Tumour Necrosis Factor-α (TNF) Receptor Subtype Signalling In Acute Lung Injury - A Therapeutic Approach

PhD thesis

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Abstract

To date no definite treatment exists for acute lung injury (ALI), only supportive therapy, among which mechanical ventilation is an important tool; however ventilation itself may exacerbate the underlying injury, termed ventilator-induced lung injury (VILI). The pro-inflammatory cytokine tumour necrosis factor alpha (TNF) has been consistently implicated in the pathogenesis of ALI/VILI, however clinical trials with anti-TNF therapy failed to improve the outcome.

TNF acts on two cell-surface receptors (TNFRs), p55 and p75, and knock-out studies in ALI/VILI indicate they have directly opposing roles, i.e. promoting or protecting against pulmonary oedema, respectively, but it remains unclear where these signalling mechanisms take place, being the endothelial or epithelial side of the alveolar-capillary membrane. This project had two main aims: to characterise the expression profile and roles of TNF receptor subtypes on each side of the alveolar-capillary membrane and to modulate ALI progression in mouse models by targeting individual TNFRs accordingly.

Characterisation studies on freshly harvested mouse pulmonary endothelial cells (PECs) using flow cytometry indicated expression of both receptors with dominance of p55. Knock-out and pharmacological inhibition studies *in vivo* and *in vitro*, respectively, found p55 to be the major mediator of TNF-induced adhesion molecule expression on PECs, with a similar but less potent role for p75 that varied across adhesion molecules. Endotoxaemia resulted in rapid shedding of p55 on PECs while expression of p75 was preserved, indicating that the relative role of the receptors may change during inflammatory conditions.

Specific inhibition of intraalveolar p55 signalling by domain antibodies significantly attenuated both pulmonary oedema and inflammation in mouse models of VILI and acid-aspiration.

These data offer new, potentially clinically applicable insights into the involvement of TNFR biology in ALI and the novel domain antibody technology may open a new therapeutic approach for patients who suffer from, or at risk of ALI.

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Declarations

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The data contained in Chapter 4 were the combined effort of Szabolcs Bertok, Michael Wilson, Andrew Bayliffe, Peter Morley, Ruud de Wildt and Masao Takata, and has been published in *Thorax* by Bertok *et al.* [2] and can be found in Appendix II.

The rest of the work is my own or otherwise referenced appropriately.

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GlaxoSmithKline Policy on the Care, Welfare and Treatment of Animals.

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1. Introduction

1.1 Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS)

1.1. Definition

ALI and its more severe form ARDS are characterised by acute onset of hypoxaemia secondary to inflammatory pulmonary oedema, and bilateral changes on chest radiograph. The entities were first described by Ashbaugh et al in 1967, who reported very similar pathologies, i.e. acute onset of tachypnoea, hypoxaemia, diffuse pulmonary infiltrates and loss of compliance, in 12 adult patients despite having varying underlying conditions ranging from pneumonia to traumatic injury which did not respond to ordinary respiratory therapy [3]. However, a uniform definition of ARDS was lacking for almost another 30 years causing uncertainties around its true incidence and clinical outcome, which prompted the American-European Consensus Conference (AECC) on ARDS in 1994 to standardise the diagnostic criteria for ALI and ARDS in order to allow comparison of epidemiological and treatment studies across the world (or at least in North America and Europe) (Table 1.1) [4].



Table 1.1 - Diagnostic criteria for acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) set by the American-European Consensus Conference on ARDS in 1994 [4]. ALI and ARDS only differ in the extent of hypoxaemia, which is more severe in the latter.

Using these criteria the incidence of ALI/ARDS in the US was estimated at 17-78.9 cases per 100,000 person-years [5-7] in different cohorts, whereas the figures are generally lower in Europe and Australia ranging from 7.2 to 34 cases per 100,000 person-years [8-10]. Mortality rates can vary in the range of 24 to 60%, depending on the severity and nature of the underlying disease and the patients' age and comorbidities [7]. Based on the ARDS Network trial 60-day mortality has declined from 36% in 1996–1997 to 26% in 2004–2005 [11], probably secondary to more wide-spread use of lung-protective ventilation strategies, conservative use of blood products and better control of nosocomial infections [12, 13], whereas the most recent ARDS Network clinical trials reported a 60-day mortality of 22% in adult patients [14, 15], which is still unacceptably high.

Although the AECC definition significantly helped to orchestrate international research efforts in the field of ALI/ARDS, its validity has been criticised. For example the interpretation of the term 'acute' may differ amongst clinicians and similar problems may arise around the determination of 'bilateral infiltrates on chest

radiograph' due to interobserver variability [16, 17]. It also ignores that the PaO_2/FiO_2 ratio is not constant over a range of FiO_2 and may vary in response to ventilator settings particularly PEEP [18-20].

In order to overcome these and other limitations, the European Society of Intensive Care Medicine, the American Thoracic Society and the Society of Critical Care Medicine drafted a revision of the AECC definitions, termed the 'Berlin definition' [21]. The main changes made to the AECC are summarised in Table 1.2, and include 1) the elimination of the term 'acute lung injury' and replacing it with three levels of ARDS severity based on PaO_2/FiO_2 ratio measured at a minimum PEEP of $5cmH_2O$, 2) defining 'acute' as ≤7 days from the predisposing insult and 3) eliminating pulmonary artery wedge pressure cut-off values as discriminating factor from cardiogenic pulmonary oedema [21].

The Berlin Definition was empirically evaluated using patient level meta-analysis of patients with ARDS from 4 multi-centre (N=4188) and 3 single-centre (N= 269) clinical data sets containing physiologic information [21]. The Berlin Definition provided significantly better predictive ability for mortality compared to the AECC definition. Therefore, it may help to better identify patients that benefit the most from treatment that have been tested in clinical trials. It also gives recommendations for clinical research to focus its attention on other treatment options that might be also beneficial in the relevant subgroups of ARDS, such as testing the feasibility and benefits of non-invasive ventilation in mild ARDS, partial extracorporeal support in moderate to severe forms, and high-frequency ventilation, inhaled nitric oxide, neuromuscular blockade and extracorporeal membrane oxygenation in the severe forms. These and other therapeutic approaches to ARDS will be discussed in more detail later in this chapter.



Table 1.2 - Comparison of the American-European Consensus Conference (AECC) definition of acute lung injury (ALI) and the Berlin definition on acute respiratory distress syndrome (ARDS). Main changes to the AECC definition include a clear definition of the term 'acute'; elimination of the term 'acute lung injury' and setting three severity levels of ARDS based on oxygenation at standardised positive end-expiratory pressure (PEEP) levels; elimination of the use of pulmonary capillary wedge pressure cut-off values to define non-cardiogenic pulmonary oedema, mainly because this is rarely measured routinely and because cardiogenic oedema and ARDS may co-exist/overlap clinically.

1.1. Aetiology

ARDS can result from a variety of insults, both from direct (pulmonary) injury, where the initial insult affects the lung parenchyma, and from an indirect (extra-pulmonary) cause, where lung injury is part of a systemic process. While pneumonia is the most

common pulmonary cause, sepsis accounts for most of the extra-pulmonary cases (Table 1.3) and overall is associated with highest risk of progression to ARDS, approximately 40 per cent [22].



Table 1.3 – Clinical disorders associated with the development of ARDS [22]. Pneumonia and sepsis are the most common direct and indirect causes of ARDS, respectively.

