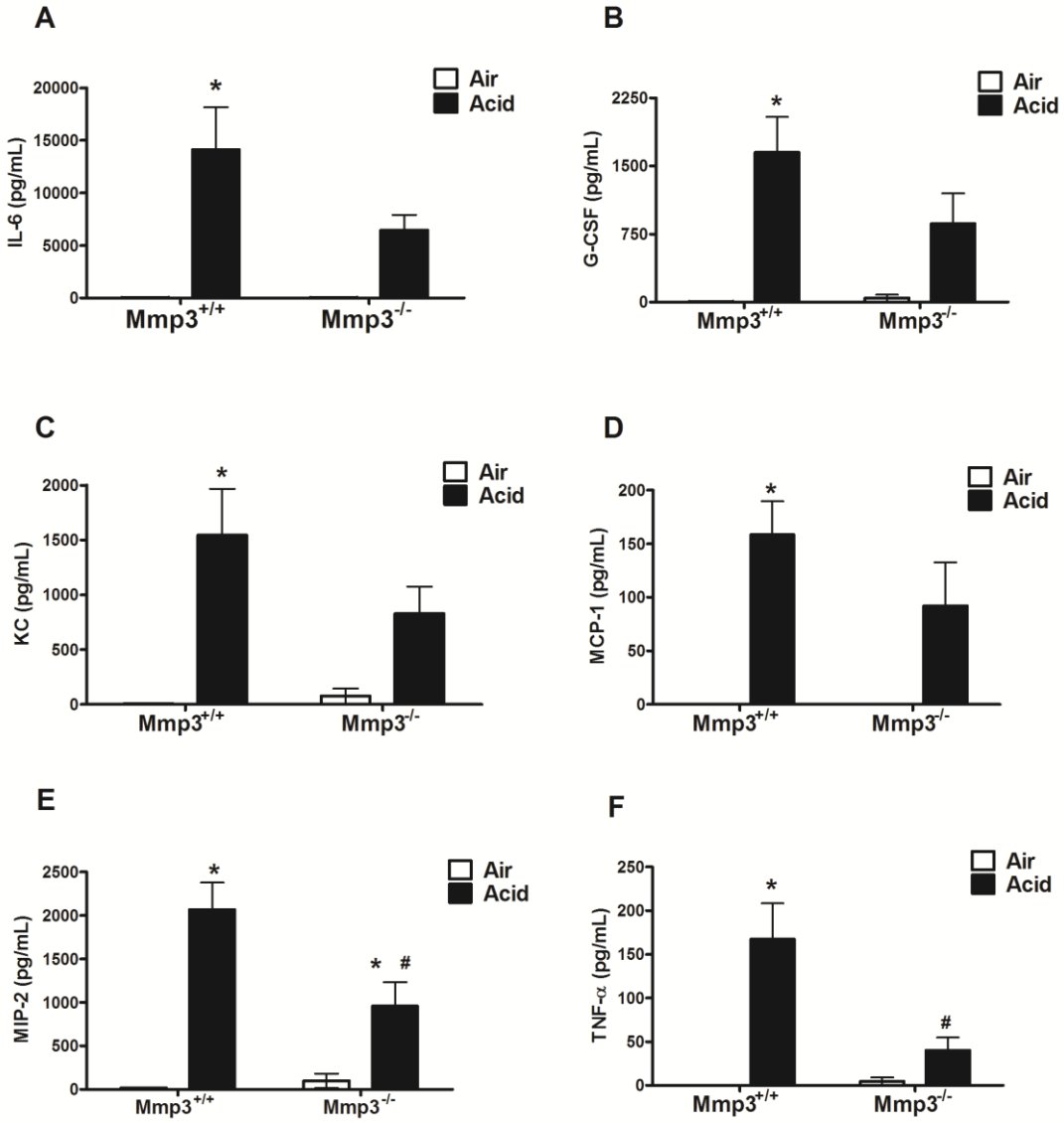

Figure 3.7 ***Experiment 2***: Cytokine and chemokine levels in lavage samples from $Mmp3^{+/+}$ and $Mmp3^{-/-}$ female mice (**A-F**), 4 hours following acid injury or air instillation. Data are expressed as mean ± SEM; n=5-6 per group. *$p<0.05$ vs Air control within the genotype; #$p<0.05$ vs $Mmp3^{+/+}$ Acid group.

IL-6= interleukin-6, G-CSF= granulocyte colony stimulating factor, KC= keratinocyte chemoattractant, MCP-1= monocyte chemotactic protein-1, MIP-2= macrophage inflammatory protein 2, and TNF-α= tumor necrosis factor alpha.

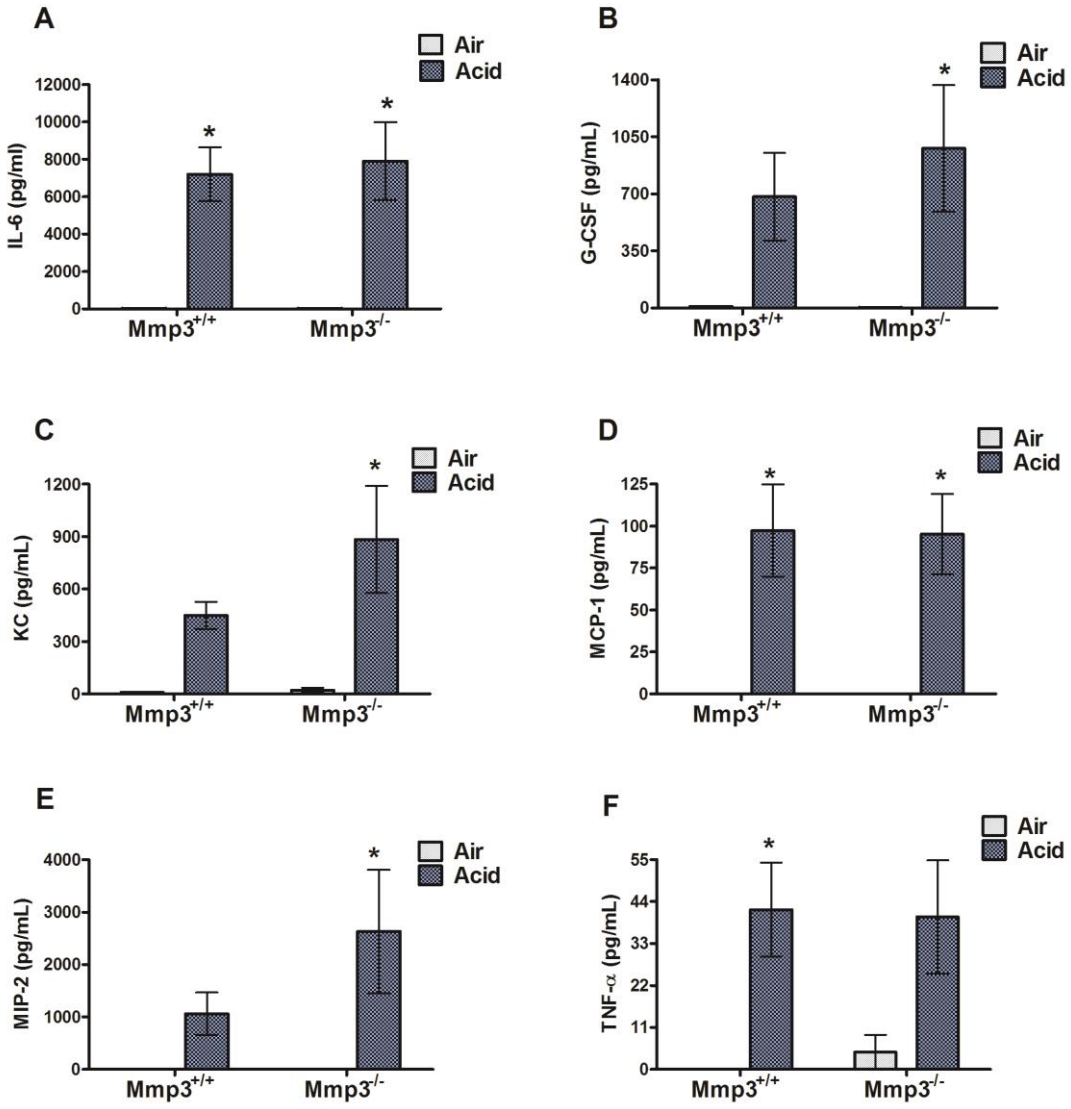

Figure 3.8 ***Experiment 2***: Cytokine and chemokine levels in lavage samples from $Mmp3^{+/+}$ and $Mmp3^{-/-}$ male mice (**A**-**F**), 4 hours following acid injury or air instillation. Data are expressed as mean ± SEM; n=5-6 per group. *$p<0.05$ vs Air control within the genotype; #$p<0.05$ vs $Mmp3^{+/+}$ Acid group.

IL-6= interleukin-6, G-CSF= granulocyte colony stimulating factor, KC= keratinocyte chemoattractant, MCP-1= monocyte chemotactic protein-1, MIP-2= macrophage inflammatory protein 2, and TNF-α= tumor necrosis factor alpha.

3.3.3. Experiment 3: *In vitro* stimulation of $Mmp3^{+/+}$ and $Mmp3^{-/-}$ bone marrow-derived macrophages

In order to investigate the observed MMP-3-related sex differences in pulmonary inflammation, inflammatory cells such as bone marrow-derived macrophages were isolated from female and male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice. Stimulation of these BMDMs with media only as a control or increasing doses of LPS (0.1µg/mL, 1µg/mL, 10µg/mL) led to step-wise increases in IL-6 in cell culture supernatant, with no significant differences related to the sex of the mice from which the cells were derived, or Mmp3 genotype (Fig. 3.9A).

A potential role of 17β-estradiol (E_2) in the MMP-3 related differences in inflammatory response was addressed in BMDMs from female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice. The results in figure 3.8B showed that stimulation of BMDMs with 1µg/mL LPS caused increased release of IL-6 in cell culture supernatant, with no additional effect stemming from E_2 co-stimulation, and no differences associated with lack of Mmp3 expression (Fig. 3.9B).

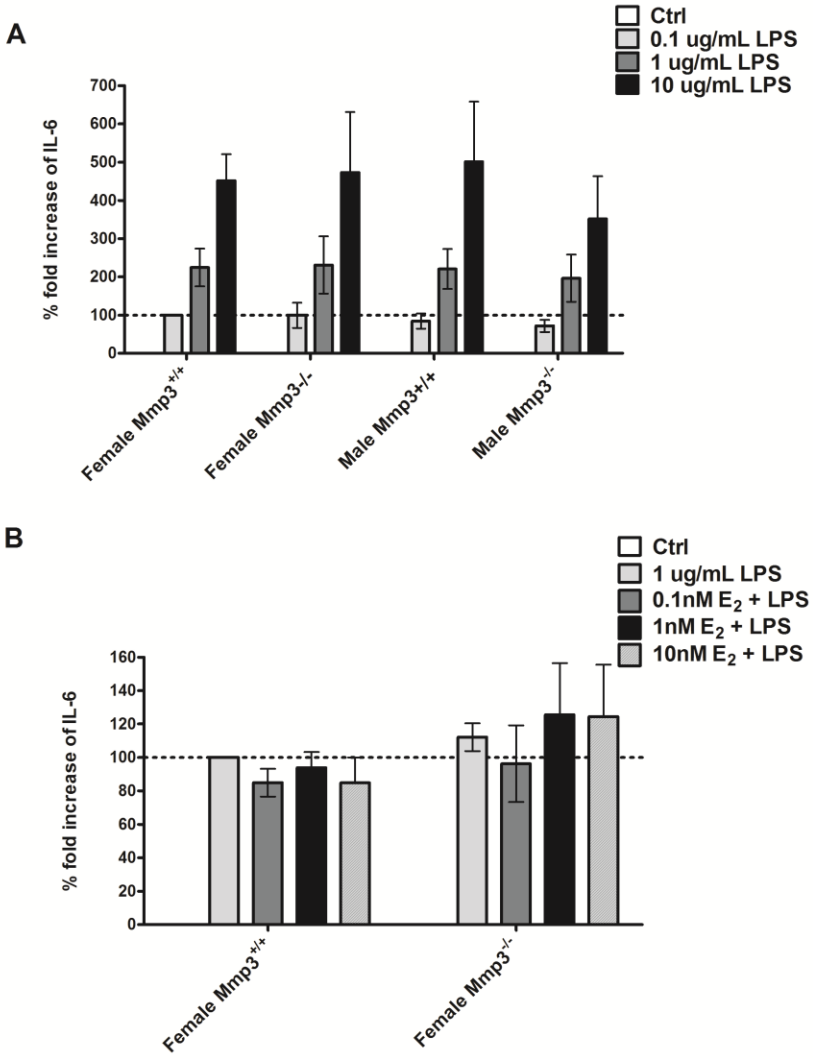

Figure 3.9 ***Experiment 3***: Stimulation of BMDMs isolated from $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice. (**A**) BMDMs from female and male mice of both genotypes were stimulated with different doses of LPS (E. Coli 0111:B4) for 18 hours. IL-6 measurements in cell culture supernatant were normalized by values for female $Mmp3^{+/+}$ BMDMs at 0.1µg/mL LPS. (**B**) BMDMs from female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice were stimulated for 18 hours with 1µg/mL LPS alone, or LPS and three different doses of E_2. IL-6 values in cell culture supernatant were normalized by values from female $Mmp3^{+/+}$ BMDMs at 1µg/mL LPS. In both experiments, release of IL-6 in the control conditions was minimal and therefore not visible on the graphs. Data are expressed as mean ± SEM and representative of three experiments performed on three independent cell isolations.

3.4. Discussion

The overall objective of this study was to investigate the role of MMP-3 in the inflammatory response associated with ALI. To this end, models of LPS- (experiment 1) and acid-induced lung injury (experiment 2) were utilized in mice lacking the expression of Mmp3, and in wild type controls. Consistent with previous experimental and clinical observations of increased MMPs expression during injury and inflammation [23, 24, 36], total MMP-3 levels were found elevated in the lavage of $Mmp3^{+/+}$ mice following LPS or acid injury. In our models, lack of Mmp3 expression did not alter baseline responses in any of the measured outcomes. Following injury, lack of Mmp3 expression did not affect total protein in lavage and did not significantly alter the recruitment of neutrophils to the injured lung. Importantly, however, the release of cytokines and chemokines in response to the different injuries appeared to be consistently mitigated by the genetic ablation of Mmp3 in female mice, while male LPS- and acid-injured $Mmp3^{-/-}$ mice showed concentrations of pulmonary inflammatory mediators comparable to male injured $Mmp3^{+/+}$ mice. Based on these data, it is concluded that MMP-3 plays a role in the pathogenesis of ALI by affecting pulmonary inflammation in a sex-dependent fashion.

The role of MMP-3 in lung injury has been previously investigated in neutrophils-dependent models of ALI, in which mice lacking Mmp3 expression demonstrated lower neutrophils infiltration and lower intra-pulmonary accumulation of proteinaceous edema in response to injury [32, 33]. Our data did not fully replicate those findings, due in part to differences in the nature of the injuries and their development, as well as potential technical limitations related to the retrieval of activated inflammatory cells and the sensitivity of the cell counting technique. In contrast to those previous studies, our experiments utilized mouse models that resembled two of the most common causes of lung injury in the patient population [5, 37]. Furthermore, in light of the association between lung cytokines/ chemokines and mortality in ALI [9, 38], the present research extended the previous observations on inflammation by analyzing the concentrations of several pulmonary inflammatory mediators. Overall, this study has investigated outcomes that are relevant in the pathogenesis of ALI, such as the development of pulmonary inflammation, and has explored sex differences in the response to lung insults. The current work, therefore,

provides an overall stronger clinical correlate about the role of MMP-3 in ALI than previously assessed.

An unanticipated but interesting outcome of the current study were the MMP-3 related sex differences in the inflammatory response. Of interest, the sex of the mice previously utilized to investigate MMP-3 in ALI was not clearly reported in those studies [32, 33]. In a general sense, therefore, our findings underscore the upcoming policy changes announced by the National Institue of Health, which recommend the inclusion of both sexes in experimental studies [39]. Such changes have been prompted by the observation that the almost exclusive use of male mice in research may have led to an under-estimation of sex-driven differences in pathophysiology, and may have negatively impacted the translation of experimental findings into clinical practice [39]. Recent studies however started to demonstrate marked sex differences in the inflammatory response, with females mounting a better and more pronounced immune response to viral and bacterial infections, and being substantially more inclined to develop autoimmune disorders than males [40, 41]. In terms of pulmonary pathophysiology, sex differences have been observed in patients affected by cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD). Specifically, prepubertal female patients with CF have a higher mortality rate than peers of the opposite sex; females also exhibit higher prevalence and risk of hospitalization due to COPD than males [42, 43]. In patients with ALI, sex differences in mortality have not been observed [2]; however, a clinical study by Heffernan et al. indicated that, following traumatic injury, female patients are more likely to develop ALI [44].