The awareness of conditions that are commonly associated with ARDS helps identifying patients who are at risk of or in the early stage of lung injury development, thus aid choosing the appropriate prophylactic and or therapeutic approach. Also, several environmental and genetic factors have been identified that contribute to the susceptibility and severity of ARDS. For example, chronic alcohol abuse has been shown to increase the risk of developing ARDS and multiple organ failure in septic shock [23, 24], whereas cigarette smoke exposure, both active and passive, has been associated with development of ARDS after blunt chest trauma [25]. Common variants of genes that regulate processes, such as inflammation, coagulation, endothelial cell function, apoptosis and generation of reactive oxygen species, which

are all involved in the pathogenesis of acute lung injury (see below), have been also linked with susceptibility to ARDS and the clinical outcome [26-31].

1.1. Pathophysiology

Although some of the clinical features might differ depending on whether ARDS originates from a pulmonary or extra-pulmonary insult [32], the main pathophysiological processes are very similar, and involve a complex interplay of cellular and molecular mechanisms leading to an imbalance of pulmonary homeostasis that can develop within hours to days from the initial insult and progress over days to weeks, and often takes months to resolve, if at all [22, 33-35]. The pathogenesis of ARDS can be separated into three main stages, and although these have some overlap, each of them has its own characteristics.

Acute phase – increased permeability oedema and inflammation

Clinically, the initial acute or exudative phase of ARDS is characterised by rapid onset of dyspnoea, hypoxaemia, respiratory failure and bilateral opacities on chest radiograph, features that are consistent with increased permeability oedema [22] and often indicate mechanical ventilation. From a respiratory physiology perspective it is associated with decreased lung compliance and increased tendency to alveolar collapse secondary to the oedema and surfactant dysfunction [36, 37], and increased dead space due to a pro-thrombotic state in the pulmonary microvasculature [38], the latter of which has been identified as a risk of death in ARDS [39]. Histology features microthrombi in the microvasculature, disrupted alveolar-capillary barrier with denuded basement membrane often lined with protein-rich eosinophilic hyaline membrane, and alveoli that are collapsed or filled with oedema fluid, inflammatory cells, and red blood cells [40, 41].

Pulmonary microvascular endothelial injury

Under normal conditions, a synchronised collaboration between the pulmonary endothelium and alveolar epithelium strictly controls fluid and molecular movement across the alveolar-capillary membrane in each direction, thus ensuring an appropriate fluid balance. Any disturbance to this finely-controlled system can result in disequilibrium and consequently lead to pulmonary oedema. Indeed, injury to and activation of the pulmonary microvascular endothelium is a key step towards the development of increased permeability oedema in the acute phase of ARDS [22]. It is important to note that oedema formation is a consequence of increased permeability rather than hydrostatic forces, thus the content of oedema fluid differs from that seen in cardiogenic oedema, which has less protein content as indicated by a lower oedema fluid protein to plasma protein ratio, a tool validated for bedside use to differentiate between the two aetiologies [42, 43].

Vascular endothelial (VE-) cadherin, an adherens junction protein, has been identified as one of the critical regulators of endothelial barrier integrity in the lung microvasculature [44, 45]. VE-cadherin is a calcium-dependent cell–cell adhesion glycoprotein functioning as a classic cadherin controlling the cohesion and organization of the intercellular junctions. Disruption of these VE-cadherin bonds destabilises vascular barrier function [44, 46]. It is regulated by cytoplasmic associations with catenins and other cytoskeletal structures, and by phosphorylation/dephosphorylation on tyrosine residues of the cadherin-catenin complex. Phosphorylation of the complex leads to internalisation of VE-cadherin compromising barrier function. In contrast, vascular endothelial receptor-type protein tyrosine phosphatase (VE-PTP) associated with VE-cadherin supports its adhesive function by maintaining a dephosphorylated state [47, 48]. It has been shown in *in*

vitro and *in vivo* studies that humoral factors, such as tumour necrosis factor alpha (TNF), vascular endothelial growth factor (VEGF), histamine, thrombin and interaction with leukocytes can all interrupt VE-cadherin bonds by inducing phosphorylation of the cadherin-catenin complex, which ultimately leads to pulmonary and extra-pulmonary oedema formation [49-51]. In contrast, prevention of dissociation of VE-PTP from VE-cadherin reduced lavage fluid protein and leukocyte content in LPS-treated mice [52].

Beside increased vascular permeability, inflammation is the other main hallmark of ARDS, in which the pulmonary microvasculature is both a target and a mediator [22]. Once activated by inflammatory stimuli such as cytokines (TNF alpha, IL-1 beta) or bacterial products (lipopolysaccharide - LPS, lipoteichoic acid - LTA), pulmonary endothelial cells express chemotactic chemokines (e.g. interleukin (IL)-8) and adhesion molecules (e.g. E- and P-selectin, vascular cell and intercellular adhesion molecule (VCAM and ICAM)-1) to facilitate pulmonary entrapment and extravasation of circulating inflammatory leukocytes and propagate a systemic inflammatory response [35, 53, 54].

Several biological markers of endothelial cell injury and activation have been studied in patients with ARDS [55]. For instance, endothelin-1 is a molecule secreted by endothelial cells upon injury [56-58] that has both vasoconstrictor and pro-inflammatory activities and is increased in the plasma of ARDS patients [59-61]. Von Willebrand factor (vWF), secreted by the Weibel-Palade granules of endothelial cells [62], is also increased in the circulation during ARDS and is an independent predictor of mortality [63, 64].

Alveolar epithelial injury

Although injury to the pulmonary endothelium is a critical step in the pathogenesis this alone appears to be insufficient to develop the characteristic changes of the acute phase [65], and injury to the alveolar epithelium is also required [22, 40, 41, 55]. Under normal conditions, the alveolar epithelium is predominantly composed of squamous, elongated type I cells that cover approximately 95% of the alveolar surface area and are prone to injury [66]. The thinness of the cells ensures rapid diffusion of oxygen and carbon-dioxide across the alveolar epithelium. Type I cells may also play a role in vectorial fluid transport across the alveolar-capillary membrane, thus contributing to the maintenance of alveolar fluid balance, although this is less well established compared to that in type II cells, which cover the rest of the alveolar surface [66]. Type II cells have a cuboidal shape and are more resistant to injury. Beside their role in alveolar fluid transport, type II cells are also the source of surfactant proteins, and are progenitors of type I cells [66].

Under normal conditions, the epithelial barrier is much tighter than the endothelial barrier, thus loss of epithelial integrity significantly contributes to alveolar oedema [35]. A study in sheep indicated that over 92% of the resistance to albumin flux across the alveolar-capillary barrier lies in the epithelial barrier [67]. This is maintained by cadherin-mediated adherens junctions (E-cadherin substitutes for VE-cadherin) and tight junctions [66]. Inflammatory cytokines, bacterial products, proteases and oxidants can all lead to disruption of this system [22].