The underlying pathology leading to sex differences in the inflammatry responses in general may encompass gonadal hormones, sex chromosomes, and anatomical differences [45]. In the context of the current study, our *in vivo* models of ALI demonstrated that a lack of Mmp3 dampened the concentrations of lavage inflammatory mediators only in female mice, with no effect in males. These differences could be related to possible inflammatory dissimilarities inherent to the response of male/female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice, however there is also evidence of a potential relationship between sex hormones, specifically 17β-estradiol, and MMP-3. Different studies have in fact shown that exposure to E_2 leads to up-regulation of Mmp3 expression by primary human osteoblasts, rat uterine

stromal cells, and human synovial fibroblasts [46–48]. These observations prompted our preliminary *in vitro* investigation into the sex-differences in the MMP-3-related inflammatory response, as shown in experiment 3. Based on the evidence of Mmp3 expression in AM [20], and the role of AM in the progression and resolution of lung injury [49, 50], two experiments were performed on isolated BMDM to evaluate: i) inherent differences in the macrophage-driven inflammatory response of male/female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice; and ii) the possibility of an hormonal influence on the MMP-3 related sex differences. The data suggested that, with respect to IL-6 secretion, there were no inherent sex-related differences between female and male derived BMDM in response to LPS and/ or 17β-estradiol.

Although those preliminary results suggest that BMDMs do not demonstrate sex differences, these experiments have some limitations and further research is required to elucidate the specific mechanisms by which the MMP-3-related sex-differences occur. For example, even though BMDMs provide a convenient, readily available and widely accepted *in vitro* model for alveolar macrophages [51, 52], some evidence suggests that AM may have properties and patterns of gene expression divergent from the ones characterizing BMDMs [53]. There is, of course, also the possibility that such MMP-3 related sex differences do not involve the AM, but rather other cell populations within the lung (i.e. alveolar type II cells, fibroblasts) or more complex, systemic interactions. Future studies may address this aspect through broader *in vitro* analyses. Additionally, ovariectomized $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice would be a useful model to determine whether estrogen- and, more generally speaking, sex hormones- have a role in MMP-3 related differences in the inflammatory response.

As mentioned, the use of two different models of ALI is a strength of the study and an important aspect in the analysis of MMP-3 role. The rationale for such experimental approach resides, firstly, in the complex pathophysiology of ALI, which is associated with multiple and different initiating insults [5, 54]. Secondly, various models of ALI could help understand whether the role of MMP-3 differs depending on the lung insult. There is indeed some evidence that other MMPs, such as MMP-8 and -9, may have roles in ALI that vary depending on the nature of the initiating insult. For example, studies on Mmp9 knock-out

mice have shown that MMP-9 plays a pathogenic role during immunoglobulin G immune-complex-induced lung injury [32], but appears to be protective from the development of ventilation-induced lung injury (VILI). Mice lacking Mmp9 expression were in fact more susceptible to the effects of injurious mechanical ventilation than wild type controls, presenting significant impairment in lung function and greater tissue damage [55]. Similarly, studies on MMP-8 have demonstrated that this protease has a protective role in models of LPS- and hyperoxia-induced lung injury while, on the other hand, it appears to favor the development of VILI [56, 57]. Overall, what emerges is the possibility that the role of matrix metalloproteinases in the pathogenesis of ALI may be dependent on the aetiology of lung injury. This evidence, combined with the multiplicity of causes leading to lung injury, undercores the necessity to perform ALI research through different injury models.

In this regard, our ALI models are characterized at different time points (18 hours in experiment 1, 4 hours in experiment 2), and by different initiating insults with dissimilar features. Intra-tracheal instillation of LPS (experiment 1) has been described to cause the activation of specific intra-cellular inflammatory pathways, with patchy areas of neutrophils infiltration and limited epithelial damage [58], as demonstrated by the mild changes in lung permeability in LPS-injured mice observed in experiment 1 (table 3.1). Conversely, intra-tracheal instillation of HCl (experiment 2) resembles more of a chemical burn, and the subsequent lung injury was associated with damage of the alveolar epithelium, neutrophil infiltration, and extensive pulmonary edema as reflected by the significant changes in total protein content shown in table 3.2. In light of such differences, it is noteworthy that the involvement of MMP-3 in pulmonary inflammation in female mice was observed consistently in both models, thereby strengthening our conclusions.

This important observation of reduced lavage cytokine and chemokine concentrations following lung injury in female $Mmp3^{-/-}$ mice has potential clinical relevance. The possibility of targeting MMP-3 to lower pulmonary levels of inflammation could improve outcomes of ALI. Within the pathophysiology of this disorder, the development of an overwhelming inflammatory response is indeed predictive of poor outcome [38, 59]. Experimental and clinical studies have shown that lung-derived inflammatory mediators

de-compartmentalized into the systemic circulation can affect the function of peripheral organs, with progression to multi organ failure and, eventually, death [10, 13, 60]. In this sense, a therapeutic approach capable of modulating the inflammation associated with lung injury would be an ideal treatment, and our study is a first step to assess whether MMP-3 could be such key target.

Aside from the aforementioned strengths, our study also has some weaknesses. For example, there may be intrinsic limitations related to the use of a mouse model that lacks Mmp3 expression as a result of gene disruption. Nonetheless, $Mmp3^{-/-}$ mice are fertile and viable, show no phenotypical alteration and appear to have normal lung function when unchallenged (chapter 4 and [20]), thereby representing a useful and valid tool for the study of MMP-3 in ALI.

Additionally, the present study did not explore the potential mechanisms responsible for the decreased inflammation in female $Mmp3^{-/-}$ mice with ALI. It has been described that MMP-3 plays a role in inflammation through direct cleavage of specific inflammatory mediators, resulting in activation, potentiation of their activity, or inactivation of such molecules, and also through the generation of chemotactic gradients for neutrophils and macrophages [23]. Our data leave open the possibility that the effect of MMP-3 on ALI-associated inflammation may result from direct or indirect proteolytic activity on a variety of inflammatory substrates. Importantly, however, new and non-enzymatic roles are emerging for MMP-3 in several disease processes [61, 62], thereby outlining a possibly more complex scenario than traditionally anticipated. More mechanistic insight about the role of MMP-3 in ALI could be gained in the future through the development of a catalytically inactive Mmp3 knock-in mouse or, when available, the administration of a highly selective MMP-3 inhibitor in models of lung injury.

In conclusion, this study expanded the knowledge of ALI pathophysiology, addressing the role of MMP-3 in the context of two, clinically relevant models of lung injury. Our findings demonstrated a sex-dependent role for MMP-3 in the overwhelming lung inflammatory response associated with ALI, and highlighted the need for a better understanding of the disease processes in the search for future therapeutic approaches.

3.5. References

1. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD: **Incidence and outcomes of acute lung injury**. *N Engl J Med* 2005, **353**:1685–1693.

2. Phua J, Badia JR, Adhikari NKJ, Friedrich JO, Fowler RA, Singh JM, Scales DC, Stather DR, Li A, Jones A, Gattas DJ, Hallett D, Tomlinson G, Stewart TE, Ferguson ND: **Has mortality from acute respiratory distress syndrome decreased over time?: A systematic review.** *Am J Respir Crit Care Med* 2009, **179**:220–227.

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

4. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS: **Acute respiratory distress syndrome: the Berlin Definition.** *JAMA* 2012, **307**:2526–2533.

5. Ware LB, Matthay MA: **The Acute Respiratory Distress Syndrome**. *N Engl J Med* 2000, **342**:1334–1349.

6. Matthay MA, Ware LB, Zimmerman GA: **The acute respiratory distress syndrome**. *J Clin Invest* 2012, **122**:2731–2740.

7. Bhatia M, Moochhala S: **Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome.** *J Pathol* 2004, **202**:145–56.

8. Goodman RB, Pugin J, Lee JS, Matthay MA: **Cytokine-mediated inflammation in acute lung injury**. *Cytokine Growth Factor Rev* 2003, **14**:523–535.

9. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A: **Inflammatory Cytokines in the BAL of Patients with ARDS. Persistent Elevation Over Time Predicts Poor Outcome**. *Chest* 1995, **108**:1303–1314.

10. Imai Y, Parodo J, Kajikawa O, de Perrot M, Fischer S, Edwards V, Cutz E, Liu M, Keshavjee S, Martin TR, Marshall JC, Ranieri VM, Slutsky AS: **Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of acute respiratory distress syndrome**. *JAMA* 2003, **289**:2104–2112.

11. O'Mahony DS, Liles WC, Altemeier WA, Dhanireddy S, Frevert CW, Liggitt D, Martin TR, Matute-Bello G: **Mechanical ventilation interacts with endotoxemia to induce extrapulmonary organ dysfunction.** *Crit Care* 2006, **10**:R136.

12. Dhanireddy S, Altemeier WA, Matute-Bello G, O'Mahony DS, Glenny RW, Martin TR, Liles WC: **Mechanical ventilation induces inflammation, lung injury, and extra-pulmonary organ dysfunction in experimental pneumonia**. *Lab Invest* 2006, **86**:790–799.

13. Walker MG, Yao LJ, Patterson EK, Joseph MG, Cepinskas G, Veldhuizen RA, Lewis JF, Yamashita CM: **The effect of tidal volume on systemic inflammation in Acid-induced lung injury**. *Respiration* 2011, **81**:333–342.

14. Chiumello D, Pristine G, Slutsky AS: **Mechanical ventilation affects local and systemic cytokines in an animal model of acute respiratory distress syndrome**. *Am J Respir Crit Care Med* 1999, **160**:109–116.

15. Stapleton RD, Wang BM, Hudson LD, Rubenfeld GD, Caldwell ES, Steinberg KP: **Causes and timing of death in patients with ARDS**. *Chest* 2005, **128**:525–532.

16. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, Slutsky AS: **Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial**. *JAMA* 1999, **282**:54–61.

17. The Acute Respiratory Distress Syndrome Network: **Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network**. *N Engl J Med* 2000, **342**:1301–1308.

18. Cepkova M, Matthay MA: **Pharmacotherapy of acute lung injury and the acute respiratory distress syndrome.** *J Intensive Care Med* 2006, **21**:119–143.

19. Bosma KJ, Taneja R, Lewis JF: **Pharmacotherapy for prevention and treatment of acute respiratory distress syndrome: current and experimental approaches.** *Drugs* 2010, **70**:1255–1282.

20. Yamashita CM, Dolgonos L, Zemans RL, Young SK, Robertson J, Briones N, Suzuki T, Campbell MN, Gauldie J, Radisky DC, Riches DW, Yu G, Kaminski N, McCulloch CA, Downey GP: **Matrix metalloproteinase 3 is a mediator of pulmonary fibrosis**. *Am J Pathol* 2011, **179**:1733–1745.

21. Davey A, McAuley DF, O'Kane CM: **Matrix metalloproteinases in acute lung injury: mediators of injury and drivers of repair**. *Eur Respir J* 2011, **38**:959–970.

22. Warner RL, Bhagavathula N, Nerusu KC, Lateef H, Younkin E, Johnson KJ, Varani J: **Matrix metalloproteinases in acute inflammation: induction of MMP-3 and MMP-9 in fibroblasts and epithelial cells following exposure to pro-inflammatory mediators in vitro**. *Exp Mol Pathol* 2004, **76**:189–195.

23. Parks WC, Wilson CL, López-Boado YS: **Matrix metalloproteinases as modulators of inflammation and innate immunity**. *Nat Rev Immunol* 2004, **4**:617–629.

24. Manicone AM, McGuire JK: **Matrix metalloproteinases as modulators of inflammation**. *Semin Cell Dev Biol* 2008, **19**:34–41.

25. Gill SE, Parks WC: **Metalloproteinases and their inhibitors: regulators of wound healing.** *Int J Biochem Cell Biol* 2008, **40**:1334–1347.

26. Yazdan-Ashoori P, Liaw P, Toltl L, Webb B, Kilmer G, Carter DE, Fraser DD: **Elevated plasma matrix metalloproteinases and their tissue inhibitors in patients with severe sepsis**. *J Crit Care* 2011, **26**:556–565.

27. O'Kane CM, McKeown SW, Perkins GD, Bassford CR, Gao F, Thickett DR, McAuley DF: **Salbutamol up-regulates matrix metalloproteinase-9 in the alveolar space in the acute respiratory distress syndrome**. *Crit Care Med* 2009, **37**:2242–2249.

28. Greenlee KJ, Werb Z, Kheradmand F: **Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted**. *Physiol Rev* 2007, **87**:69–98.

29. Parks WC, Shapiro SD: **Matrix metalloproteinases in lung biology.** *Respir Res* 2001, **2**:10–19.

30. Kong MYF, Gaggar A, Li Y, Winkler M, Blalock JE, Clancy JP: **Matrix metalloproteinase activity in pediatric acute lung injury.** *Int J Med Sci* 2009, **6**:9–17.

31. Fligiel SE, Standiford T, Fligiel HM, Tashkin D, Strieter RM, Warner RL, Johnson KJ, Varani J: **Matrix metalloproteinases and matrix metalloproteinase inhibitors in acute lung injury**. *Hum Pathol* 2006, **37**:422–430.

32. Warner RL, Beltran L, Younkin EM, Lewis CS, Weiss SJ, Varani J, Johnson KJ: **Role of stromelysin 1 and gelatinase B in experimental acute lung injury**. *Am J Respir Cell Mol Biol* 2001, **24**:537–544.

33. Nerusu KC, Warner RL, Bhagavathula N, McClintock SD, Johnson KJ, Varani J: **Matrix metalloproteinase-3 (stromelysin-1) in acute inflammatory tissue injury**. *Exp Mol Pathol* 2007, **83**:169–176.

34. Mudgett JS, Hutchinson NI, Chartrain NA, Forsyth AJ, Mcdonnell J, Singer II, Bayne EK, Flanagan J, Kawka D, Shen CF, Stevens K, Chen H, Trumbauer M, Visco DM: **Susceptibility of stromelysin 1-deficient mice to collagen-induced arthritis and cartilage destruction**. *Arthritis Rheum* 1998, **41**:110–121.

35. Manicone AM, Birkland TP, Lin M, Betsuyaku T, van Rooijen N, Lohi J, Keski-Oja J, Wang Y, Skerrett SJ, Parks WC: **Epilysin (MMP-28) restrains early macrophage recruitment in Pseudomonas aeruginosa pneumonia.** *J Immunol* 2009, **182**:3866–3876.

36. Khokha R, Murthy A, Weiss A: **Metalloproteinases and their natural inhibitors in inflammation and immunity.** *Nat Rev Immunol* 2013, **13**:649–665.

37. Erickson SE, Martin GS, Davis JL, Matthay MA, Eisner MD: **Recent trends in acute lung injury mortality: 1996-2005.** *Crit Care Med* 2009, **37**:1574–1579.

38. Parsons PE, Eisner MD, Thompson BT, Matthay MA, Ancukiewicz M, Bernard GR, Wheeler AP, and the NHLBI Acute Respiratory Distress Syndrome Clinical Trials Network: **Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury**. *Crit Care Med* 2005, **33**:1–6.

39. Clayton JA, Collins FS: **Policy: NIH to balance sex in cell and animal studies.** *Nature* 2014, **509**:282–283.

40. Klein SL: **Immune cells have sex and so should journal articles.** *Endocrinology* 2012, **153**:2544–2550.

41. Furman D, Hejblum BP, Simon N, Jojic V, Dekker CL, Thiébaut R, Tibshirani RJ, Davis MM: **Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination.** *Proc Natl Acad Sci U S A* 2014, **111**:869–874.

42. Rosenfeld M, Davis R, FitzSimmons S, Pepe M, Ramsey B: **Gender gap in cystic fibrosis mortality.** *Am J Epidemiol* 1997, **145**:794–803.

43. Prescott E, Bjerg AM, Andersen PK, Lange P, Vestbo J: **Gender difference in smoking effects on lung function and risk of hospitalization for COPD : results from a Danish longitudinal population study**. *Eur Respir J* 1997, **10**:822–827.

44. Heffernan DS, Dossett LA, Lightfoot MA, Fremont RD, Ware LB, Sawyer RG, May AK: **Gender and acute respiratory distress syndrome in critically injured adults: a prospective study.** *J Trauma* 2011, **71**:878–883.

45. Fish EN: **The X-files in immunity: sex-based differences predispose immune responses**. *Nat Rev Immunol* 2008, **8**(September):737–744.

46. Garcia AJ, Tom C, Guemes M, Polanco G, Mayorga ME, Wend K, Miranda-Carboni GA, Krum SA: **ERα signaling regulates MMP3 expression to induce FasL cleavage and osteoclast apoptosis.** *J bone Miner Res* 2013, **28**:283–290.

47. Russo LA, Peano BJ, Trivedi SP, Cavalcanto TD, Olenchock BA, Caruso JA, Smolock AR, Vishnevsky O, Gardner RM: **Regulated expression of matrix metalloproteinases, inflammatory mediators, and endometrial matrix remodeling by 17beta-estradiol in the immature rat uterus.** *Reprod Biol Endocrinol* 2009, **7**:124.

48. Yamaguchi A, Nozawa K, Fujishiro M, Kawasaki M, Takamori K, Ogawa H, Sekigawa I, Takasaki Y: **Estrogen inhibits apoptosis and promotes CC motif chemokine ligand 13 expression on synovial fibroblasts in rheumatoid arthritis.** *Immunopharmacol Immunotoxicol* 2012, **34**:852–857.

49. Aggarwal NR, King LS, D'Alessio FR: **Diverse macrophage populations mediate acute lung inflammation and resolution.** *Am J Physiol Lung Cell Mol Physiol* 2014, **306**:L709–L725.

50. Frank JA, Wray CM, McAuley DF, Schwendener R, Matthay MA: **Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury.** *Am J Physiol Lung Cell Mol Physiol* 2006, **291**:L1191–L1198.

51. Tanino Y, Chang MY, Wang X, Gill SE, Skerrett S, McGuire JK, Sato S, Nikaido T, Kojima T, Munakata M, Mongovin S, Parks WC, Martin TR, Wight TN, Frevert CW: **Syndecan-4 regulates early neutrophil migration and pulmonary inflammation in response to lipopolysaccharide.** *Am J Respir Cell Mol Biol* 2012, **47**:196–202.

52. Gill SE, Gharib SA, Bench EM, Sussman SW, Wang RT, Rims C, Birkland TP, Wang Y, Manicone AM, McGuire JK, Parks WC: **Tissue inhibitor of metalloproteinases-3 moderates the proinflammatory status of macrophages.** *Am J Respir Cell Mol Biol* 2013, **49**:768–777.

53. Kapetanovic R, Fairbairn L, Downing A, Beraldi D, Sester DP, Freeman TC, Tuggle CK, Archibald AL, Hume DA: **The impact of breed and tissue compartment on the response of pig macrophages to lipopolysaccharide.** *BMC Genomics* 2013, **14**:581.

54. Wheeler AP, Bernard GR: **Acute lung injury and the acute respiratory distress syndrome: a clinical review.** *Lancet* 2007, **369**:1553–1564.

55. Albaiceta GM, Gutiérrez-Fernández A, Parra D, Astudillo A, García-Prieto E, Taboada F, Fueyo A: **Lack of matrix metalloproteinase-9 worsens ventilator-induced lung injury.** *Am J Physiol Lung Cell Mol Physiol* 2008, **294**:L535–L543.

56. Quintero PA, Knolle MD, Cala LF, Zhuang Y, Owen CA: **Matrix metalloproteinase-8 inactivates macrophage inflammatory protein-1 alpha to reduce acute lung inflammation and injury in mice.** *J Immunol* 2010, **184**:1575–1588.

57. Albaiceta GM, Gutierrez-Fernández A, García-Prieto E, Puente XS, Parra D, Astudillo A, Campestre C, Cabrera S, Gonzalez-Lopez A, Fueyo A, Taboada F, López-Otin C: **Absence or inhibition of matrix metalloproteinase-8 decreases ventilator-induced lung injury.** *Am J Respir Cell Mol Biol* 2010, **43**:555–563.

58. Matute-Bello G, Frevert CW, Martin TR: **Animal models of acute lung injury**. *Am J Physiol Cell Mol Physiol* 2008, **295**:L379–L399.

59. Parsons PE, Matthay MA, Ware LB, Eisner MD: **Elevated plasma levels of soluble TNF receptors are associated with morbidity and mortality in patients with acute lung injury.** *Am J Physiol Lung Cell Mol Physiol* 2005, **288**:L426–L431.

60. Markovic N, McCaig LA, Stephen J, Mizuguchi S, Veldhuizen RA, Lewis JF, Cepinskas G: **Mediators released from LPS-challenged lungs induce inflammatory responses in liver vascular endothelial cells and neutrophilic leukocytes**. *Am J Physiol liver Physiol* 2009, **297**:G1066–G1076.

61. Kessenbrock K, Dijkgraaf GJP, Lawson DA, Littlepage LE, Shahi P, Pieper U, Werb Z: **A role for matrix metalloproteinases in regulating mammary stem cell function via the Wnt signaling pathway.** *Cell Stem Cell* 2013, **13**:300–313.

62. Correia AL, Mori H, Chen EI, Schmitt FC, Bissell MJ: **The hemopexin domain of MMP3 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with HSP90β.** *Genes Dev* 2013, **27**:805–817.

CHAPTER 4:

Analysis of surfactant and lung mechanics in a mouse model of lung injury lacking matrix metalloproteinase-3 expression.

Valeria Puntorieri, Lynda A. McCaig, Scott Milos, Stephanie Aigbe, Chris Howlett, James F. Lewis, and Ruud A.W. Veldhuizen.

4.1. Introduction

Acute lung injury (ALI) is a life threatening disorder initiated by a variety of insults to the lung, among which bacterial pneumonia is one of the most common [1]. No effective pharmacological therapy is currently available for the treatment of patients with ALI, and mortality is still extremely high at approximately 40% [2, 3]. The clinical criteria of ALI include an acute onset (within days) of respiratory failure due to pulmonary edema with no evidence of left ventricular failure, and hypoxemia, the latter of which is defined by the ratio of partial pressure of arterial oxygen over the fraction of inspired oxygen (PaO_2 / F_IO_2) being lower than 300 mmHg [4].

In order to develop novel therapies for ALI, better insight into the various complex pathophysiological alterations is required. One of the biggest challenges in this regard is the poor understanding of the relationship between the primary physiological alterations related to lung mechanics and oxygenation, as determined clinically as part of the definition of ALI, and the underlying inflammatory processes that appear to influence the mortality associated with the disease [1, 5–7]. The objective of this study was to utilize an experimental model, namely the Mmp3 deficient mouse, in which altered inflammatory processes in response to lung injury have been demonstrated [8, 9], and study the potential changes in lung mechanics and its associated changes to pulmonary surfactant in this setting.

MMP-3 belongs to a family of zinc-dependent proteases that have important roles in innate immunity and inflammation [10, 11]. In the previous chapter, the role of this protease in lung injury was investigated in mice lacking Mmp3 expression ($Mmp3^{-/-}$) and in wild type controls ($Mmp3^{+/+}$) exposed to two different models of ALI (chapter 3, page 92). The results from both injury models highlighted a role for MMP-3 in the pathogenesis of this disorder. Specifically, the data showed a sex-dependent effect of MMP-3 on the pulmonary inflammatory response, with female $Mmp3^{-/-}$ mice having lower lavage concentrations of cytokines and chemokines following injury than female $Mmp3^{+/+}$ controls in both models of ALI.

Although these data constitute an important step towards a better understanding of the inflammatory response occurring in ALI, the role of MMP-3 in other aspects of the complex pathophysiology of ALI, such as lung mechanics, still needs to be investigated. It is clear that the hypoxemia defining ALI stems from many physiological and biochemical processes occurring within the lung, including decreased lung distensibility and increased work of breathing, which together with pulmonary edema, result in poor oxygenation [12]. The underlying mechanisms responsible for the alterations in the mechanical properties of the lung reside, to some extent, in the dysfunction of lung surfactant, an important substance that prevents alveolar collapse at the end of expiration [1, 13].

Lung surfactant is essential to facilitate the work of breathing [13]. Surfactant is a lipid-protein mixture secreted into the alveolar space, where it exists in two distinct structural forms: the functionally active large aggregates, and the inactive small aggregates, the latter existing as vesicular structures within the airspace [14]. Adsorbing at the air-liquid interface, the surfactant film reduces surface tension, thereby ensuring stability of the alveoli at low lung volumes and maintaining lung compliance [13]. The relationship between inactivation of lung surfactant and physiological alterations of lung function in ALI has been demonstrated in many different animal models of lung injury [12, 15–17]. In conjunction with *in vitro* analyses of surfactant samples isolated from injured lungs, these studies also have shown that the impairment in the surface tension reducing properties of the large aggregates, and the increased proportion of small aggregates relative to LA as injury progresses are associated with lower compliance and oxygenation [12, 15–17]. The mechanisms responsible for these surfactant alterations are several, and include functional inactivation by extravasated plasma proteins such as albumin and fibrinogen, and degradation and inactivation of surfactant components by proteases secreted by bacteria or inflammatory cells [18–20].

Based on this information, it was hypothesized that MMP-3 contributes to alterations to lung mechanics and surfactant function during ALI. This hypothesis was tested by exposing $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice of both sexes to an 18-hour model of lipopolysaccharide (LPS)-induced lung injury, followed by histological assessment of lung injury, measurement of pulmonary mechanics, and *in vitro* analyses of lung surfactant.

4.2. Methods

4.2.1. Animal procedures and experimental design

Mice lacking the expression of Mmp3 ($Mmp3^{-/-}$) were originally generated as previously described by Mudgett JS et al [21]. Two breeding pairs were kindly donated to our laboratory by Dr. G.P. Downey (National Jewish Hospital, Denver, Colorado), and utilized to obtain a pathogen-free colony via back-crossing with Mmp3 wild type mice ($Mmp3^{+/+}$) on a C57BL/6 background. All mice were housed in an animal research facility, with ad libitum access to food and water and daily exposed to a 12 hours light/dark cycle. All procedures were reviewed and approved by the Animal Use Subcommittee at Western University (protocol #: 2010-272).

A total of 39 $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice (17.9 ± 0.4 weeks old) of both sexes were exposed to an 18-hour model of LPS-induced lung injury previously described (chapter 3) and utilized for the analyses of lung mechanics and histology. Surfactant analyses were performed on samples isolated from a cohort of mice (14.5 ± 0.4 weeks old) utilized in chapter 3 (details in section 3.2) and exposed to the same injury model.

4.2.2. Lipopolysaccharide-induced lung injury

Female and male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice were randomized to receive an intratracheal (i.t.) instillation of LPS (from E.Coli, 0111:B4, 20 µg/mouse; Sigma, St. Louis, Mo., USA), or saline (0.15M NaCl solution) as a control. Based on the evidence from our previous study (chapter 3), data from female and male mice were analyzed separately leading, for each sex, to the following experimental groups: i) $Mmp3^{+/+}$ Saline, ii) $Mmp3^{+/+}$ LPS, iii) $Mmp3^{-/-}$ Saline, iv) $Mmp3^{-/-}$ LPS.

For a more detailed description of the instillation and other experimental procedures related to this model, please refer to chapter 3, section 3.2, page 95.

4.2.3. Analysis of lung mechanics

Eighteen hours after LPS instillation, mice received an intraperitoneal injection of sodium pentobarbital (110 mg/kg; Lundbeck, Valby, Denmark) with a euthanasing dose. When the

appropriate depth of anesthesia was reached, mice were placed in a supine position, and the lower abdomen was exposed through a small midline incision. The inferior vena cava was identified, and 50 µL of heparin (Sandoz, Boucherville, QC, Canada) were injected intravenously over a 1 minute-period, followed by mice exsanguination. A tracheostomy was subsequently performed, and an endotracheal tube was secured in the trachea. Mice were then returned to a prone position, and connected to a FlexiVent rodent ventilator (Scireq, Montreal, Quebec, Canada) for *ex vivo* measurements of lung function, via Flexiware software controlled maneuvers. Immediately after connecting the endotracheal tube to the FlexiVent ventilator, mice were exposed to 2 minutes of *ex vivo* mechanical ventilation (Vt = 10 mL/kg, RR =150 breaths/min, PEEP = 0 cmH_2O), before being exposed to 4 different maneuvers. These software-controlled procedures were performed with 10 seconds intervals of *ex vivo* ventilation. In order to standardize volume history, the first maneuver performed was a deep inflation of the lungs from PEEP value to a maximal pressure of 30 cmH_2O. This maneuver, representing the total lung capacity (TLC), also allowed for the determination of the inspiratory capacity (IC). A single frequency (150 bpm or 2.5Hz) forced oscillation technique (FOT) was subsequently performed, in which a sinusoidal waveform was over imposed on the mouse lungs. The signal output of this analysis can be implemented onto a single compartment model using linear regression, leading to determination of compliance (Crs), elastance (Ers), and resistance (Rrs) of the whole respiratory system. The third maneuver consisted of the application of a broad-band FOT (over a range of mutually prime frequencies); the resulting input impedance of the respiratory system was fit onto the constant phase model to determine values of airway resistance (R_N), tissue damping (G), and tissue elastance (H). Lastly, software-controlled pressure-volume (PV) curves were performed via step-wise increases in pressure, in order to calculate quasi-static lung compliance (Cst) and elastance (Est). An overview of maneuvers and outcomes of interest in this study is provided in Table 4.1. Once all measurements had been performed, *ex vivo* ventilation was delivered for an additional 10 seconds, before disconnecting the endotracheal tube from the ventilator and proceeding with lung isolation for histological analysis.

FlexiVent Maneuver	Description of maneuver	Outcomes	
TLC	Volume history; estimate of IC	**IC**	Inspiratory capacity
Single frequency FOT	Assessment of mechanics for the whole respiratory system	**Crs**	Dynamic compliance
		Ers	Dynamic elastance
		Rrs	Dynamic resistance
Broadband FOT	Discrimination between central and peripheral contributions to lung mechanics	**R_N**	Airway resistance
		G	Tissue damping
		H	Tissue elastance
PV curve	Traditional assessment of quasi-static lung mechanical properties	**Cst**	Quasi-static compliance
		Est	Quasi-static elastance

Table 4.1: Summary of the maneuvers and outcomes of respiratory mechanics relevant in this thesis work. TLC, total lung capacity; FOT, forced oscillation technique; PV curve, pressure-volume curve.

4.2.4. Histology

Following measurements of lung mechanics, the lungs and heart were isolated en block. Briefly, the diaphragm was cut and a midline incision was performed to open the rib cage and expose the lungs. Lungs, heart, and trachea with the previously placed endotracheal tube intact were carefully removed, connected to a manometer and air inflated to a pressure of 15 cmH_2O. A tight knot was then performed to occlude the trachea, the endotracheal tube removed, and the isolated heart-lungs block immersed in a fixative solution of 4% paraformaldehyde for at least 24 hours. Fixed lungs were rinsed twice, for 20 minutes and under gentle rocking, with phosphate buffered saline, before being placed in increasingly higher concentrations of ethanol (v/v %; 30% then 50% ethanol for 20 minutes each, at room temperature with gentle rocking), and kept in 70% ethanol at 4˚C until further processing, kindly performed by Dr. Chris Howlett (Dept. of Pathology and Laboratory Medicine, Western University, Canada). Once wax embedded, lungs were sectioned into slices and stained with hematoxylin and eosin (H&E) stain. The development of lung injury

was subsequently assessed through analysis and scoring of the lung tissue slides by a pathologist blinded to the experimental conditions.

The scoring system applied to the histological sections for the semi-quantitative assessment of lung injury is shown in Table 4.2, while figure 4.1 (A-D) provides representative pictures, from lung sections used in this experiment, exemplifying the degree of lung injury for each point of the scoring system. The lowest score assigned was 0, representing a normal histological section with no significant inflammatory or structural alterations (Fig. 4.1A), proceeding up to 3, which was assigned to lung sections with substantial neutrophil infiltrate and structural damage (Fig. 4.1D).

Score	Evaluation Criteria
0	No significant neutrophil infiltrate/inflammatory changes
1	Focal peribronchial interstitial or alveolar neutrophilic infiltrate
2	Patchy interstitial neutrophilic infiltrate, +/- alveolar involvement; septal thickening
3	Diffuse interstitial and/or alveolar neutrophilic infiltrate, or patchy interstitial and/or alveolar neutrophil infiltrate with parenchymal destruction

Table 4.2: Scoring system utilized for the assessment of lung injury on H&E stained lung sections.