Although leukocytes (and molecules as well) can migrate passively through a quasi-physically damaged epithelial barrier, there is evidence that alveolar epithelial cells (AECs) can actively control and assist this process, which may be relevant in the early stages of the syndrome when the alveolar epithelium may still be physically intact. This is mediated, in part, by induced expression of chemotactic cytokines by

AECs, such as IL-8 (or its rodent functional homologues, macrophage inflammatory protein (MIP)-2 and keratinocyte-derived chemokine (KC)) and monocyte chemotactic protein (MCP)-1 [68-71], that will lead to a build-up of chemokine gradient across the epithelial membrane and prompt neutrophils and monocytes to marginate to the lung microvasculature, extravasate and migrate to the alveolar space [68]. This latter step is facilitated and mediated by induced epithelial expression of adhesion molecules, such as ICAM-1 [72, 73] and VCAM-1 [74] and the interaction with their counterpart ligands on leukocytes. Beside the role in neutrophil adherence, surface expressed ICAM-1 on epithelial cells also facilitates alveolar macrophage and neutrophil phagocytic activity [75, 76]. On the other hand, soluble form of ICAM-1 (sICAM-1) released by alveolar epithelial cells can activate alveolar macrophages and enhance their TNF and MIP-2 production, thus further increasing leukocyte accumulation in the lung and intensifying injury [77]. Moreover, sICAM-1 (which may have both endothelial and epithelial origin) is elevated in pulmonary oedema fluid and plasma of ARDS patients, the latter of which is associated with poor outcome [78].

Neutrophils

Histology on ARDS lungs indicate a marked accumulation of neutrophils [40, 41], and these cells also dominate in the pulmonary oedema and bronchoalveolar lavage fluid (BALF) obtained from these patients [55]. Also, several animal models of ARDS are neutrophil dependent [79-83].

Leukocyte migration into the alveolar compartments involves several steps, which are regulated in part, by specific endothelial-leukocyte adhesion molecules [84]. This is different from that happening in the systemic circulation where the initial

interaction between circulating neutrophils and endothelium appears to be transient, resulting in the rolling of leukocytes along the vessel wall in which selectins such as P- and E-selectin expressed on endothelial cells play a role [85]. The rolling neutrophils then become activated by local factors generated by the endothelium, resulting in their arrest and firm adhesion to the vessel wall. This step is mainly regulated by VCAM-1 and ICAM-1 and their counterpart ligands on neutrophils. Finally, the neutrophils transmigrate through the endothelium [85].

In the lung, however, much of the leukocyte margination and transmigration takes place through pulmonary capillaries which are too narrow to allow rolling, and adhesion molecules appear not to mediate the initial events, but are important to keep leukocytes in the lungs [86]. Under normal conditions, neutrophils have to deform to pass through the pulmonary microvasculature [87]. The deformability of neutrophils however is adversely affected by the pro-inflammatory mediators released by the lung of ARDS patients, such as leukotriene B_4, IL-8, C5a and endotoxin, which will result in stiffer neutrophils and entrapment in the pulmonary microvasculature [87-90]. IL-8 will also prompt the entrapped neutrophils to leave the vasculature and enter the alveoli [91]. Neutrophils can migrate through the endothelium at the site of inter-endothelial junctions or alternatively using a trans-cellular route [92]. Cell-to-cell interactions between neutrophils and pulmonary endothelial cells as well as the release of pro-inflammatory mediators by neutrophils induce cytoskeletal changes in the endothelial cells, resulting in a transient remodelling of intercellular junctions, allowing easier penetration through the endothelium [93-95].

Once retained and activated in the lung parenchyma, leukocytes can release products that will ultimately lead to further damage to the alveolar-capillary barrier

and propagate lung injury. These include proteolytic enzymes, oxidative radicals, cytokines and different growth factors [96]. Elastase, the predominant protease released by neutrophils in ARDS, will cause damage to the alveolar-capillary membrane by destruction of the extracellular matrix and induce apoptosis in alveolar epithelial cells, thus facilitating further migration of neutrophils from the microvasculature to the alveolar space [55, 97]. Elastase was found to be elevated both in plasma and bronchoalveolar lavage fluid from patients who are at risk for or already have established ARDS [98, 99]. Other neutrophil derived proteases, such as gelatinases and collagenase are also elevated in the bronchoalveolar lavage fluid of ARDS patients [100-104].

Monocytes

Although recruitment of neutrophils to the lung is a key event in the development of ALI/ARDS [22], and studies investigating the role of leukocytes in ARDS pathogenesis predominantly focused on these cells [105-110], ARDS can also develop in neutropenic patients [111, 112], suggesting that other leukocyte subsets might be also important in the pathogenesis. Indeed, there is accumulating evidence from our group and others, that monocytes may also play an important role [113-119]. While it can be argued that neutropenic patients are often monocytopenic as well, at least in the aforementioned study by Sivan *et al.* 10 out of 26 neutropenic patients had moderate infiltration of monocytes on post-mortem lung histology [111]. Moreover, these observations only underline the importance of neutrophil independent mechanisms (both cellular and humoral) in ARDS pathogenesis.

Monocytes are a pluripotent, heterogeneous population of cells of the mononuclear phagocytic system. Upon activation, they can release a variety of

inflammatory mediators, including TNF. These cells exist in two phenotypically distinct subsets based on their expression of Gr-1 (specifically the Ly6C epitope) in mice and CD14 and CD16 in humans [120, 121]. Immature monocytes - defined in mice as Gr-1^{high} and CD14^{high}/CD16^{-} in humans - enter the circulation from the bone marrow and migrate to the site of inflammation and injury [120, 122, 123], and hence are called the inflammatory subset. During maturation of monocytes there is a change to less inflammatory phenotype – Gr-1^{low} and CD14^{low}/CD16^{high} in mice and humans, respectively - [124] and these cells differentiate into tissue resident macrophages and dendritic cells [120, 125].

O'Dea *et al.* have shown in mice that inflammatory Gr-1^{high} monocytes marginate to the lung during early endotoxaemia and by promoting pulmonary endothelium activation contribute to the progression of ALI [115]. This was found to be mediated by TNF, expressed on these lung-marginated inflammatory monocytes. Furthermore, Wilson and co-workers have also demonstrated in a mouse two-hit LPS/VILI model that recruitment of inflammatory monocytes exacerbated lung injury [116]. In a more recent study in mice, Dhaliwal and co-workers demonstrated that monocytes play an important role in the second-phase neutrophil emigration in established acute lung injury, thus contribute to injury development [118]. On the contrary, D'Alessio et al showed in a model of LPS-induced lung injury, that monocytes are important regulators of injury resolution but did not investigate the individual role of monocyte subsets [117].

These findings indicate that monocytes are also important in the pathogenesis of ARDS, and more investigation should be dedicated to them in the future in order to better understand the inflammatory mechanisms underlying ALI.

Platelets

There is piling evidence suggesting that platelets also play a significant role in the pathogenesis of acute respiratory distress syndrome [110, 126, 127]. In sepsis, platelets roll along and firmly adhere to capillary endothelial cells in the pulmonary circulation that is mainly mediated by platelet P-selectin and an unknown counter-receptor on the endothelium [126]. This adherence mediated activation of platelets results in subsequent release of chemokines and lipid mediators, which then either activate attached leukocytes or induce further activation of endothelial cells by releasing pro-inflammatory mediators [110]. Platelet activation is associated with increased expression of P-selectin [128], which mediates further capturing of freely rolling neutrophils in the pulmonary capillaries. The physical interaction between these two cell types leads to a reciprocal activation and results in the release of thromboxane A2 [110], which then further activates endothelial cells and induces the expression of adhesion molecules, polymerisation of actin, and contraction, which ultimately results in vascular leak and pulmonary oedema formation [110]. When this interaction is prevented by elimination or blockade of platelet P-selectin, pulmonary margination of neutrophils is significantly reduced and mice are protected from acid-induced lung injury [110].