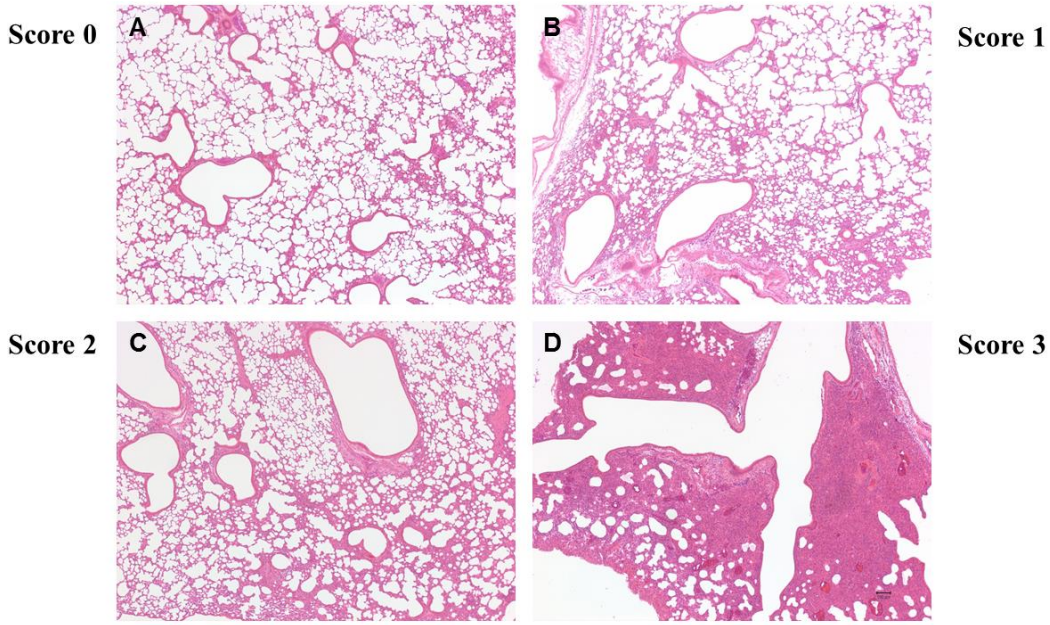

Figure 4.1: Representative images of the histology score utilized for the analyses of lung sections isolated from female and male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice, 18 hours post intra-tracheal instillation of LPS or saline. (**A**) Score 0, (**B**) score 1, (**C**) score 2, (**D**) score 3. Slides stained with hematoxylin and eosin; magnification, 40x. Scale bar = 100µm shown in the bottom right corner of panel **D.**

4.2.5. Lung lavage isolation and surfactant analysis

Lung lavage samples were isolated from the cohort of mice described in chapter 3. Experimental details about lung lavage isolation are provided in chapter 3, section 3.2.4, page 97. Following centrifugation of whole lung lavage at 380 g at 4˚C for 10 min., the supernatant was collected and termed total surfactant (TS). Part of TS was aliquoted and utilized for the measurements of inflammatory mediators and protein content described in chapter 3; 1 mL of TS was centrifuged at 40,000 g at 4˚C for 15 min. to separate the heavier large aggregate (LA) sub-fraction (pellet) from the small aggregate (SA) sub-fraction (supernatant). The LA pellet was resuspended in 0.3 mL of 0.9% NaCl. The remaining volume of TS was utilized for analysis of total surfactant pool sizes. TS, LA, and SA were stored frozen at -80˚C until further analysis. The phospholipid content of TS, LA, and SA was determined via phosphorous assay on the lipid component extracted by the Bligh and Dyer method, as previously described [22, 23], and corrected by body weight. The percent LA indicates the percent proportion of LA over the sum of LA and SA.

4.2.6. Biophysical analysis of surfactant activity

Individual crude LA samples were centrifuged at 21,000 g at 4˚C for 15 minutes, and the resulting pellets were resuspended in a buffer solution (2.5 mM HEPES, 1.5 mM $CaCl_2$, pH 7.2) to a final phospholipid concentration of 1 mg/mL. For each of these LA samples, 9 to 10 µL were utilized for the analysis of their surface tension reducing properties through a computer controlled constrained sessile drop surfactometer, as previously described [24]. This system is composed of a light source (Advanced Illuminator CS410, Rochester, VT, USA) and a microscope connected to a camera (1.3 Megapixel CMOS monochrome camera), all directed at a pedestal on which LA samples are deposited, thereby forming a drop. The pedestal, which has the top and lateral sides arranged in an approximately 60˚ angle, has a sharp edge that prevents the drop from spilling over. It is also provided with a central hole, which is connected to a motor-driven syringe controlled by a computer software and utilized for the cyclic compression and expansion of the surfactant drop. Twenty-five to thirty cycles were performed per minute, at 37˚C, for each LA sample. All processes of image recording and image analysis were automated. Images were recorded at 10 frames per second throughout the duration of the thirty cycles. All images were

sequentially analyzed via the axisymmetric drop shape analysis, providing values of surface tension and surface area for each picture during the complete dynamic cycles. Values of minimum surface tension at cycles 1-10, 15, 20, and 25 were subsequently analyzed. The percent surface area compression necessary to reach minimum surface tension at cycle 10 was also measured for each drop.

4.2.7. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). The GraphPad statistical software (La Jolla, CA, USA) was used to perform statistical analyses. A two-way ANOVA (variables: genotype and treatment) was utilized to analyze the data, followed by a one-way ANOVA with a Tukey's post-hoc test. $P<0.05$ was considered statistically significant.

4.3. Results

Lung histology

Representative pictures of lung histology with a summary of the histological score are shown in Figure 4.2 for female mice, and Figure 4.3 for male mice. Overall, no differences were found in the lung histology of Saline-instilled female (Fig. 4.2A, C, E) or male mice (Fig. 4.3A, C, E), indicating that the genotype itself had no effect on the histological indications of lung injury.

Based on the scoring system outlined in table 4.2, the analysis of lung histological sections from female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice showed a significantly higher histology score in LPS-instilled mice compared to their respective Saline controls (Fig. 4.2E), with increased cellularity and parenchymal damage following LPS injury (Fig. 4.2B, D) than after saline instillation (Fig. 4.2A, C). No effect of the genotype was observed (Fig. 4.2 A-E).

In male mice, the increases in the histology score of LPS-injured mice reached significance only in the $Mmp3^{-/-}$ group compared to Saline controls (Fig. 4.3E). The extent of lung injury in LPS-instilled male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice was mild to moderate, with peribronchial infiltrates, thickening of the septa and/or patchy areas of more pronounced inflammation following injury (Fig. 4.3B, D) compared to saline instillation (Fig. 4.3 A, C). No effect of the genotype was observed (Fig. 4.3E).

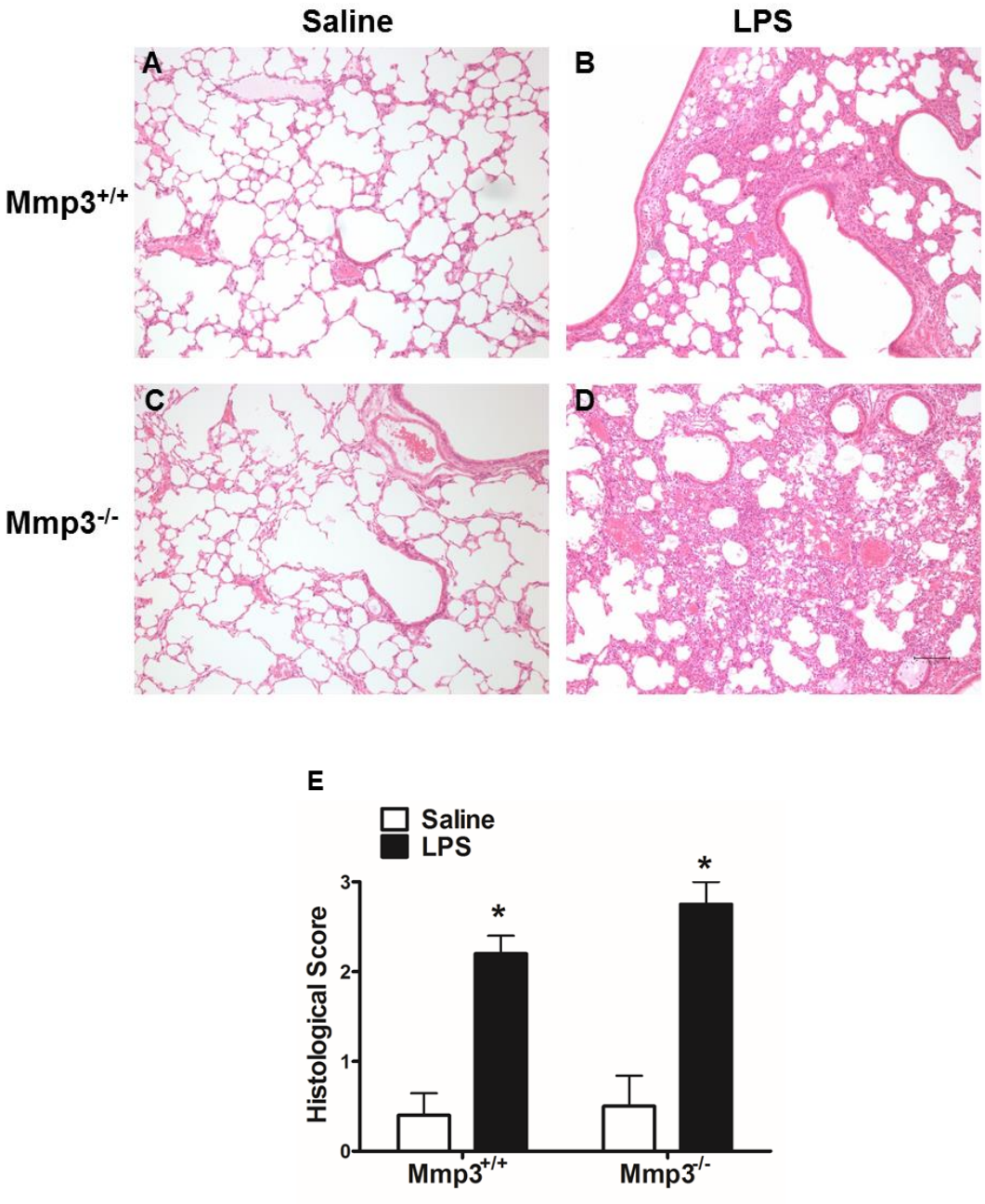

Figure 4.2: Hematoxylin-eosin stained pictures representative of lung sections from female $Mmp3^{+/+}$ (**A, B**) and $Mmp3^{-/-}$ (**C, D**) mice, 18 hours after saline (**A, C**) or LPS (**B, D**) instillation. Magnification, 100x. (**E**) Quantitative histological assessment of lung injury. Analyses were performed on lung sections from female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice, according to the scoring system described above. Data are expressed as mean ± SEM; n=4-6 per group; *$p<0.05$ vs Saline control. Scale bar = 100µm shown in the bottom right corner of panel **D.**

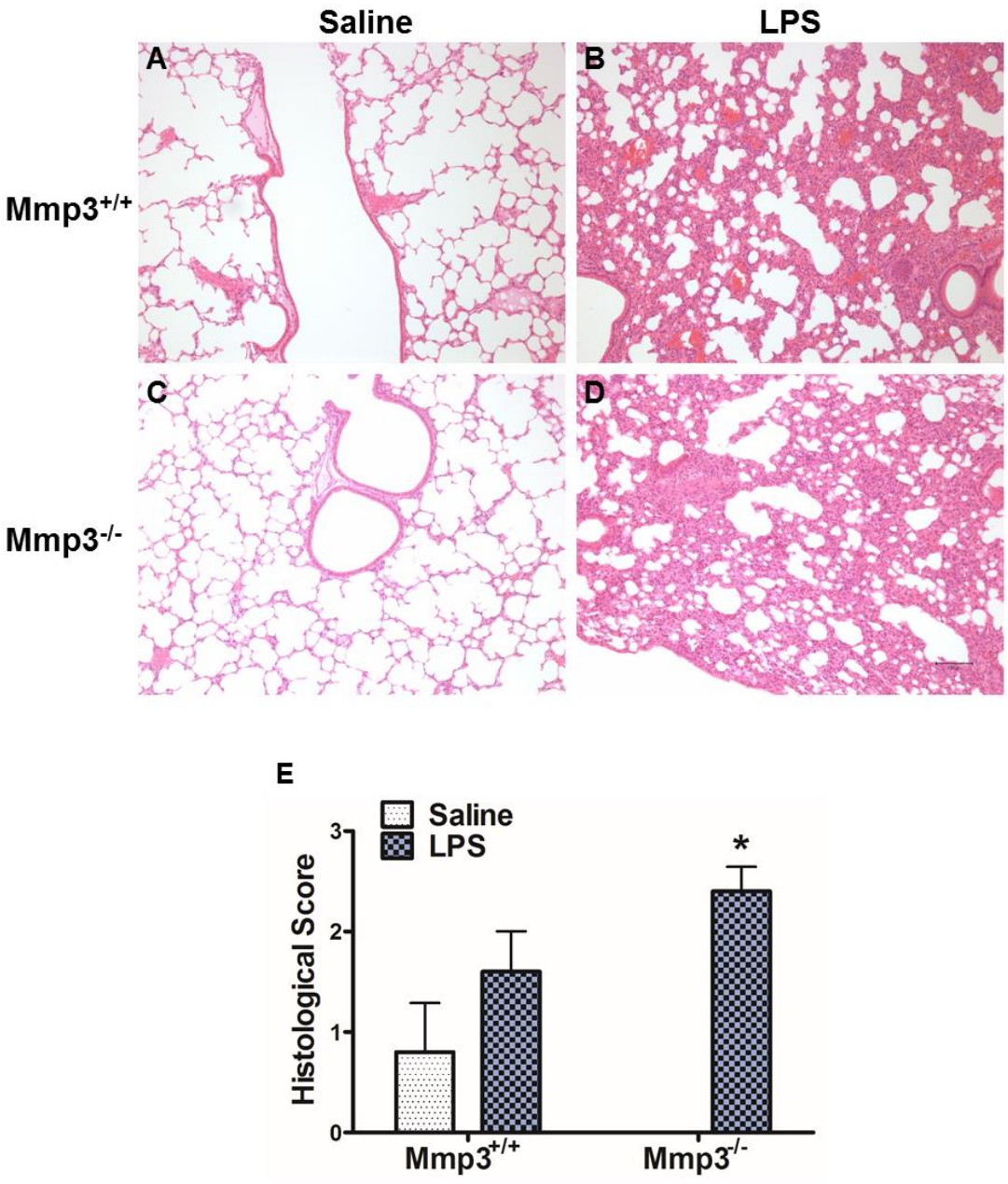

Figure 4.3: Representative hematoxylin-eosin stained pictures of lung sections from male $Mmp3^{+/+}$ (**A, B**) and $Mmp3^{-/-}$ (**C, D**) mice, 18 hours after saline (**A, C**) or LPS (**B, D**) instillation. Magnification, 100x. (**E**) Quantitative histological assessment of lung injury. Analyses were performed on lung sections from male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice, according to the scoring system described above. Data are expressed as mean ± SEM; n=4-5 per group; *$p<0.05$ vs Saline control. Scale bar = 100µm shown in the bottom right corner of panel **D.**

Lung mechanics

Results from the assessment of respiratory mechanics in female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice are shown in figure 4.4A-I. No differences due to genotype were observed in Saline controls, for any of the outcomes. LPS-instilled mice showed a reduction in inspiratory capacity (IC; Fig. 4.4G) that reached statistical significance only in the LPS-injured $Mmp3^{-/-}$ group, compared to the Saline controls. Analysis of dynamic compliance (Crs), dynamic elastance (Ers), and dynamic resistance (Rrs) for the whole respiratory system demonstrated alterations of these parameters only in $Mmp3^{-/-}$ mice following LPS injury. Specifically, Crs was significantly reduced, while Ers and Rrs were significantly increased in female LPS-injured $Mmp3^{-/-}$ mice compared to Saline (Fig. 4.4A, B, C respectively).

LPS-induced lung injury also led to a significant reduction in quasi-static compliance (Cst; Fig. 4.4D) and a significant increase in quasi-static elastance (Est; Fig. 4.4E) in female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice, compared to Saline-instilled controls. Lastly, no changes were observed overall for measurements of central airway resistance (R_N; Fig. 4.4F), while LPS instillation led to significantly higher tissue resistance (tissue damping, G; Fig 4.4H) and elastance (H; Fig. 4.4I) only in female $Mmp3^{-/-}$ mice compared to Saline controls. No statistical differences due to genotype were observed in LPS-instilled female mice, for any of the measurements performed (Fig. 4.4A-I).

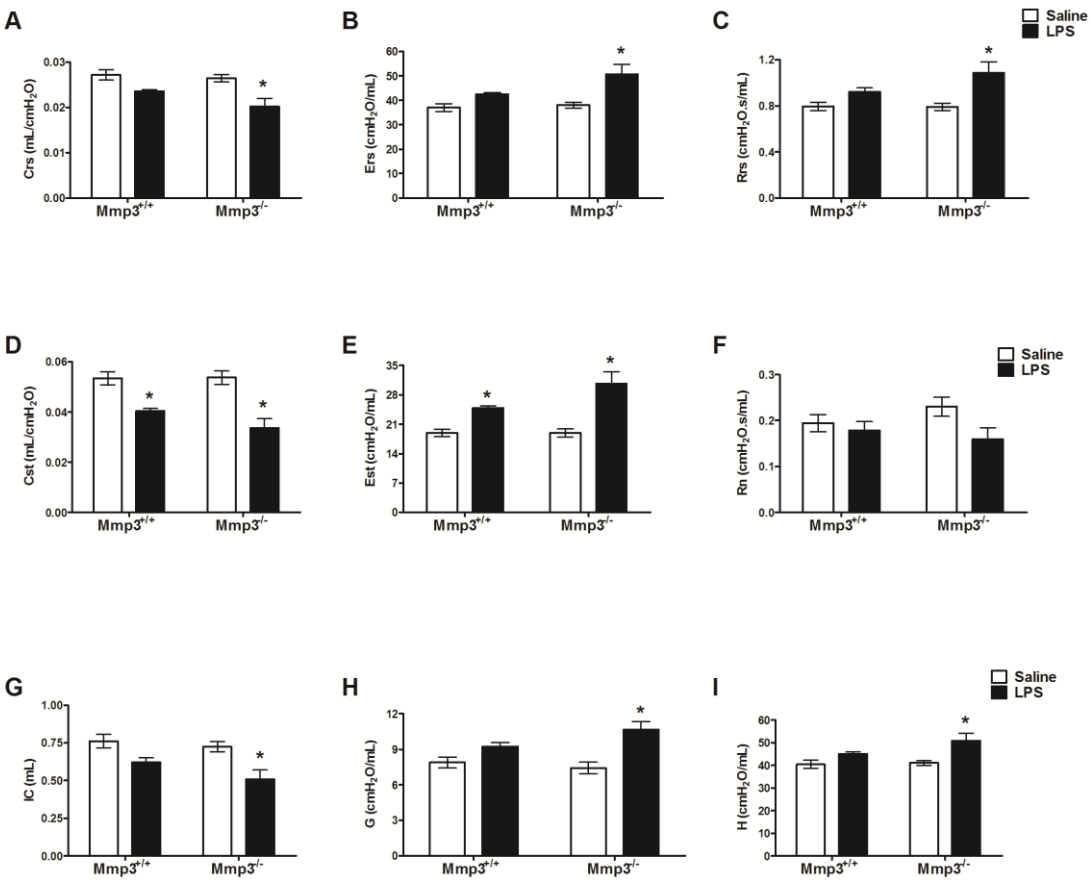

Figure 4.4: Analysis of respiratory mechanics in female $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice, at 18 hours post LPS or saline instillation. (**A**) Lung compliance (Crs), (**B**) elastance (Ers), and (**C**) resistance (Rrs) of the whole respiratory system were obtained through a single frequency forced oscillation technique (FOT). (**D**) Quasi-static compliance (Cst) and (**E**) elastance (Est) resulted from P-V curves performed via step-wise increases in pressure. Broadband FOT was utilized to obtain values of (**F**) central airway resistance (R_N), (**H**) tissue damping (G), and (**I**) tissue elastance (H). (**G**) IC, inspiratory capacity. Data are expressed as mean ± SEM; n=4-6 per group; *$p<0.05$ vs Saline control.

Respiratory mechanics were also analyzed in male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice (Fig. 4.5A-I). No differences were present due to genotype between Saline controls. Following LPS instillation, no significant changes were observed in inspiratory capacity (Fig. 4.5G), dynamic compliance, elastance, and resistance of the respiratory system (Fig. 4.5A, B, and C respectively) compared to Saline-instilled mice in both genotypes. A decrease in quasi-static compliance and an increase in quasi-static elastance was observed in male, LPS-instilled $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice compared to Saline controls (Fig. 4.5D, E); however, these differences did not reach statistical significance. Additionally, measurements of airway resistance (Fig. 4.5F), tissue damping indicative of tissue resistance (Fig. 4.5H), and tissue elastance (Fig. 4.5I) did not show any significant change due to LPS injury. Lastly, no effect of the genotype was observed for any of the performed measurements in male, LPS-injured mice (Fig. 4.5A-I).

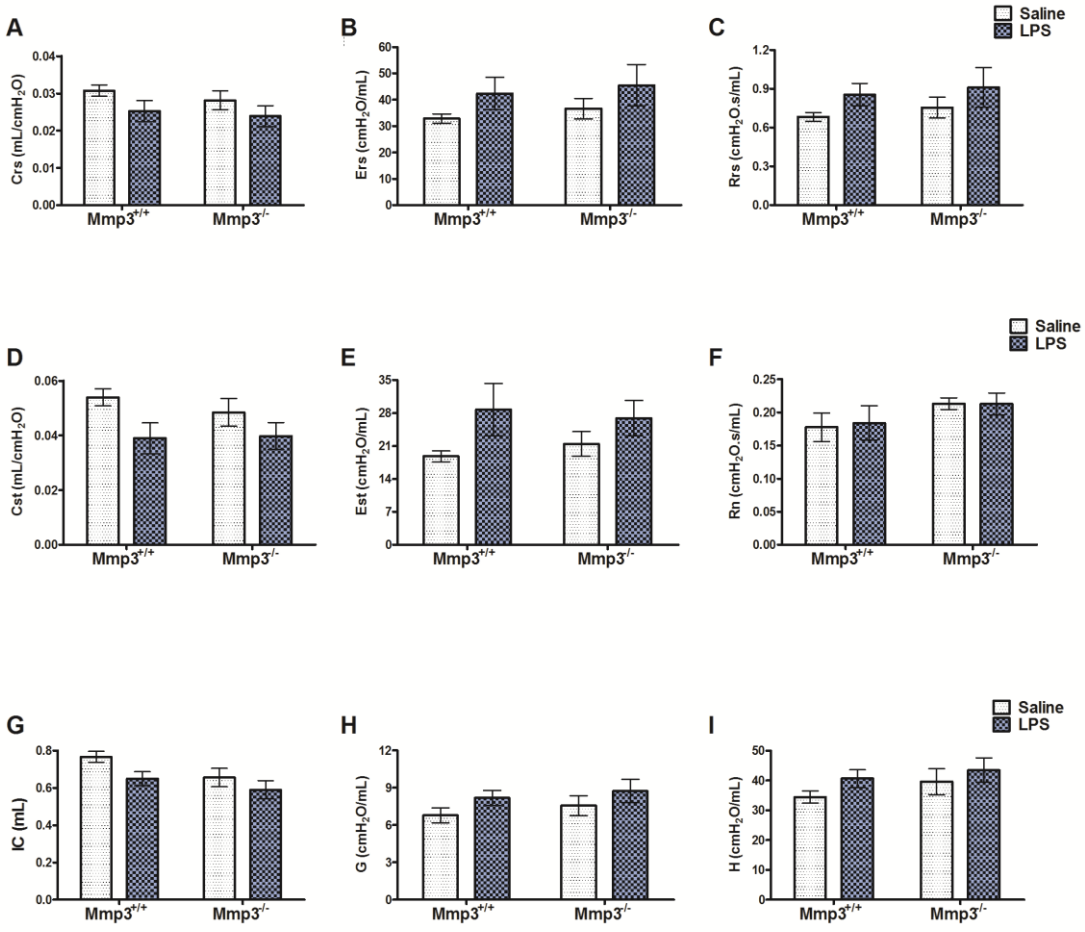

Figure 4.5: Analysis of respiratory mechanics in male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice, at 18 hours post LPS or saline instillation. (**A**) Lung compliance (Crs), (**B**) elastance (Ers), and (**C**) resistance (Rrs) of the whole respiratory system were obtained through a single frequency forced oscillation technique (FOT). (**D**) Quasi-static compliance (Cst) and (**E**) elastance (Est) resulted from P-V curves performed via step-wise increases in pressure. Broadband FOT was utilized to obtain values of (**F**) central airway resistance (R_N), (**H**) tissue damping (G), and (**I**) tissue elastance (H). (**G**) IC, inspiratory capacity. Data are expressed as mean ± SEM; n=4-6 per group; *$p<0.05$ vs Saline control.

Surfactant analysis and biophysical activity

The analysis of surfactant sub-fractions is shown in figure 4.6A-D for both sexes. Overall, there were no differences in surfactant pool sizes between Saline instilled mice as a result of the different genotype. This was observed in both female (Fig. 4.6A, B) and male mice (Fig. 4.6C, D).

In female mice, no differences were found in TS and SA pool sizes following LPS injury, or as a result of lacking Mmp3 expression (Fig. 4.6A). The amount of LA (Fig. 4.6A) and the percent LA (Fig. 4.6B) were significantly higher in female, LPS-injured $Mmp3^{+/+}$ mice compared to Saline control, while no increases in the LA sub-fraction (Fig. 4.6A) and percent LA (Fig. 4.6B) were found in female, $Mmp3^{-/-}$ mice exposed to LPS injury. Furthermore, female LPS-instilled $Mmp3^{-/-}$ mice had significantly lower LA amounts than LPS-injured $Mmp3^{+/+}$ mice (Fig. 4.6A).

In male mice, no significant differences were observed in the amounts of TS, LA, or SA between LPS-injured and Saline-instilled mice; moreover, there was no effect of the genotype on male surfactant pool sizes (Fig. 4.6C). In line with these observations, there were no significant changes in the percent LA retrieved following LPS injury or in mice lacking Mmp3 (Fig. 4.6D).

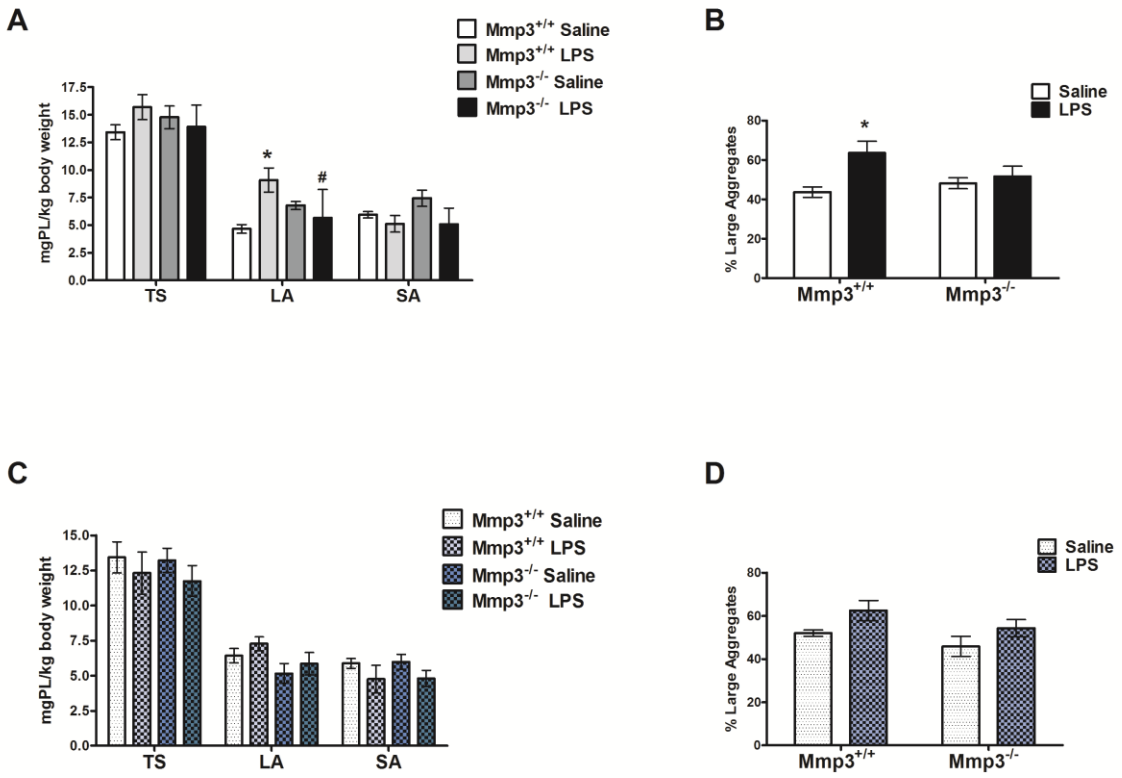

Figure 4.6: Surfactant pool sizes (**A, C**) and percent large aggregates (**B, D**) measured in female (**A, B**) and male (**C, D**) $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice, 18 hours after LPS or saline instillation. (**A, C**) Total surfactant (TS), large aggregate (LA), and small aggregate (SA) sub-fractions were measured in isolated lung lavage samples. The data represent the amount of phospholipids normalized by body weight. Within each sub-fraction, *$p<0.05$ vs Saline control of the same genotype; #$p<0.05$ vs $Mmp3^{+/+}$ LPS. (**B, D**) The data represent the proportion of LA over the sum of LA and SA. *$p<0.05$ vs Saline control of the same genotype. Data are expressed as mean ± SEM; n=5-6 in each group.

The biophysical activity of LA samples was evaluated using a constrained sessile drop surfactometer. The minimum surface tension achieved by LA samples during repeated cycles of compression-expansion is shown in Table 4.3. Minimum surface tension of LA samples from Saline instilled mice did not differ between genotypes in both female and male mice.

No differences were found in the surface activity of LA samples from female, LPS-injured mice compared to Saline controls at cycles 1, 5, and 10 of dynamic compression-expansion (Table 4.3). Furthermore, no effect of the genotype on LA biophysical activity was observed for any of the cycles (Table 4.3).

LA samples from male, LPS-injured mice also reached values of minimum surface tension that were not significantly different from Saline-instilled controls (Table 4.3); moreover, lack of Mmp3 expression did not affect the surface activity of LA in male mice at cycles 1, 5, and 10 (Table 4.3).

The surface tension reducing properties of LA samples were further assessed through measurements of the percent area compression necessary to achieve minimum surface tension at a given cycle of compression-expansion (cycle 10). The data in Table 4.4 show no significant differences in percent area compression at baseline between Saline-instilled mice, and in LPS-instilled mice of either sex compared to the respective Saline-instilled controls. Additionally, no effect of the genotype was observed in LPS-instilled female or male mice (Table 4.4).

Minimum Surface Tension (mN/m)				
Cycle #		**1**	**5**	**10**
Female mice	**$Mmp3^{+/+}$ Saline**	6.1±0.4	5.7±0.4	5.8±0.6
	$Mmp3^{+/+}$ LPS	5.6±0.4	4.8±0.2	4.6±0.6
	$Mmp3^{-/-}$ Saline	5.7±0.2	5.5±0.6	6.0±1.0
	$Mmp3^{-/-}$ LPS	5.0±0.2	5.3±0.6	4.8±1.0
Male mice	**$Mmp3^{+/+}$ Saline**	6.7±0.4	5.0±0.4	4.7±0.7
	$Mmp3^{+/+}$ LPS	5.6±0.2	5.5±0.3	4.9±0.7
	$Mmp3^{-/-}$ Saline	7.0±0.7	6.4±0.8	6.6±1.1
	$Mmp3^{-/-}$ LPS	7.1±0.3	4.9±0.4	4.6±0.7

Table 4.3: Surface activity of LA samples at cycle #1, #5, and #10 of dynamic compression-expansion cycles, performed via a constrained sessile drop surfactometer. Data are expressed as mean ± SEM; n=5-6 in each group.

% Area Compression (cycle 10)	**$Mmp3^{+/+}$**		**$Mmp3^{-/-}$**	
	Saline	**LPS**	**Saline**	**LPS**
Female mice	13.1±0.8	13.5±1.0	13.1±0.4	13.3±1.0
Male mice	15.9±0.3	15.3±0.5	16.6±0.7	16.8±0.4

Table 4.4: Percent area compression at cycle #10 of dynamic compression-expansion, needed to achieve minimum surface tension. Measurements performed via a constrained sessile drop surfactometer. Data are expressed as mean ± SEM; n=5-6 in each group.

4.4. Discussion

The objective of the present study was to investigate whether MMP-3, previously shown to modulate the pulmonary inflammatory response in models of lung injury (Chapter 3), could affect the changes in histology, respiratory mechanics, and surfactant function associated with ALI. Analysis of lung histological sections from female and male $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice demonstrated alterations in the alveolar architecture and increased neutrophil infiltration in response to LPS instillation, which was consistent with previous observations of this injury model. Notably, lungs from female mice exposed to LPS were less compliant and had greater elastic recoil compared to Saline controls, while such changes in respiratory mechanics were less prominent and not statistically significant in male mice given LPS versus control. Lastly, isolated surfactant samples retained good biophysical properties across all experimental groups. No effect of Mmp3 ablation was observed for the majority of the measured outcomes following LPS instillation, with the exception of LA pool size in female, LPS-injured mice. Furthermore, no differences due to genotype were observed at baseline between Saline-instilled mice in both sexes. Based on these data, and on the observations from our previous study (chapter 3), it was concluded that although MMP-3 impacts inflammation in LPS-induced injury in female mice, this protease does not impact pulmonary mechanics and surfactant function associated with lung injury.