On the other hand, platelets are an important source of sphingosine-1 phosphate (S1P), a lipid mediator that has been shown to play an important role in regulating inter-endothelial cell junctions and consequently in the maintenance/restoration of vascular integrity [129, 130].

Alveolar macrophages

Alveolar macrophages (AMs) are members of the mononuclear phagocyte system (MPS), along with monocytes. AMs are present in the alveoli and alveolar ducts and

serve as the first line of defence in the lungs [131]. They actively phagocytise and kill invading airborne and blood-borne pathogens and regulate the inflammatory reactions in the lungs [132-135]. In order to induce and potentiate the inflammatory and immune processes, AMs release cytokines (TNF, IL-1 and -6, IFN-gamma), chemokines (CXC chemokines such as IL-8 or its rodent homologue MIP-2 and KC, CC chemokines such as MCP-1, MIP-1alpha and beta) and prostaglandin-E2 during the initial phase of lung inflammation [135-137]. The cytokines TNF and IL-1 will induce expression of adhesion molecules (such as E-selectin, VCAM-1 and ICAM-1) on both endothelial and epithelial cells to facilitate migration of leukocytes across the alveolar-capillary membrane [135]. Up-regulated ICAM-1 on alveolar epithelial cells will enhance the adhesiveness of alveolar macrophages that subsequently results in intensified production of cytokines and chemokines and enhanced phagocytic function [75, 76, 135]. Release of IL-6 by AMs also facilitates the secretion of inflammatory mediators by alveolar epithelial and pulmonary endothelial cells [135]. Production of IL-8 (or MIP-2 and KC in rodents) attracts neutrophils to the lung parenchyma and alveolar space, MCP-1 enhances lung margination of monocytes and neutrophils, whereas MIP-1 alpha and beta will further intensify the production of CXC chemokines by macrophages in an autocrine manner [135].

There is large amount of evidence to suggest that AMs play a pivotal role in regulating the pro- and anti-inflammatory events during sepsis-related ARDS [138, 139]. It has been suggested that, neutrophils recruited to the alveolar space by AM-induced or derived chemokines stimulate the release of IL-10 from the AMs [140-143], a powerful suppressor of the NF-kappaB (NFkB) inflammatory pathway. Thus, whilst AMs play an important role in initiating the inflammatory response in the early stage of ARDS, they also counteract at later stages during the pathogenesis by

triggering an anti-inflammatory response. This temporal dichotomy is perhaps demonstrated by depletion studies. For example, in more acute animal models of ARDS, such as ventilator-induced lung injury [144, 145] and lung ischaemia-reperfusion injury [146] depletion of AMs by clodronate-liposomes ameliorates injury, while in more chronic models, such as polymicrobial sepsis [147] and intra-alveolar activation of the Fas/FasL pathway by agonist antibodies [148] lung injury is intensified. On the other hand, it is also possible that this differential effect of AM depletion is more related to other model differences.

Surfactant dysfunction

Pulmonary surfactant is important in maintaining lung homeostasis because of its role in reducing work of breathing by decreasing surface tension in the alveoli, and in host defence [149, 150]. It is composed of phospholipids (80%), cholesterol (10%) and four surfactant-associated proteins, namely SP-A, B, C and D. The components of surfactant mainly responsible for its biophysical properties are dipalmitoyl-phosphatidyl-choline (DPPC) and SP-B and –C [151], whereas SP-A and –D play a major role in host defence [152-154], although SP-A can also reverse the inhibitory effect of blood-borne proteins (fibrinogen and albumin) on the surface activity of surfactant in vitro [155].

Surfactant is synthesised by type II AECs and exocytosed in lamellar bodies into the alveoli, which then solve to form tubular myelin-like structures [156-158] that adsorb to the air-liquid interface [159, 160]. These lamellar bodies and myelin-like structures give the denser subfraction of surfactant obtained by lung lavage, and are so called large aggregates (LAs) [161, 162]. They reduce surface tension to levels that will prevent alveolar collapse during exhalation, and thus decrease breathing

effort during inhalation [163, 164], and will also prevent alveolar flooding due to hydrostatic forces. Secondary to the repeated expansion and surface contraction that comes with respiration, this fine surfactant film continuously breaks up into small vesicles which are then endocytosed by type II alveolar epithelial cells, where they are recycled for the synthesis of new surfactant, or by alveolar macrophages in which case they are permanently removed from the air spaces [165-167]. These vesicular structures form the less dense fraction of the lavaged surfactant called small aggregates (SAs). As opposed to the LAs, these do not contain all the proteins and lipids required to reduce surface tension [163, 164]. Within the normal lung, the ratio of LA:SA is consistent and maintained by a dynamic process of on-going secretion of LAs, conversion of LAs into SAs during respiration, and the clearance and/or recycling of SAs by AMs and type II AECs, respectively [165, 168].

The physiological features observed in ARDS, such as hypoxaemia and decreased compliance are both consequences of a dysfunctional surfactant system [150]. Changes in the surfactant obtained from ARDS patients include an imbalance of the different phospholipids and decreased levels of surfactant-associated proteins [36, 169]. These changes are, in part, a result of the damage to the type II AECs and their metabolic dysfunction, as well as of the increased alveolar permeability leading to accumulation of proteins [170, 171], enzymes (e.g. proteases, phospholipases) [172-178] and oxidants (ROS, RNS) [179-182] that either biophysically or chemically inhibit surfactant function. Decreased synthesis of LAs by dysfunctional type II AECs and increased conversion of LAs into SAs triggered by mechanical ventilation [183-187] will result in a proportionally lower amount of functional LAs relative to non-functional SAs [188, 189], which ultimately leads to alveolar instability. While the role of surfactant in host defence is well established *in vitro* and in animal models, it

remains unknown how these alterations affect the immune response and susceptibility to further insult in ARDS patients.

Inflammatory cytokines/chemokines

A dysregulated inflammatory response is central to the pathogenesis of acute lung injury. Activation of the inflammatory cascade starts with recognition of microbial products (so called pathogen-associated molecular patterns [PAMPs], e.g. lipoteichoic acid [LTA], LPS, flagellin, double-stranded RNA [dsRNA]) and/or cell injury-associated molecules (so called danger-associated molecular patterns [DAMPs], e.g. heat-shock proteins, high-mobility group box 1 [HMGB1], hyaluronan, oxidised phospholipids, DNA) by pattern recognition receptors (PRR) expressed on pulmonary cells (such as AECs, AMs, pulmonary endothelial cells [PECs], dendritic cells) [190]. Toll-like receptors (TLRs) are the most well-known members of PRR, whereas others include the cytosolic NOD-like receptors (NLRs), RIG-I–like receptors (RLRs), and DNA sensors [190]. These receptors have a close downstream link with the inflammatory transcription factor NFkB and interferon regulatory factor (IRF). Upon ligand binding, these transcription factors become activated leading to the production of inflammatory cytokines TNF, IL-1 beta, IL-6, IFN-beta and chemokine IL-8, predominantly by AMs [190]. IFN-beta enhances the production of antimicrobial peptides by AMs and AECs in an autocrine and paracrine fashion, respectively, whereas TNF and IL-1 beta will trigger the release of other CC (e.g. MCP-1, MIP-1 alpha and beta) and CXC chemokines (e.g. KC, MIP-2) by AECs and PECs. These chemokines are then retained locally by matrix heparan sulphate proteoglycans establishing a chemokine gradient which will result in the recruitment of neutrophils and monocytes to the lung microvasculature and alveolar space [190].