The contribution of MMP-3 to lung injury has been previously demonstrated in models of ALI initiated by intra-tracheal instillation of immunoglobulin G, or instillation of the chemokine MIP-2 [8, 9]. In those models, lack of Mmp3 mitigated the development of injury mainly through reduced recruitment of inflammatory neutrophils to the lung [8, 9]. In our previous study (chapter 3), lack of Mmp3, while not affecting lavage neutrophil numbers, led to decreased inflammatory mediators in the lavage of female mice exposed to LPS-induced ALI. This same injury model has been utilized in the current work to investigate the interplay between pulmonary inflammation, surfactant, and lung mechanics in order to gain a better understanding of ALI pathophysiology. In ALI, this relationship appears to be very complex with various experimental evidence supporting an important role for surfactant in modulating pulmonary inflammation, but with other evidence

supporting a marked effect of inflammation on surfactant composition and function and, consequently, lung mechanics. Furthermore, in some other studies, inflammation and surfactant alterations appear to occur independently.

For example, modalities of mechanical ventilation (MV), the main supportive therapy in ALI, that are considered to be injurious for the lung can lead to surfactant impairment and decreased compliance, and have been associated with increased pulmonary inflammation [12, 25, 26]. Interestingly, maintaining an active surfactant system in this scenario has been shown to reduce pulmonary inflammation [27]. Lung surfactant, comprised of phospholipids and surfactant associated proteins (surfactant proteins A-D), also has important immuno-modulatory properties within the alveolar space [13, 28]. Specifically, surfactant protein C (SP-C) has been recently shown to have an anti-inflammatory role in a model of chronic LPS exposure, as mice lacking SP-C expression had more intense lung inflammation compared to wild type [29]. Additionally, numerous evidence support the role of SP-A and SP-D in host defense and inflammation. SP-A has been shown to modulate levels of inflammatory mediators such as TNF-α and, interestingly, mice lacking the expression of SP-A have higher lavage concentrations of IL-6 and TNF-α following lung injury [28, 30]. Thus, there is strong evidence supporting the concept that pulmonary surfactant can impact inflammatory responses.

Conversely, and more relevant to the current study, inflammatory mediators released in the alveolar space during ALI can affect lung surfactant metabolism, and inhibit the expression of surfactant proteins such as SP-A and SP-B with potential consequences on surfactant biophysical function [31–33]. Moreover, proteases from inflammatory cells or bacteria present within the alveolar space have been shown to degrade surfactant associated proteins, thereby altering aggregate conversion and increasing surfactant minimum surface tension values [19, 20, 34]. Whether any of the four surfactant associated proteins could be a potential substrate for MMP-3 is currently not known. In our experiment, the lower concentration of inflammatory mediators released in the alveolar space of female $Mmp3^{-/-}$ mice after LPS injury (Chapter 3) did not appear to have a major impact on surfactant function and lung mechanics when compared to $Mmp3^{+/+}$ mice. These data, in part, reproduced the findings by Nakamura et al. which demonstrated that mice lacking the

expression of Il6, a major cytokine and marker of ALI, had changes in surfactant and lung function following lung injury that were not different from the ones affecting injured, wild type animals [35]. Yamashita et al. also showed that, in mice with aberrant inflammatory processes due to absence of Apolipoprotein E, the elevated lavage concentrations of IL-6, KC, MCP-1, and TNF-α after lung injury did not correlate with physiological outcomes measured in the study [36]. Indeed, surfactant pool sizes, percent LA, oxygenation, and lung distensibility were not different from the ones measured in control injured mice, which experienced only minor inflammatory changes [36]. Together, these data underscore the complex nature of the relationship between inflammation and surfactant. It is likely that the specific cause of lung inflammation, the different cell types involved, as well as the nature and concentrations of the inflammatory mediators have all an impact on these processes; future studies are required to clarify these aspects.

Although no major effect of genotype was observed in the present study, the biochemical and functional changes to lung surfactant in this model are of general interest to our understanding of ALI. In our analysis of surfactant pool sizes, a significant *increase* in the active LA sub-fraction and percent LA was observed in female, LPS-instilled $Mmp3^{+/+}$ compared to $Mmp3^{+/+}$ Saline control, while female LPS-instilled $Mmp3^{-/-}$ mice maintained LA pool sizes comparable to $Mmp3^{-/-}$ Saline control. Similar observations were reported by Nakamura et al. and Malloy et al. in spontaneously breathing animals exposed to a cecal ligation and perforation model of sepsis-induced ALI [16, 26, 37, 38]. In these studies, no differences were found in LA pool sizes between control and septic animals; moreover, as reported by Malloy and colleagues, the percent LA relative to TS was significantly increased in spontaneously breathing septic mice compared to uninjured controls [16]. Interestingly, the application of mechanical ventilation to septic lungs led to alterations in surfactant aggregates more reflective of the surfactant alterations observed in ALI patients all of whom are, in fact, mechanically ventilated [16, 39]. Such changes in aggregates consist in a relative decrease in LA and increase in SA. This has been shown to result from the increased conversion of LA into SA during lung injury, process that is influenced by changes in tidal volumes and consequent changes in the alveolar surface area [40]. In other words, the larger tidal volumes delivered with mechanical ventilation, compared to spontaneous breathing, cause larger surface area changes and greater LA to SA conversion,

leading to decreased percent LA. Even though tidal volumes were not measured in the current work, it could be speculated that the tidal volume in female mice was reduced following LPS injury, thereby preserving LA pool sizes.

Importantly, preservation of the LA pool sizes may have partially overcome inhibition of surfactant by leaked serum proteins, or released proteases. In this sense, our biophysical surfactant analyses demonstrated that LA samples from mice of both sexes and genotypes retained good surface tension reducing properties, even following LPS injury. These findings seemingly conflict with the notion that decreased compliance in ALI results from impaired surfactant function; however, regional variability in LPS-induced lung injury should also be taken into consideration when interpreting these data. As surfactant is routinely isolated through lavage of the whole lung, impaired material originating from the more injured areas may ultimately combine with surfactant from functional, non-injured alveoli. It becomes more difficult, therefore, to detect surfactant impairments, when present, under all the aforementioned circumstances.

Despite such limitations, the biophysical analysis of surfactant constitutes an innovative aspect of this study. This is indeed the first time that the surface activity of surfactant samples from $Mmp3^{-/-}$ mice has been analyzed, both at baseline (Saline group) and post-lung injury (LPS group). Moreover, accurate and sensitive determination of surface tension was obtained through the use of a constrained sessile drop surfactometer, a new technology that also provides rigorous control of experimental settings such as temperature and humidity of the chamber where the sessile drop is contained [24, 41]. Most importantly, the constrained sessile drop surfactometer requires the use of small sample volumes, thereby overcoming the necessity of sample-pooling that is typical of traditional systems of surfactant analysis (see chapter 2, captive bubble surfactometer) and allowing measurements of surface tension in individual mouse LA samples.

In addition to analyzing surfactant, one of the goals of this study was to expand the knowledge of the respiratory mechanics in $Mmp3^{-/-}$ mice, both at baseline and following lung injury. Previous measurements of lung compliance were performed in $Mmp3^{-/-}$ mice following bleomycin-induced fibrosis [42]. That study showed greater compliance in bleomycin-instilled $Mmp3^{-/-}$ mice compared to $Mmp3^{+/+}$ controls, and highlighted a role

for MMP-3 in the development of lung fibrosis following acute inflammation [42]. Aside from this particular study, the information related to the changes in respiratory mechanics associated with lung injury in $Mmp3^{-/-}$ mice is very limited. Our investigation addressed this knowledge gap through the use of the FlexiVent mechanical ventilator, a system that provides reliable and reproducible measurements of lung mechanics, task that is otherwise difficult to accurately perform in spontaneously breathing mice [43, 44]. In general, it was observed that lack of Mmp3 did not affect respiratory mechanics at baseline or following injury in both sexes. Measurements of quasi-static compliance and quasi-static elastance in our study illustrated changes in female, LPS instilled mice that were consistent with mechanical alterations typical of ALI. Additionally, a significant decrease in dynamic compliance (Crs), and significant increases in elastance and resistance of the whole respiratory system (Ers, Rrs) were only observed in female, LPS injured $Mmp3^{-/-}$ mice compared to Saline $Mmp3^{-/-}$ controls, but not within the $Mmp3^{+/+}$ groups. These mechanical changes were possibly related to modifications in the lung parenchyma, as suggested by the increases in tissue resistance (G) and tissue elastance (H), which are representative of the dissipative and elastic properties of the lung tissue, but no significant changes in the resistance of the central airways (R_N). It could be speculated that such changes observed in female, LPS injured $Mmp3^{-/-}$ mice compared to Saline $Mmp3^{-/-}$ controls stemmed from a slower repair process in the $Mmp3^{-/-}$ mice. It has been previously shown that early contraction of dermal wounds occurred more slowly *in vivo* in $Mmp3^{-/-}$ mice compared to $Mmp3^{+/+}$ mice, and that $Mmp3^{-/-}$ lung fibroblasts demonstrated impaired contraction *in vitro* [45, 46]. Nevertheless, $Mmp3^{-/-}$ mice exposed to bleomycin injury were protected from developing pulmonary fibrosis, suggesting that the overall repair process is ultimately functional in these mice [42].

Lastly, while LPS-induced lung injury appeared to elicit significant responses in female mice for most of the measured outcomes, instillation of LPS in male mice did not always yield significant changes compared to their respective Saline controls, as particularly evident in the analyses of respiratory mechanics and surfactant pool sizes. These findings are consistent with observations noted in chapter 3, in which the inflammatory response of male mice exposed to the same 18 hour model of LPS-induced lung injury appeared to be of lower magnitude than the one elicited in $Mmp3^{+/+}$ female mice. A formal statistical

comparison between male and female mice, however, has not been performed in either studies, and further investigations are required to appropriately address the issue of sex differences in the response to injury.

In conclusion, this study investigated the interplay between pulmonary inflammation, lung mechanics and surfactant function in a mouse model of ALI, specifically expanding our knowledge of the role of MMP-3 in the modulation of physiological outcomes of ALI. The results from the current study suggest that MMP-3 did not appear to participate in such modulation, and that the decrease in lavage inflammatory mediators (i.e. IL-6, G-CSF, MIP-2, and TNF-α) previously observed in female $Mmp3^{-/-}$ mice with lung injury (Chapter 3) did not correlate with superior lung mechanics and surfactant function following LPS-induced ALI. The specific interplay between alveolar cytokines and chemokines, lung surfactant and pulmonary function requires further investigation. Given the correlation between soluble inflammatory mediators and outcomes of ALI, a better understanding of these relationships could ultimately result in more effective treatments and an improved mortality.

4.5. References

1. Ware LB, Matthay MA: **The Acute Respiratory Distress Syndrome**. *N Engl J Med* 2000, **342**:1334–1349.

2. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD: **Incidence and outcomes of acute lung injury**. *N Engl J Med* 2005, **353**:1685–1693.

3. Rubenfeld GD, Herridge MS: **Epidemiology and outcomes of acute lung injury**. *Chest* 2007, **131**:554–562.

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

5. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, Slutsky AS: **Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial**. *JAMA* 1999, **282**:54–61.

6. The Acute Respiratory Distress Syndrome Network: **Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network**. *N Engl J Med* 2000, **342**:1301–1308.

7. Stapleton RD, Wang BM, Hudson LD, Rubenfeld GD, Caldwell ES, Steinberg KP: **Causes and timing of death in patients with ARDS**. *Chest* 2005, **128**:525–532.

8. Nerusu KC, Warner RL, Bhagavathula N, McClintock SD, Johnson KJ, Varani J: **Matrix metalloproteinase-3 (stromelysin-1) in acute inflammatory tissue injury**. *Exp Mol Pathol* 2007, **83**:169–176.

9. Warner RL, Beltran L, Younkin EM, Lewis CS, Weiss SJ, Varani J, Johnson KJ: **Role of stromelysin 1 and gelatinase B in experimental acute lung injury**. *Am J Respir Cell Mol Biol* 2001, **24**:537–544.

10. Parks WC, Wilson CL, López-Boado YS: **Matrix metalloproteinases as modulators of inflammation and innate immunity**. *Nat Rev Immunol* 2004, **4**:617–629.

11. Manicone AM, McGuire JK: **Matrix metalloproteinases as modulators of inflammation**. *Semin Cell Dev Biol* 2008, **19**:34–41.

12. Maruscak AA, Vockeroth DW, Girardi B, Sheikh T, Possmayer F, Lewis JF, Veldhuizen RAW: **Alterations to surfactant precede physiological deterioration during high tidal volume ventilation.** *Am J Physiol Lung Cell Mol Physiol* 2008, **294**:L974–L983.

13. Goerke J: **Pulmonary surfactant: functions and molecular composition**. *Biochim Biophys Acta* 1998, **1408**:79–89.

14. Brackenbury AM, Malloy JL, McCaig LA, Yao LJ, Veldhuizen RA, Lewis JF: **Evaluation of alveolar surfactant aggregates in vitro and in vivo**. *Eur Respir J* 2002, **19**:41–46.

15. Bailey TC, Maruscak AA, Petersen A, White S, Lewis JF, Veldhuizen RA: **Physiological effects of oxidized exogenous surfactant in vivo: effects of high tidal volume and surfactant protein A**. *Am J Physiol Cell Mol Physiol* 2006, **291**:L703–L709.

16. Malloy JL, Veldhuizen RAW, Lewis JF: **Effects of ventilation on the surfactant system in sepsis-induced lung injury**. *J Appl Physiol* 2000, **88**:401–408.

17. Welk B, Malloy JL, Joseph M, Yao LJ, Veldhuizen A: **Surfactant treatment for ventilation-induced lung injury in rats: effects on lung compliance and cytokines.** *Exp Lung Res* 2001, **27**:505–520.

18. Seeger W, Grube C, Gunther A, Schmidt R: **Surfactant inhibition by plasma proteins: differential sensitivity of various surfactant preparations**. *Eur Respir J* 1993, **6**:971–977.

19. Malloy JL, Veldhuizen RAW, Thibodeaux BA, Callaghan RJO, Wright JR: **Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions**. *Am J Physiol Lung Cell Mol Physiol* 2005, **288**:L409–L418.

20. Beatty AL, Malloy JL, Wright JR: **Pseudomonas aeruginosa Degrades Pulmonary Surfactant and Increases Conversion In Vitro**. *Am J Respir Cell Mol Biol* 2005, **32**:128–134.

21. Mudgett JS, Hutchinson NI, Chartrain NA, Forsyth AJ, Mcdonnell J, Singer II, Bayne EK, Flanagan J, Kawka D, Shen CF, Stevens K, Chen H, Trumbauer M, Visco DM: **Susceptibility of stromelysin 1-deficient mice to collagen-induced arthritis and cartilage destruction**. *Arthritis Rheum* 1998, **41**:110–121.

22. Bligh EG, Dyer WJ: **A rapid method of total lipid extraction and purification**. *Can J Biochem Physiol* 1959, **37**:911–917.

23. Duck-Chong CG: **A rapid sensitive method for determining phospholipid phosphorous involving digestion with magnesium nitrate.** *Lipids* 1979, **14**:492–497.

24. Yu LMY, Lu JJ, Chan YW, Ng A, Zhang L, Hoorfar M, Policova Z, Grundke K, Neumann AW: **Constrained sessile drop as a new configuration to measure low surface tension in lung surfactant systems.** *J Appl Physiol* 2004, **97**:704–15.

25. Chiumello D, Pristine G, Slutsky AS: **Mechanical ventilation affects local and systemic cytokines in an animal model of acute respiratory distress syndrome**. *Am J Respir Crit Care Med* 1999, **160**:109–116.

26. Nakamura T, Malloy J, Mccaig L, Yao L, Joseph M, Lewis J, Veldhuizen R: **Mechanical ventilation of isolated septic rat lungs : effects on surfactant and inflammatory cytokines**. *J Appl Physiol* 2001, **91**:811–820.

27. Yamashita C, Forbes A, Tessolini JM, Yao LJ, Lewis JF, Veldhuizen RA: **Protective effects of elevated endogenous surfactant pools to injurious mechanical ventilation**. *Am J Physiol Cell Mol Physiol* 2008, **294**:L724–L732.

28. Wright JR: **Immunoregulatory functions of surfactant proteins**. *Nat Rev* 2005, **5**:58–68.

29. Glasser SW, Maxfield MD, Ruetschilling TL, Akinbi HT, Baatz JE, Kitzmiller JA, Page K, Xu Y, Bao EL, Korfhagen TR: **Persistence of LPS-induced lung inflammation in surfactant protein-C-deficient mice.** *Am J Respir Cell Mol Biol* 2013, **49**:845–854.

30. LeVine AM, Whitsett JA, Gwozdz JA, Richardson TR, Fisher JH, Burhans MS, Korfhagen TR: **Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung**. *J Immunol* 2000, **165**:3934–3940.

31. Ikegami M, Whitsett JA, Chroneos ZC, Ross GF, Reed JA, Bachurski CJ, Jobe AH: **IL-4 increases surfactant and regulates metabolism in vivo.** *Am J Physiol Lung Cell Mol Physiol* 2000, **278**:L75–L80.

32. Mallampalli RK, Ryan AJ, Salome RG, Jackowski S: **Tumor necrosis factor-alpha inhibits expression of CTP:phosphocholine cytidylyltransferase.** *J Biol Chem* 2000, **275**:9699–9708.

33. Pryhuber GS, Khalak R, Zhao Q: **Regulation of surfactant proteins A and B by TNF-alpha and phorbol ester independent of NF-kappa B.** *Am J Physiol* 1998, **274**(2 Pt 1):L289–L295.

34. Mariencheck WI, Alcorn JF, Palmer SM, Wright JR: **Pseudomonas aeruginosa Elastase Degrades Surfactant Proteins A and D**. *Am J Respir Cell Mol Biol* 2003, **28**:528–537.

35. Nakamura T, Moyer BZ, Veldhuizen RA, Lewis JF: **Interleukin-6 has no effect on surfactant or lung function in different lung insults.** *Exp Lung Res* 2006, **32**:27–42.

36. Yamashita CM, Fessler MB, Vasanthamohan L, Lac J, Madenspacher J, McCaig L, Yao L, Wang L, Puntorieri V, Mehta S, Lewis JF, Veldhuizen RA: **Apolipoprotein E-deficient mice are susceptible to the development of acute lung injury.** *Respiration* 2014, **87**:416–427.

37. Malloy J, McCaig L, Veldhuizen R, Yao LJ, Joseph M, Whitsett J, Lewis J: **Alterations of the endogenous surfactant system in septic adult rats**. *Am J Respir Crit Care Med* 1997, **156**(2 Pt 1):617–623.

38. Malloy JL, Veldhuizen RAW, McCormack FX, Korfhagen TR, Whitsett J a, Lewis JF: **Pulmonary surfactant and inflammation in septic adult mice: role of surfactant protein A**. *J Appl Physiol* 2002, **92**:809–816.

39. Veldhuizen RA, McCaig LA, Akino T, Lewis JF: **Pulmonary surfactant subfractions in patients with the acute respiratory distress syndrome**. *Am J Respir Crit Care Med* 1995, **152**(6 Pt 1):1867–1871.

40. Ito Y, Veldhuizen RA, Yao LJ, McCaig LA, Bartlett AJ, Lewis JF: **Ventilation strategies affect surfactant aggregate conversion in acute lung injury**. *Am J Respir Crit Care Med* 1997, **155**:493–499.

41. Zuo YY, Veldhuizen RAW, Neumann AW, Petersen NO, Possmayer F: **Current perspectives in pulmonary surfactant--inhibition, enhancement and evaluation.** *Biochim Biophys Acta* 2008, **1778**:1947–1977.

42. Yamashita CM, Dolgonos L, Zemans RL, Young SK, Robertson J, Briones N, Suzuki T, Campbell MN, Gauldie J, Radisky DC, Riches DW, Yu G, Kaminski N, McCulloch CA, Downey GP: **Matrix metalloproteinase 3 is a mediator of pulmonary fibrosis**. *Am J Pathol* 2011, **179**:1733–1745.

43. Irvin CG, Bates JHT: **Measuring the lung function in the mouse: the challenge of size.** *Respir Res* 2003, **4**:4.

44. Glaab T, Taube C, Braun A, Mitzner W: **Invasive and noninvasive methods for studying pulmonary function in mice.** *Respir Res* 2007, **8**:63.

45. Bullard KM, Lund L, Mudgett JS, Mellin TN, Hunt TK, Murphy B, Ronan J, Werb Z, Banda MJ: **Impaired wound contraction in stromelysin-1-deficient mice.** *Ann Surg* 1999, **230**:260–265.

46. Bullard KM, Mudgett J, Scheuenstuhl H, Hunt TK, Banda MJ: **Stromelysin-1-Deficient Fibroblasts Display Impaired Contraction in Vitro**. *J Surg Res* 1999, **84**:31–34.

CHAPTER 5:

General discussion and future directions

5.1. Summary and discussion of major findings

The overall objective of this work was to advance the knowledge of ALI, a pulmonary inflammatory disorder associated with a complex pathophysiology and lacking any effective pharmacological therapy [1, 2]. One of the major challenges facing the development of treatments for this disease is to effectively compromise between supporting the impaired lung function through mechanical ventilation (MV) on one hand, and limiting the propagation of lung inflammation, which is necessary to improve outcomes of ALI, on the other hand [3–5].

In an attempt to pursue our objective and alter ALI progression, attention was placed on two key elements of ALI pathophysiology that are common to all ALI patients: the effects of MV on the injured lung, and the development of a pulmonary inflammatory response following a lung insult. In order to perform our investigations, we utilized clinically relevant models of ALI (chapters 2, 3, and 4) resembling gastric acid aspiration and bacterial infection, two of the most common causes of direct lung injury [6]. *Ex vivo* MV was also utilized for part of our research, as ventilation can worsen lung injury and contribute to the severity of pulmonary and systemic inflammation which, in turn, is highly correlated with mortality in ALI [4, 7].

In **chapter 2**, exogenous surfactant administration was investigated as a lung-targeted therapy potentially mitigating the pro-inflammatory effects of MV. The results from this chapter convincingly demonstrated that, even though exogenous surfactant was safe and overall well-tolerated, it was not effective in down-modulating the inflammatory response associated with ventilation in our models of ALI.

These observations have led us to some important considerations. Firstly, the inability of exogenous surfactant to reduce lung and systemic inflammation in ALI may offer insight into the results of clinical trials in which surfactant treatment failed to improve mortality [8]. In light of the association between inflammation and mortality in ALI, however, it appeared necessary to broaden our research interest to identify other molecules, such as matrix metalloproteinase-3 (MMP-3), involved in inflammatory processes that could be targeted to limit the overwhelming inflammation in ALI. Secondly, while the results from

chapter 2 were consistent with previous experimental studies on exogenous surfactant [9, 10], they conflicted with evidence suggesting that elevated *endogenous* surfactant has the potential to dampen the inflammation associated with different models of lung injury, while also preserving compliance and oxygenation [11, 12]. Therefore, there appeared to be complex inter-connections between pulmonary inflammation, lung surfactant, and respiratory mechanics, which we believed required further elucidation.

As mentioned, the role MMP-3 was investigated in the context of LPS-induced and acid-induced ALI (**chapter 3**) on the basis of a well described role for this protease in inflammation and repair [13]. Our findings confirmed a role for MMP-3 in the pulmonary inflammatory response associated with ALI, but also pointed out unanticipated sex differences in such response. Specifically, lack of Mmp3 expression reduced the concentrations of alveolar cytokines and chemokines following lung injury only in female mice, but not in males. Importantly, results from **chapter 4** suggested that the mitigation of the inflammatory process observed in female, $Mmp3^{-/-}$ mice did not seem to correlate with improvements in physiological parameters related to respiratory mechanics and lung surfactant. The alteration observed in surfactant and lung mechanics were consistent with models of LPS-induced ALI, with no differences between genotypes.

Based on the knowledge acquired from these series of studies, the purpose of this final chapter is to discuss observations not addressed in the previous chapters, and to briefly provide an overview of potential future directions that could complement and expand the findings of this thesis work.

5.2. Future directions

5.2.1. Exogenous surfactant as a vehicle for anti-inflammatory molecules

The lack of efficacy of exogenous surfactant administration in our models of lung injury, and indeed in clinical trials of patients with ALI, has been disappointing. Nevertheless, these findings do not preclude the potential usefulness of this therapy in the treatment of lung injury. Exogenous surfactant can improve compliance and gas exchange, and these features are quite important within the pathogenesis of a disorder associated with

endogenous surfactant impairment such as ALI. As noted above, however, down-regulation of the overwhelming inflammatory response is also required for optimal therapy. In this regard, combination therapy of surfactant with anti-inflammatory agents may be a novel therapeutic approach for ALI. In this approach, the ability of surfactant preparations to reach the more distal regions of the lung and spread across the alveolar surface will enhance the delivery of anti-inflammatory molecules to the distal areas of the injured lung [14].

In the context of ALI, an anti-inflammatory molecule that could be utilized to improve the immuno-modulatory properties of exogenous surfactant is SP-A. This collectin has important functions in innate immunity and inflammation, and mice lacking SP-A expression experience higher lung concentrations of inflammatory mediators in models of lung injury [15, 16]. Importantly, SP-A is not present in exogenous surfactant preparations due to the purification process. The lack of SP-A in the exogenous surfactant utilized in our experiments may account, at least in part, for the absence of beneficial effects from surfactant treatment on inflammation observed in chapter 2. Future studies could therefore evaluate the efficacy of an exogenous surfactant preparation supplemented with SP-A in clinically relevant models of LPS-induced ALI or acid injury. Interestingly, both the oxidative stress, generated during acid injury, and LPS can activate TLR-4, a cell surface receptor expressed by alveolar macrophages and epithelial cells and involved in the production of pro-inflammatory cytokines [17, 18]. This is important, since SP-A has been shown to interact with TLR-4 preventing binding of smooth LPS, and it can also modulate TLR-4 expression and cellular localization [17, 19]. Supplementing exogenous surfactant with SP-A, therefore, could provide superior immuno-modulation associated with lower pulmonary and systemic inflammation, and consequently improved outcomes of ALI. It should be noted, however, that obtaining sufficient amounts of SP-A for clinical usage may be problematic as animal sources may lead to immunological issues, and synthesis of human SP-A, which is a complex glycosylated octadecamer, is currently not feasible.

Despite the above limitations, it would be of interest to complement our findings from the study in chapter 2 by testing the effects of an exogenous surfactant-SP-A preparation on the inflammatory response subsequent to acid injury and MV. Considering that a recent

study suggested a role for SP-A in limiting the translocation of inflammatory mediators from the lung to the circulation [20], the isolated and perfused mouse lung set up would be ideal to deliver *ex vivo* MV. This system, in fact, allows for the collection and measurement of the lung-derived mediators released in the circulation throughout MV, alongside the monitoring of respiratory mechanics and lung inflammation.

Whereas SP-A is an endogenous anti-inflammatory component of surfactant, it is also possible to supplement exogenous surfactant with other drugs to allow for optimal delivery. An important aspect of these approaches is to establish that the drug does not interfere with the function of surfactant and, vice versa, that the surfactant does not alter the properties of the anti-inflammatory drug. An example of this approach is currently being investigated in our laboratory as a potential therapy for the treatment of *Pseudomonas Aeruginosa* lung infection. In these studies, an exogenous surfactant is fortified with a small anti-microbial, anti-inflammatory peptide, capable of directly killing bacteria without causing antibiotic resistance [21]. Preliminary experiments appear promising, showing maintenance of surfactant and antimicrobial properties, safety and tolerability of this "fortified" surfactant, and excellent efficacy in the clearance of bacterial infection (B. Banaschewski, personal communication).

Although a specific MMP-3 inhibitor has not yet been reported, a similar approach could be used if such compound became available. In this scenario exogenous surfactant could be used to deliver an MMP-3 inhibitor specifically to the injured lung, assuming that the surfactant-inhibitor preparation maintains both surfactant properties and pharmacological inhibition. Such an approach would overcome inherent limitations stemming from the use of a knock out mouse model. Importantly, this would allow us to study the effect of MMP-3 inhibition on lung inflammation *after* the development of lung injury, thereby resembling a more clinically relevant scenario.

5.2.2. Sex differences and the inflammatory response

One of the most interesting observations stemming from this thesis work was related to the sex-differences observed in our models of ALI (chapters 3 & 4). Aside from the MMP-3 related differences already discussed in chapter 3, a general examination of our data led to

the observation of different inflammatory responses to lung injury in wild type female compared to wild type male mice. As the *a priori* experimental questions in those studies were related to MMP-3, the female-male differences were not analyzed statistically; nonetheless, our results would suggested the presence of sexual dimorphism in the response to lung injury, which could be interesting to investigate in future studies.

Male-female differences in immunity and inflammation have been described in several clinical and experimental studies. These investigations have demonstrated that, following infections, female mice have higher lung concentrations of inflammatory mediators, and mount a more robust innate and humoral responses than males [22–24]. This greater responsiveness in female subjects may favor a faster and more effective clearance of the invading pathogens than occurs in males, providing lower frequency of disease. With respect to pulmonary involvement, some studies have in fact shown greater susceptibility and incidence of respiratory infections [25], and greater risk of post trauma pneumonia in male patients compared to female patients [26, 27]. Interestingly, the risk of death among patients with post-injury pneumonia was greater in women than men [27]. Similar findings have also been described by Sakr and colleagues, who reported that female gender was an independent risk factor associated with death among patients with severe sepsis [28]. This clinical evidence suggests that the heightened immune response in females can be a ‘double-edge sword’, as it can potentially exacerbate injury and lead to poorer outcomes. As pneumonia and sepsis are two of the most common causes of ALI, there is a good rationale for more experimental investigations aimed at exploring sex differences in ALI.

Some indications in this regard can be found in our studies, in which a seemingly more robust pulmonary release of cytokines and chemokines such as IL-6, G-CSF, and TNF-α was observed in wild type females compared to males after LPS-induced lung injury, and was even more pronounced in mice exposed to acid instillation. To draw definitive conclusions and complement the observations of this thesis, the rigorous exploration of male-female differences in inflammation during ALI should be the objective of future studies. The first step would consist in exposing female and male mice to either LPS- or acid-induced ALI, and then analyzing outcomes of lung injury similar to the ones described throughout this thesis. Multiple cytokines and chemokines would be assayed in lavage

samples to evaluate sex differences in inflammation; moreover, time-course analyses could shed light onto possible variability in lung injury development. If the presence of sexual dimorphism in the inflammatory response to lung injury is confirmed, it would then be important to understand the underlying biological and physiological mechanisms, which could be related to the activities of steroid hormones [22]. To this end, outcomes of lung injury and inflammation would be measured in both sham and gonadectomized mice exposed to LPS and acid instillation. Subsequently, differences in the inflammatory response could be investigated following hormonal replacement. Altogether, these approaches would be helpful for unravelling the potential interactions between gonadal hormones and inflammatory processes in the pathogenesis of ALI. If such interactions truly occur, it will then be necessary to consider sex and gender as important variables within the treatment of this disease.

5.2.3. MMP-3 and Surfactant

While the above proposed studies would primarily focus on the effects of surfactant therapy or sex differences on the inflammatory response, the possibility that mediators of inflammation involved in ALI may affect surfactant (and therefore, lung function) should also be considered. This issue of the complex and elusive relationship between the different components – inflammation, surfactant, and lung mechanics – participating in the pathogenesis of ALI has been addressed in the context of the altered inflammatory response to injury in $Mmp3^{-/-}$ mice (chapter 4). One aspect, however, that has not been investigate yet is the specific interaction of the protease MMP-3 with surfactant proteins, such as SP-A. As mentioned, this collectin has an important role in innate host defense; however SP-A also contributes to the biophysical properties of surfactant, by reducing surfactant inhibition stemming from plasma protein or oxidative alterations [29, 30]. In this sense, alterations and/ or degradation of SP-A may not only affect the host defense properties, but can also lead to poorer biophysical function of surfactant, as shown by Malloy et al. in relation to the effects of *P. Aeruginosa* protease IV on SP-A [31].

These interesting observations prompted preliminary *in vitro* experiments in our lab, performed through incubation of human SP-A with recombinant human MMP-3. Our preliminary results suggest that this protease can degrade free SP-A, but cannot cleave

SP-A when bound to surfactant lipids (data not shown). These initial findings are intriguing, and it would be interesting to validate them further through a series of *in vitro* experiments assessing, for example, the time course and dose response of SP-A degradation by MMP-3. Moreover, to answer the question of *in vivo* relevance of such potential interaction, lavage samples from $Mmp3^{+/+}$ and $Mmp3^{-/-}$ mice exposed to lung injury could be analyzed to assess the state of SP-A degradation after injury-driven increases in lavage MMP-3, and the effect on overall surfactant function. Given the important roles of MMP-3 and SP-A in the modulation of the inflammatory response in ALI, and the biophysical alterations stemming from SP-A degradation, a more in-depth knowledge of such interplay could be beneficial to a better treatment of this disorder.