These recruited cells then cause further activation of the local pulmonary cells amplifying the inflammatory response.

However, as the inflammatory response progresses, several endogenous anti-inflammatory mediators are also released. For example, IL-10 (also known as human cytokine synthesis inhibitory factor [CSIF]), IL-11 and IL-13, predominantly expressed by monocytes/macrophages and T-cells, can suppress NFkB by inhibiting the hydrolysis of IkB-alpha, thus preventing NFkB to translocate to the nucleus and initiate transcription of pro-inflammatory mediators [191-193]. Exogenous administration of these substances in animal models of acute lung injury attenuated injury and improved survival [194-197]. Expression of autoantibodies against IL-8 neutralises the chemotactic effect of IL-8 [198] and levels of IL-8:anti-IL-8 autoantibody complexes in the BALF better predict clinical outcome of ARDS than IL-8 levels alone [199]. Level of IL-1 receptor antagonist (IL-1Ra), the natural antagonist to IL-1 beta, is also elevated in the BALF of patients with ARDS, and may play an important role in the resolution of the fibroproliferative process of ARDS (see below) [200]. Soluble receptors of TNF are also present in the alveolar space of ARDS and may dampen the inflammatory response by preventing TNF binding to its membrane receptors [22]. Therefore in summary one can say that the balance between these pro- and anti-inflammatory mediators may be more important in determining the overall inflammatory response and the clinical outcome of ALI/ARDS than the absolute levels of one or several specific pro-inflammatory cytokines [201]. The detailed involvement of all cytokines/chemokines is beyond the scope of this study, but the actions of TNF will be explored in more detail in a separate section.

Oxidative injury and redox imbalance

There is mounting evidence in the literature that reactive oxygen and nitrogen species (ROS and RNS, respectively) are key modulators in the pathogenesis of ARDS, particularly in mediating pulmonary endothelial cell dysfunction [202-205]. For example, patients with ARDS have increased levels of hydrogen peroxide (H_2O_2) in their exhaled breath condensate (EBC) [202], myeloperoxidase (MPO) and oxidised $alpha_1$-antitrypsin in their BALF [204], and show increased plasma lipid peroxidation [203, 205].

The majority of ROS during the development of ARDS is generated by the inflammatory response, primarily by neutrophils and monocytes/macrophages [35]. In response to the appropriate stimuli (e.g. LPS, TNF, IL-1, platelet activating factor [PAF]), these cells release large amounts of superoxide anion ($\bullet O_2^-$) produced by xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase. In addition to $\bullet O_2^-$, XO can also produce H_2O_2 [206]. Superoxide is dismutated by superoxide dismutase (SOD) forming H_2O_2 which plays a central role in bacterial killing [207].

Nitric oxide (NO) is a free radical and signalling molecule with multiple physiological effects. It is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS). There are three NOS isozymes: neuronal NOS (nNOS or NOS-1), cytokine-inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). The main sources of NO in the lung are iNOS and eNOS. While eNOS is constitutively active in pulmonary endothelial cells and produces a moderate amount of NO that is mainly involved in regulating vascular functions, iNOS which is mainly expressed by AMs, PMNs and type II AECs, is only activated by different inflammatory cytokines (e.g. TNF) and has a high output providing an opportunity for NO to react with $\bullet O_2^-$ leading to the production of peroxynitrite ($ONOO^-$). $ONOO^-$ is

also a highly active radical that can cause DNA and protein damage leading to apoptosis and cell necrosis. Patients who died from ARDS show markers of peroxynitrite formation in their lungs [208].

In addition to the inflammatory response, the therapeutic administration of oxygen to patients with ARDS may also increase the production of ROS and exacerbate the injury [209-212]. However, it is not only the excess production of free radicals but the deprivation of the endogenous antioxidant systems and the resultant imbalance of the pulmonary redox state that leads to lung injury [206].

Thiols are an important group of antioxidants, among which glutathione (GSH) is one of the most important. GSH exerts its antioxidant effect by 1) directly scavenging free radicals, 2) regenerating the active (reduced) forms of Vitamins C and E, 3) maintaining critical protein thiols, and 4) acting as a co-factor for the detoxifying enzymes glutathione-peroxidase (GPX) and glutathione-S-transferase (GST) [213]. To undertake these functions, GSH needs to be in its reduced form, which depends on the activity of glutathione reductase. However, the activity of this enzyme can reduce in oxidative stress such as that occurs in ARDS patients, who therefore have 500,000-fold less plasma thiols than controls [214], and their GSH level both in plasma and BALF is also depleted [215, 216].

ROS/RNS can activate transcription factor NFkB via p38 and ERK MAPKs [217], but also the activator protein (AP)-1 family, which leads to production of inflammatory proteins, AEC apoptosis and increased vascular and epithelial permeability in the lung [218, 219]. It has been also demonstrated, that IL-1-beta and TNF-induced activation of hypoxia-inducible factor (HIF)-1-alpha, the transcription factor that regulates the production of VEGF and iNOS, is mediated by induction of

intracellular ROS. This is quite important as both VEGF and increased iNOS activity is associated with endothelial barrier dysfunction in the lung [220, 221].

In summary, excess production of free radicals in conjunction with a depleted antioxidant system favours a pro-oxidant state in the lung which ultimately leads to injury by different mechanisms, including direct cell injury and activation of signalling pathways involved in inflammation and apoptosis.

Coagulation pathway

Intra-alveolar fibrin deposition is a histological hallmark of ARDS resulting in abnormalities in gas exchange [41]. Besides its effects on gas exchange, activation of the coagulation pathway is also closely linked with the inflammatory response seen in sepsis and ARDS [38]. Firstly, activation of the coagulation pathway through tissue factor (TF) leads to thrombin formation on the endothelial surface, which then activates inflammatory leukocytes by binding to their protease-activated receptors (PARs) with a subsequent up-regulation of inflammatory mediator production and release [222]. Secondly, inflammatory mediators produced during the pathogenesis of sepsis/ALI/ARDS can induce TF expression and down-regulate its natural inhibitors protein C and S, which eventually results in thrombin formation and deposition of fibrin [223].

TF is the most potent initiator of the coagulation pathway. Tissue factor pathway inhibitor (TFPI) expressed by endothelial cells is the endogenous inhibitor of TF, and can be found in the plasma and on the surface of platelets [224]. The balance between TF and TFPI will determine the degree of TF-initiated coagulation. Several cell types in the lung can produce TF, including AMs [225], AECs [226, 227] and PECs, and its production is up-regulated in response to pro-inflammatory

cytokines (IL-1beta, TNF and IFN-gamma) *in vitro* [228], and in animal models of direct [229, 230] and indirect [231] lung injury that is in part mediated by the NFkB pathway [232]. In the clinical setting, patients who are at risk for ARDS and those with established syndrome have increased TF-dependent pro-coagulant activity in the alveolar compartment compared to healthy controls regardless of the aetiology [229, 233, 234], indicating the importance of TF in the pathogenesis of ALI/ARDS.

Protein C (PC) is one of the most important regulators of the coagulation pathway. When PC encounters the thrombomodulin (TM)-thrombin complex on the cell surface, it is transformed into its activated form (APC) [235]. Once activated, APC suppresses further generation of thrombin by proteolytic inactivation of coagulation factors Va and VIIIa [236, 237]. Activation of PC is further amplified when it binds to the endothelial cell PC receptor (EPCR) [238]. APC also plays an important role in regulating the inflammatory response during sepsis/ALI. *In vitro*, APC supresses TNF production by monocytes through inhibition of the NFkB-pathway [239, 240], and inhibits neutrophil activation and leukocyte chemotaxis *in vivo* [241, 242]. Patients with sepsis or ALI/ARDS have reduced PC levels and ability to produce APC that is associated with ventilator dependency, higher prevalence of ARDS and increased mortality [243-245].