5.3. Concluding remarks

The Acute Lung Injury/ Acute Respiratory Distress Syndrome is a life- threatening condition with a mortality of 40%, high morbidity, and an incidence around 60-80 new cases for 100,000 persons every year. Despite many decades of intensive research, the main treatment in ALI is only supportive and involved in the disease pathogenesis.

The findings presented in this thesis have contributed to the knowledge of ALI pathophysiology by: i) evaluating potential treatments in relation to outcomes of ALI clinically related to mortality; ii) identifying a potential target (MMP-3) in the regulation of the lung inflammatory response; iii) outlining possible sex-differences in the response to lung injury. Overall, these findings underscored the challenges faced in the identification of suitable targets and therapies for the treatment of this disorder. Such challenges result in part from the very complex and mutual influences between inflammation, lung surfactant, and overall lung function within the pathophysiology of ALI. Further experimental and clinical efforts, therefore, need to be channeled into ALI/ ARDS research to provide more effective treatment of this disorder and decrease mortality.

5.4. References

1. Ware LB, Matthay MA: **The Acute Respiratory Distress Syndrome**. *N Engl J Med* 2000, **342**:1334–1349.

2. Yamashita CM, Lewis JF: **Emerging therapies for treatment of acute lung injury and acute respiratory distress syndrome**. *Expert Opin Emerg Drugs* 2012, **17**:1–4.

3. Goligher E, Ferguson ND: **Mechanical ventilation: epidemiological insights into current practices**. *Curr Opin Crit Care* 2009, **15**:44–51.

4. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, Slutsky AS: **Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial**. *JAMA* 1999, **282**:54–61.

5. Parsons PE, Matthay MA, Ware LB, Eisner MD: **Elevated plasma levels of soluble TNF receptors are associated with morbidity and mortality in patients with acute lung injury.** *Am J Physiol Lung Cell Mol Physiol* 2005, **288**:L426–L431.

6. Erickson SE, Martin GS, Davis JL, Matthay MA, Eisner MD: **Recent trends in acute lung injury mortality: 1996-2005.** *Crit Care Med* 2009, **37**:1574–1579.

7. The Acute Respiratory Distress Syndrome Network: **Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network**. *N Engl J Med* 2000, **342**:1301–1308.

8. Kesecioglu J, Haitsma JJ: **Surfactant therapy in adults with acute lung injury/acute respiratory distress syndrome.** *Curr Opin Crit Care* 2006, **12**:55–60.

9. Wu H, Kobayashi T, Wan Q, Shi W, Qian H, Cui X, Li W: **Effects of surfactant replacement on alveolar overdistension and plasma cytokines in ventilator-induced lung injury.** *Acta Anaesthesiol Scand* 2010, **54**:354–361.

10. Welk B, Malloy JL, Joseph M, Yao LJ, Veldhuizen A: **Surfactant treatment for ventilation-induced lung injury in rats: effects on lung compliance and cytokines.** *Exp Lung Res* 2001, **27**:505–520.

11. Yamashita C, Forbes A, Tessolini JM, Yao LJ, Lewis JF, Veldhuizen RA: **Protective effects of elevated endogenous surfactant pools to injurious mechanical ventilation**. *Am J Physiol Cell Mol Physiol* 2008, **294**:L724–L732.

12. Walker MG, Tessolini JM, McCaig L, Yao LJ, Lewis JF, Veldhuizen RA: **Elevated endogenous surfactant reduces inflammation in an acute lung injury model**. *Exp Lung Res* 2009, **35**:591–604.

13. Parks WC, Wilson CL, López-Boado YS: **Matrix metalloproteinases as modulators of inflammation and innate immunity**. *Nat Rev Immunol* 2004, **4**:617–629.

14. Haitsma JJ, Lachmann U, Lachmann B: **Exogenous surfactant as a drug delivery agent**. *Adv Drug Deliv Rev* 2001, **47**:197–207.

15. Borron P, McIntosh JC, Korfhagen TR, Whitsett JA, Taylor J, Wright JR: **Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo.** *Am J Physiol Lung Cell Mol Physiol* 2000, **278**:L840–L847.

16. LeVine AM, Whitsett JA, Gwozdz JA, Richardson TR, Fisher JH, Burhans MS, Korfhagen TR: **Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung**. *J Immunol* 2000, **165**:3934–3940.

17. Sender V, Stamme C: **Lung cell-specific modulation of LPS-induced TLR4 receptor and adaptor localization.** *Commun Integr Biol* 2014, **7**:1–9.

18. Imai Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, van Loo G, Ermolaeva M, Veldhuizen R, Leung YHC, Wang H, Liu H, Sun Y, Pasparakis M, Kopf M, Mech C, Bavari S, Peiris JSM, Slutsky AS, Akira S, Hultqvist M, Holmdahl R, Nicholls J, Jiang C, Binder CJ, Penninger JM: **Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury.** *Cell* 2008, **133**:235–249.

19. Yamada C, Sano H, Shimizu T, Mitsuzawa H, Nishitani C, Himi T, Kuroki Y: **Surfactant protein A directly interacts with TLR4 and MD-2 and regulates inflammatory cellular response. Importance of supratrimeric oligomerization.** *J Biol Chem* 2006, **281**:21771–21780.

20. Truscott EA, McCaig LA, Yao L-JJ, Veldhuizen RAW, Lewis JF: **Surfactant protein-A reduces translocation of mediators from the lung into the circulation**. *Exp Lung Res* 2010, **36**:431–439.

21. Veldhuizen EJA, Brouwer EC, Schneider VAF, Fluit AC: **Chicken cathelicidins display antimicrobial activity against multiresistant bacteria without inducing strong resistance.** *PLoS One* 2013, **8**:e61964.

22. Klein SL: **Immune cells have sex and so should journal articles.** *Endocrinology* 2012, **153**:2544–2550.

23. Fish EN: **The X-files in immunity: sex-based differences predispose immune responses**. *Nat Rev Immunol* 2008, **8**(September):737–744.

24. Robinson DP, Lorenzo ME, Jian W, Klein SL: **Elevated 17β-estradiol protects females from influenza A virus pathogenesis by suppressing inflammatory responses.** *PLoS Pathog* 2011, **7**:e1002149.

25. Falagas ME, Mourtzoukou EG, Vardakas KZ: **Sex differences in the incidence and severity of respiratory tract infections.** *Respir Med* 2007, **101**:1845–1863.

26. Gannon CJ, Pasquale M, Tracy JK, McCarter RJ, Napolitano LM: **Male gender is associated with increased risk for postinjury pneumonia.** *Shock* 2004, **21**:410–414.

27. Napolitano LM, Greco ME, Rodriguez A, Kufera JA, West RS, Scalea TM: **Gender differences in adverse outcomes after blunt trauma.** *J Trauma* 2001, **50**:274–280.

28. Sakr Y, Elia C, Mascia L, Barberis B, Cardellino S, Livigni S, Fiore G, Filippini C, Ranieri VM: **The influence of gender on the epidemiology of and outcome from severe sepsis.** *Crit Care* 2013, **17**:R50.

29. Cockshutt AM, Weitz J, Possmayer F: **Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro.** *Biochemistry* 1990, **29**:8424–8429.

30. Bailey TC, Maruscak AA, Petersen A, White S, Lewis JF, Veldhuizen RA: **Physiological effects of oxidized exogenous surfactant in vivo: effects of high tidal volume and surfactant protein A**. *Am J Physiol Cell Mol Physiol* 2006, **291**:L703–L709.

31. Malloy JL, Veldhuizen RAW, Thibodeaux BA, Callaghan RJO, Wright JR: **Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions**. *Am J Physiol Lung Cell Mol Physiol* 2005, **288**:L409–L418.

APPENDIX 1:

UWO animal use sub-committee protocol approval

-------- Original Message --------
Subject: eSirius Notification - New Animal Use Protocol is APPROVED2010-272::1
Date: Thu, 17 Feb 2011 15:54:24 -0500
From: [redacted] (eSiriusWebServer)
To:
CC:

*** THIS IS AN EMAIL NOTIFICATION ONLY. PLEASE DO NOT REPLY ***

AUP Number: 2010-272.
AUP Title: Studies Utilizing The Ipml Set-up

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Studies Utilizing The Ipml Set-up" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2010-272::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Floyd, Katherine
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Western

Dec. 22, 2006

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2010

Dear Dr. Lewis:

Your Animal Use Protocol form entitled:
Studies Utilizing the Isolated Perfused Mouse Lung

Funding Agency CIHR - Grant # MOP-11666

has been approved by the University Council on Animal Care. This approval is valid from **Dec. 22, 2006 to Dec. 31, 2007.** The protocol number for this project is **#2006-124-12 and replaces #2002-086-08**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YR.

Species	Strain	Other Detail	Pain Level	Animal # Total for 1 Year
Mouse	Various	mature M/F	D	450

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (661-2111 ext. 86770) in case of difficulties or if you require copies.
SOP's are also available at http://www.uwo.ca/animal/acvs
310 Holding Period Post-Admission
320 Euthanasia
321 Criteria for Early Euthanasia/Rodents
330 Post-Operative Care/Rodent
343 Surgical Prep/Rodent/Recovery Surgery

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - J. Lewis, L. McCaig, D. Forder
Approval Letter - L. McCaig, D. Forder

The University *of* Western Ontario
Animal Use Subcommittee/University Council on Animal Care
Health Sciences Centre • London, Ontario • CANADA - N6A 5C1
Phone: 519-661-2111 ext. 86770 • Fax: 519-661-2028 • www.uwo.ca/animal

APPENDIX 2:

Information about copyright release for publication

00528100 re:Permission

Inbox x Work x Work/Thesis x

"Dunncan Suarez" Jun 19

to me

Dear Dr. Puntorieri

Thank you for contacting BioMed Central.

The article you refer to is an open access publication. Therefore you are free to use the article for the purpose required, as long as its integrity is maintained and its original authors, citation details and publisher are identified.

For detailed information about the terms please refer to the open access license:

http://www.biomedcentral.com/about/license.

If you have any questions please do not hesitate to contact me.

Best wishes

Dunncan Suarez
Customer Services

www.biomedcentral.com
----------Your Question/Comment ----------

Hello,

I am writing to obtain information on your permissions policy.

I am the primary author on a publication from BMC Pulmonary Medicine (2013, shown below) and I wish to use it, in its entirety, in my PhD thesis.

Could you please suggest what would be the best procedure to follow in order to obtain permission?

Puntorieri V, Hiansen JQ, McCaig LA, Yao LJ, Veldhuizen RA, Lewis JF. "The effects of exogenous surfactant administration on ventilation-induced inflammation in mouse models of lung injury."
BMC Pulm Med. 2013 Nov 20;13:67.

Best regards,
Valeria Puntorieri

CURRICULUM VITAE

Name: Valeria Puntorieri

Post-secondary Education and Degrees:

University of Turin
Turin, Italy
2000-2003 B.Sc. Biotechnology

University of Turin
Turin, Italy
2003-2005 M.Sc. Medical Biotechnology

The University of Western Ontario
London, Ontario, Canada
2009- Ph.D. Candidate

Honours and Awards:

Lawson Internal Research Fund award -
Lawson Health Research Institute, 2009

First place Poster Presentation Award– Research Day,
Department of Medicine, 2010

Teaching Assistant Award Nominee – Western University
2010/2011/2013

Leadership Award Nominee – Lawson Health Research Institute
2011

Related Work Experience

Research Assistant – University of Turin
Department of Anesthesiology and Intensive Care
2005-2008

Teaching Assistant –Physiology 3130y
Department of Physiology and Pharmacology
Western University
2009-2011

Teaching Assistant –Human Physiology 2130
Department of Physiology and Pharmacology
Western University
2011-2014

Publications:

Manuscripts

Valeria Puntorieri, Lynda A. McCaig, Li-Juan Yao, James F. Lewis, Cory M. Yamashita, and Ruud A.W. Veldhuizen. **Lack of matrix metalloproteinase-3 in mouse models of lung injury ameliorates the pulmonary inflammatory response in females but not in male mice**. *In preparation*

Cory M Yamashita, Michael B Fessler, Lakshman Vasanthamohan, Joanne Lac, Jennifer Madenspacher, Lynda McCaig, Lijuan Yao, Lefeng Wang, **Valeria Puntorieri**, Sanjay Mehta, Jim F Lewis, Ruud A W Veldhuizen. **Apolipoprotein E-deficient mice are susceptible to the development of acute lung injury.** *Respiration*. 2014 Mar 22.

Valeria Puntorieri, Josh Qua Hiansen, Lynda A McCaig, Li-Juan Yao, Ruud AW Veldhuizen and James F Lewis. **The effects of exogenous surfactant administration on ventilation-induced inflammation in mouse models of lung injury**. *BMC Pulmonary Medicine*, 2013 Nov 20;13(1):67.

Fanelli V, **Puntorieri V**, Assenzio B, Martin EL, Elia V, Bosco M, Delsedime L, Del Sorbo L, Ferrari A, Italiano S, Ghigo A, Slutsky AS, Hirsch E, Ranieri VM. **Pulmonary-derived phosphoinositide 3-kinase gamma (PI3Kγ) contributes to ventilator-induced lung injury and edema.** *Intensive Care Med*. 2010 Nov; 36(11):1935-45. Epub 2010 Aug 19.

Martin EL, Souza DG, Fagundes CT, Amaral FA, Assenzio B, **Puntorieri V**, Del Sorbo L, Fanelli V, Bosco M, Delsedime L, Pinho JF, Lemos VS, Souto FO, Alves-Filho JC, Cunha FQ, Slutsky AS, Ruckle T, Hirsch E, Teixeira MM, Ranieri VM. **PI3K{gamma} kinase activity contributes to sepsis and organ damage by altering neutrophil recruitment**. *Am J Respir Crit Care Med.*, 2010 Sep 15;182(6):762-73.

Fanelli V., Mascia L., **Puntorieri V**., Assenzio B., Elia V., Fornaio G., Martin E.L., Grasso S., Bosco M., Delsedime L., Ranieri V.M. **Pulmonary atelectasis during low stretch ventilation: "open lung versus "lung rest" strategy.** *Crit Care Med.*, 2009 Mar; 37(3):1046-53.

Cantaluppi V., Assenzio B., Pasero D., Mauriello-Romanazzi G., Beltramo S., Figliolini F., Pacitti A., Lanfranco G., **Puntorieri V**., Martin E.L., Mascia L., Biancone L., Segoloni G.P., Camusi G., Ranieri V.M. **Polymyxin-B hemoperfusion inactivates circulating proapoptotic factors.** *Intensive Care Medicine*, 2008 Sep; 34(9):1638-45.

Fonsato V., Buttiglieri S., Deregibus MC., **Puntorieri V**., Bussolati B., Camussi G. (2006). **Expression of Pax2 in human renal tumor-derived endothelial cells sustains apoptosis resistance and angiogenesis.** *American Journal of Pathology*, 2006 Feb; 168(2):706-13.

Abstracts

Valeria Puntorieri, Lynda A. McCaig, Li-Juan Yao, Stephanie Aigbe, Jim Lewis, Cory M. Yamashita, Ruud A.W. Veldhuizen. **The role of matrix metalloproteinase 3 in the Acute Respiratory Distress Syndrome.** Matrix Metalloproteinases Gordon Research Conference, Barga (Italy), May 2013.

Valeria Puntorieri, LeFeng Wang, Li-Juan Yao, Sanjay Mehta, Rudolf A.W. Veldhuizen, James F. Lewis, Cory Yamashita. **The effect of oxidized low-density lipoprotein on pulmonary endothelial function in vitro.** *Am. J. Respir. Crit. Care Med.,* May 2012; 185: A2108.

Valeria Puntorieri, Lynda A. McCaig, LiJuan Yao, Ruud A. Veldhuizen, and James F. Lewis. **Systemic inflammation in acute lung injury using an isolated and perfused mouse lung model.** *Am. J. Respir. Crit. Care Med.,* May 2011; 183: A5236.

Puntorieri V., Martin E. L., Pasero D., Del Sorbo L., Ranieri V. M. **PMX treatment of septic patients improved viability of pulmonary epithelial cells**. *Intensive Care Medicine* 2008

Puntorieri V., Martin E. L., Del Sorbo L., Ranieri V. M.. **Lipopolysaccharide induces alterations in lung epithelial cells indirectly through macrophages**. *Intensive Care Medicine* 2008

Puntorieri V, Martin EL, Assenzio B, Del Sorbo L, Ranieri VM. **Indirect effects of LPS on pulmonary A549 epithelial cells via THP-1 differentiated macrophages**. *Am. J. Crit. Care Med*., 2007, 177: A623.