In ALI/ARDS there is a shift toward a pro-coagulant state, where up-regulation of TF and down-regulation of APC is accompanied by inhibition of fibrinolysis by an increased activity of the plasminogen activator inhibitor (PAI-1) system. Plasminogen activator (PA) and PAI-1 are the most important regulators of fibrinolysis. During sepsis there is an initial up-regulation of fibrinolytic activity, shortly after followed by a prolonged increase in circulating PAI-1 [246] favouring the persistence of fibrin and

microvascular thrombosis, leading to organ ischaemia and dysfunction that is associated with higher mortality [247].

Similarly to the TF and PC pathways, PAI-1 has been also linked with inflammation. For example, TNF has been shown to induce PAI-1 expression by endothelial cells *in vitro*, through activation of the NFkB pathway [248]. On the contrary, blockade of TNF by monoclonal antibodies did not attenuate the increase in PAI-1 levels in a rabbit model of endotoxaemia, suggesting that other mechanisms are also likely to be involved [249]. Also, human AMs exposed to endotoxin convert to an anti-fibrinolytic phenotype and increase PAI-1 activity, and AMs in lung biopsy from ARDS patients also exhibit increased expression of PAI-1 [250]. There is also a change in the haemostatic phenotype of pulmonary microvascular endothelial cells (PECs) [251]. PECs isolated from ARDS patients express constitutively more PAI-1 than controls and have lower fibrinolytic activity as expressed by tPA:PAI-1 ratio [251]. Patients with ARDS have increased levels of PAI-1 in their pulmonary oedema fluid as well as in their circulation and demonstrate reduced fibrinolytic activity [234, 252-254] associated with higher mortality [244]. Nevertheless, it is important to realise that this anti-fibrinolytic profile in sepsis and ARDS may play an important role in detaining infection.

Fibrosing alveolitis

After the initial exudative phase of ALI/ARDS, some patients display an uncomplicated course with rapid resolution [22]. Others, however, develop pulmonary fibrosis observed as soon as 5 to 7 days after the onset of ALI/ARDS [22, 40, 41]. This process is considered to be a result of a maladaptive reparative response to injury, characterised by proliferation of myofibroblasts and fibroblasts,

prolonged presence of interstitial inflammatory leukocytes, pronounced vascularisation and deposition of connective tissue in the alveolar space [35]. Fibrosing alveolitis is associated with increased risk of death [255], and those dying from ARDS have increased lung content of collagen type II and III as well as fibronectin [256]. It is thought that the molecular mechanisms that will determine whether a patient develops fibrosing alveolitis or not may be set early in the course of ARDS, and likely reflects the severity of the initial lung injury. For example, alveolar levels of pro-collagen III can be elevated even at the time of diagnosis and are associated with increased mortality [257-259]. Pro-inflammatory cytokines, such as IL-1 may be important in promoting the initiation of fibrogenesis [260]. For instance, lavage fluid from patients with early ARDS is mitogenic for human fibroblasts in vitro, which is dependent on active IL-1 [261, 262].

Resolution of lung injury

Resolution of lung injury happens in phases which may overlap in time and depends on several humoral and cellular factors. To start with, intrapulmonary inflammation must cease for a successful resolution process. This involves scavenging of the chemotactic cytokines/chemokines [263, 264], so that recruitment of neutrophils to the lung parenchyma can be halted. Then the neutrophils already present in the lung have to go through apoptosis [265] and be cleared via phagocytosis by resident and recruited macrophages [119, 266]. It has been recently demonstrated that a specific subtype of regulatory T-cells (Treg lymphocytes) also appears in the alveolar space in gradually increasing numbers peaking around 10 days from the onset of lung injury [267, 268], and play an important role in the resolution phase through TGF-beta mediated mechanisms [267].

At the same time, to restore the adequate gas exchange, pulmonary oedema must resolve. This is driven by vectorial transport of sodium and chloride from the alveoli to the lung interstitium, creating an osmotic gradient, which prompts water to follow passively through transcellular water channels, the aquaporins (AQ), predominantly AQ5 expressed on type I cells [66]. From the interstitium, oedema fluid is then removed by the lymphatic circulation. Sodium is transported basolaterally by sodium-potassium ATPase (NaKATPase), which exchanges 3 intracellular Na^+ for 2 extracellular K^+ in an energy consuming process that generates a gradient allowing sodium transport across the apical sodium channels, such as the amiloride-sensitive epithelial sodium channel (ENaC) and other amiloride-insensitive sodium channels (e.g. cyclic nucleotide-gated cation channels (CNG)) [66]. Chloride is transported by trans- and paracellular pathways. The rate of alveolar fluid clearance increases substantially in the presence of cAMP, which enhances the expression and activity of ENaC and NaKATPase and the opening of the cystic fibrosis conductance regulator (CFTR) on the apical surface to facilitate chloride uptake [66]. In addition to water, soluble and insoluble protein must also be removed from the alveolar space. Insoluble proteins are predominantly removed by endocytosis and transcytosis by AECs and phagocytosis by AMs, whereas soluble proteins are removed primarily by diffusion between AECs [269]. Patients with intact alveolar fluid clearance require shorter period of mechanical ventilation, have improved oxygenation and a better survival rate [270].

In order to achieve a net fluid clearance, the alveolar epithelial barrier also needs to be restored. Re-epithelialisation of the epithelial surface is accomplished by differentiation of type II epithelial cells into type I cells, which is facilitated by growth factors, such as keratinocyte growth factor (KGF) and hepatocyte growth factor [271,

272]. Recent evidence indicates that bronchoalveolar and bone marrow-derived stem cells also contribute to epithelial repair [273-276]. Bone marrow-derived stem cells are likely recruited by stromal-derived factor-1 and other chemokines and cytokines (e.g. IL-8, TNF, macrophage inhibitory factor (MIF), TGF-beta) released by the injured lung, as they express the relevant receptors for these factors [277-281]. The migration of bone marrow-derived endothelial progenitor cells to the injured lung is mediated by CXCL1 and 2 chemokines [282], and increased number of these cells in the circulation of patients with ALI/ARDS is apparently associated with improved survival [273].

1.2 Mechanical ventilation and Ventilator-Induced Lung Injury

Patients who develop ALI/ARDS - of any aetiology – require positive pressure ventilation in order to maintain appropriate gas exchange. However there is accumulating evidence that mechanical ventilation has its own deleterious effects, and can cause high-stretch related injury also known as ventilator induced lung injury (VILI)[283].

Although it has been recognised clinically for many decades that ventilation with high airway pressures can cause alveolar rupture and air leak into the surrounding tissues, leading to pneumothorax, pneumomediastinum, pneumopericardium, subcutaneous emphysema and air emboli [284, 285], the concept of VILI mainly originates from animal models, where more subtle lung injury (i.e. without alveolar air leak) can be induced by overdistention of healthy lung units using high inspiratory pressures. The first example of this is the seminal study by Webb and Tierney which showed that rats ventilated with a PIP of 45cmH_2O (without PEEP) developed more severe and more rapid pulmonary oedema than those ventilated with a PIP of 30cmH_2O, whereas ventilation with a PIP of 14cmH_2O for 1 hour caused no histological changes in the lungs [286]. Similar findings were later observed in rabbits [287], sheep [288] and dogs [289].

VILI models show the same pathophysiological features as clinical ARDS, such as pulmonary oedema, epithelial damage and hyaline membrane formation [290], making VILI indistinguishable from ARDS in the clinical setting. Mechanical ventilation, through VILI can exacerbate the existing injury, and generate a vicious cycle of inflammatory mechanisms that eventually leads to systemic inflammatory response syndrome (SIRS) and multi-organ failure (MOF)[291].

VILI and the associated injuries are not solely due to excessive lung inflation, but a combination of insults with various mechanisms, namely: volutrauma, atelectrauma and biotrauma. These mechanisms now will be discussed.

Volutrauma

In early animal studies of VILI, it was suggested that injury occurs in response to high airway pressures, it was relatively soon realised that it is the associated overdistention of the lung rather than the pressure *per se* that drives pulmonary oedema formation. This was elegantly demonstrated in a study by Dreyfuss *et al.* who compared the effects of low and high V_T ventilation in the presence of high and low peak airway pressures in rats [292]. Low V_T–high PIP ventilation was achieved by thoraco-abdominal strapping of the animals, whereas high V_T–low PIP ventilation was accomplished by using an iron lung. The authors concluded that it was the excessive V_T and the resultant end-inspiratory lung volume and overdistention that caused pulmonary oedema, rather than excessively high inspiratory pressure *per se*, which led to the term *volutrauma* as opposed to *barotrauma* [293, 294]. These findings were also confirmed in rabbits and lambs using similar protocols [295, 296].

Subsequent animal studies evaluated the effect of mechanical ventilation with overdistention on pre-injured lungs to resemble the clinical situation, particularly because VILI occurs less readily in ventilated patients with healthy lungs [297, 298]. In perfused rabbit lung models a combination of oleic acid pre-injury with relatively high tidal volume has been shown in a variety of studies to result in increased capillary filtration co-efficient and wet:dry weight ratio, whereas the two insults individually did not [299, 300]. One of these groups also demonstrated that inactivation of surfactant by dioctyl-succinate enhanced the increase in capillary

filtration co-efficient caused by high-stretch ventilation [301]. Dreyfuss *et al.* demonstrated the synergistic effect of pre-existing lung injury and high-stretch ventilation in an *in vivo* rat model where lungs were damaged with alpha-naphtylthiourea and ventilated with high V_T (45ml/kg) [302]. The combination of the two insults resulted in more severe permeability oedema than that predicted by summing up their individual effects, indicating synergism.

One mechanism by which increased permeability occurs in high-stretch ventilation is the mechanical injury caused to the pulmonary endothelial and epithelial cells by excessive stretching forces, so called stress-failure. During mechanical ventilation an internal tension develops in the alveolar structure with each inspiration that is equal to the transpulmonary pressure (airway pressure minus pleural pressure) and is also called stress [303]. This stress is associated with elongation of the fibres of the extracellular matrix as well as the cells (i.e. endothelial and epithelial) of the alveoli anchored to it, and this is called strain. If stress exceeds the elastic properties of this structure it results in stress-failure. Fu *et al.* demonstrated in rabbit lungs that an increase in transpulmonary pressure from 5 to 20cmH_2O resulted in a significant increase in endothelial and epithelial ruptures [304]. This observation points out that while high peak inspiratory pressure *per se* may not be an important determinant of pulmonary oedema (see study by Dreyfuss *et al.* above), transpulmonary pressure (stress) is, and it is equally important to volume (strain).

Beside the mechanical injury, high-stretch ventilation can also activate cellular mechanisms (a concept often referred to as mechanotransduction) that may result in increased pulmonary permeability. For example, Parker *et al.* demonstrated in isolated perfused rat lungs that blockade of stretch-activated cation channels with

gadolinium administered to the perfusate prevented high-stretch induced increase in microvascular permeability [305]. Their findings suggested that increased microvascular permeability was mediated by active cellular mechanisms dependent on intracellular calcium as opposed to passive mechanical injury to the alveolar-capillary barrier. Other active mechanisms, such as p38 MAPK-mediated alveolar cell apoptosis and actin polymerisation were also demonstrated to mediate alveolar-capillary barrier dysfunction associated with VILI [306, 307]. Several plasma membrane-associated molecules have been identified to sense mechanical stress (hence called mechanoreceptors), including adhesion molecules, cytoskeleton and associated proteins and mechanosensitive ion channels, all of which can activate downstream signalling pathways [308].

Atelectrauma – Injury at low lung volumes

In addition to volutrauma at high lung volumes there is great amount of evidence that lung injury can also occur during ventilation at low lung volumes (meaning low absolute lung volumes not low tidal volumes). This injury is thought to be related to the repetitive opening-closing of collapsed distal lung units. It is important to note that in a healthy lung, alveoli do not collapse at the end of expiration due to the low surface tension maintained by the surfactant lining and the physiological PEEP. In ARDS, however, because of surfactant dysfunction secondary to the interaction with pulmonary oedema fluid and the effects of mechanical ventilation (i.e. increased breakdown, impaired *de novo* synthesis), lungs are more susceptible to develop atelectasis. Once the airways collapsed, the amount of force required to reopen them is enormous and inversely proportional to their diameter, i.e. the more distal the airway the more force (pressure) is needed. Another aspect to this is that in normal

lungs, adjacent alveoli (except the ones adjacent to the pleura) are always surrounded by other alveoli, and the forces exerted on one lung unit are transmitted to those around it [309]. This support is called interdependence, and helps to maintain the homogeneity of the lung. However, this also implies that if an alveolus collapses, the forces applied to its walls by the surrounding open alveoli will be greatly increased in order to help re-expansion. It has been calculated that at a transpulmonary pressure of 30cmH_2O, re-expansion pressures can reach as high as 140cmH_2O, which results in a potentially injurious stress at the interface of collapsed and expanded groups of alveoli [310]. Indeed, it has been shown in autopsies from ARDS patients that pseudocysts and cavities were predominantly around atelectatic areas [311]. Therefore, the repetitive opening and closing of small airways leads to excessive shear-stress and subsequent injury to lung units, particularly if present in each breath [312].

The main approach to minimise the deleterious effect of atelectrauma is by the application of PEEP. Webb and Tierney were the first to show that alveolar oedema was reduced by applying 10cmH_2O of PEEP during ventilation of rats with 45cmH_2O PIP [286]. This was later replicated by Dreyfuss *et al.*, who also demonstrated that this positive effect of PEEP was associated with preservation of the alveolar epithelium [292].

While PEEP increases the end-expiratory volume and keeps lung units open, if the tidal volume applied is the same, PEEP will also increase end-inspiratory volume thus favouring overdistention. Conversely, if application of PEEP is accompanied by reduction of V_T and end-inspiratory volume is kept constant, oedema formation is reduced [313], as demonstrated by the studies of Webb and Tierney, and Dreyfuss *et al*. Therefore setting the right level of PEEP is very

important to avoid overdistension of lung units, in which the pressure/volume (P/V) curve can be helpful.

The P/V curve has two characteristic points, namely the lower and upper inflection points (LIP and UIP, respectively). The LIP represents the recruitment pressure and lung volume at which lung units start to re-open and lung expansion rapidly begins, which is then followed by linear increases in volume with increasing pressures until the UIP is reached, where the lung is near maximum capacity determined by elastic properties of its fibrous structure [312, 314, 315]. Beyond this point, however, no further recruitment occurs and lung units are endangered to over-distension. Therefore in an ideal ventilation strategy tidal ventilation would take place on the steep portion of the static P/V curve, between the LIP and UIP [315, 316]. This may be achieved by setting the PEEP above the LIP, which should keep the alveoli open at end-expiration thus prevent repeated opening and closing and the associated shear stress in distal airways. This approach of setting PEEP is central to the protective, 'open-lung' ventilation strategy [317], which will be discussed in more detail later under 'Therapeutic approaches to Acute Lung Injury'.

Biotrauma and Multiple System Organ Failure (MSOF)

Beside some of the detrimental effects on respiratory physiology, mechanical stresses associated with ventilation can also initiate cellular and molecular mechanisms that lead to an inflammatory response, termed biotrauma [318]. An increasing body of evidence from both experimental and clinical studies indicates that the degree of inflammation depends on the mode of mechanical ventilation [319-322]. It has been also suggested that the inflammatory response is not limited to the lung but may also trigger and propagate a systemic inflammatory response,

contributing to MSOF [291], the major cause of death in this patient population [323-326]. Therefore, understanding how mechanical ventilation influences these cellular and molecular processes is crucial, as they may represent potential therapeutic targets in the battle against ARDS-associated mortality.

Most of the cellular and humoral factors involved in the inflammatory response associated with ARDS have been already discussed earlier, but here we will focus on them in relation to VILI.

Pulmonary inflammation induced by mechanical ventilation

1) Cellular mechanisms

The study by Hamilton *et al.* in 1983 was the first to demonstrate that injurious mechanical ventilation is associated with an increased inflammatory response. The authors compared the effects of high-frequency oscillation and conventional ventilation strategies in surfactant depleted rabbits [327]. The authors noted that the conventional ventilation strategy was not only associated with a significantly worse lung function and histological lung injury compared to HFO, but also with neutrophil infiltration to the alveoli and interstitial space [327]. To dissect out the role of neutrophils in lung injury associated with conventional injurious ventilation, in a subsequent study Kawano *et al.* subjected neutrophil-depleted rabbits to the same ventilation protocol, and found that in marked contrast to controls, the neutrophil-depleted animals did not develop significant lung injury [328]. However, when neutrophils were re-introduced to neutrophil-depleted rabbits, this resulted in pulmonary dysfunction [328], indicating that lung injury due to surfactant dysfunction and VILI was mediated, at least in part, by neutrophils. Choudhury *et al.* later also demonstrated in mice that injurious high-stretch ventilation *per se* without any

underlying lung injury induces early pulmonary sequestration of neutrophils preceding the physiological signs of lung injury [106]. The authors also found that this phenomenon was associated with stretch-induced inflammatory events leading to neutrophil stiffening and was mediated by L-selectin-dependent mechanisms [106]. In a subsequent study the authors also demonstrated that neutrophil accumulation in the lung secondary to pure mechanical-stretch is mediated, at least in part, by intra-alveolar TNF signalling [109].

Although these and other studies clearly demonstrate that injurious mechanical ventilation strategies are associated with lung inflammation characterised by neutrophil margination, more recent evidence suggest that monocytes are also involved in VILI. Wilson and colleagues showed that healthy mice subjected to injurious mechanical ventilation (high V_T and zero PEEP [ZEEP]) had an increased number of the inflammatory subset Gr-1^{high} monocytes in their pulmonary vasculature compared to control [116]. Pre-treatment with a subclinical dose of intravenous LPS (20ng) 2 hours prior to the start of high-stretch ventilation increased the number of marginated monocytes and significantly aggravated lung injury, whereas monocytes depletion with intravenously administered clodronate liposomes 24h prior to high-stretch ventilation significantly attenuated pulmonary oedema and dysfunction both in LPS-treated and non-treated mice [116].

Alveolar macrophages (AMs) are also key players in VILI. Imanaka *et al.* showed an increased expression of ICAM-1 and CD11b on AMs recovered from rats exposed to high-pressure ventilation [107]. Moreover, Frank *et al.* demonstrated that depletion of AMs significantly attenuates permeability pulmonary oedema in a rat model of VILI [145]. Interestingly, the authors also found that interaction of AMs with alveolar epithelial cells was required for AM activation [145]. These studies clearly

indicate that AMs become activated during high-stretch ventilation and significantly contribute to VILI.

2) Humoral factors

Several clinical investigators have observed that conventional as compared to lung-protective ventilation strategies can increase the level of pro-inflammatory mediators in the lung, such as IL-1-beta, IL-6, IL-8 and TNF [329-331] and that pulmonary inflammation is associated with poor outcome in ARDS patients [332, 333]. Experimental studies also confirmed that injurious mechanical ventilation results in production of a wide range of inflammatory mediators in the lung [109, 319, 322, 334-337]. For example, Tremblay *et al.* examined the effects of injurious mechanical ventilation using an *ex vivo* isolated rat lung model [335]. The authors found that lungs from rats subjected to injurious ventilation strategies (high end-inspiratory and/or low end-expiratory lung volume) had increased levels of the inflammatory cytokines/chemokines TNF, IL-1-beta, IL-6, MIP-2, IFN-gamma and IL-10 in their lavage fluid, and showed an increased expression of mRNA for c-*fos* (an early response gene with a stretch sensitive promoter) and TNF in the lung homogenate [335]. Independently from this finding, the same year Takata *et al.* published similar observations in rabbits subjected to saline lavage followed by HFO or conventional mechanical ventilation (CMV), demonstrating that CMV was associated with increased expression of TNF mRNA in lavage cells [322]. Experimental 'blocking' studies indicated that these inflammatory mediators are not only produced in response to high-stretch ventilation but also have a pathogenic role. For example, blockade of IL-1beta and TNF signalling significantly attenuated the inflammatory response (and in some cases lung dysfunction and pulmonary oedema as well)

associated with VILI in different *in vivo* studies both with pre-injured and healthy subjects [109, 334, 338, 339].

Subsequent studies then aimed to identify the source and mechanisms by which these mediators are produced in the lung in response to stretch. Focus therefore turned to *in vitro* setups where individual cell types of the lung parenchyma could be exposed to stretch forces. For instance, isolated foetal rat lung cells exposed to cyclic stretch (5% elongation with 40cycles/min) produced MIP-2 within 1 hour from the start of insult, and this was associated with cell injury (as shown by production of lactate dehydrogenase in the supernatant) but independent from stretch-activated cation channels as demonstrated by Mourgeon *et al. [340]*. Pugin *et al.* identified alveolar macrophages as the main source of TNF, IL-6, IL-8, IL-10 and matrix-metalloproteinase-9 (MMP-9) in response to prolonged (8 and 24h) cyclic stretch, whereas the alveolar type II cell-like A549 epithelial cells produced only a small amount of IL-8 [341]. The authors also found that this response to stretch was associated with activation of NFkB in AMs and there was a synergistic effect between stretch and LPS, suggesting that mechanical ventilation is particularly more deleterious in pre-injured or infected lungs. In a similar study, Vlahakis and colleagues subjected A459 cells to cyclic stretch and measured the production of IL-8 [342]. They found that when cells were stretched by 30% they released ~50% more IL-8 than the static controls, however when were stretched by only 20% the cells did not produce IL-8 [342]. This may give an explanation to the observation of Pugin and co-workers [341], as in their study cells were only stretched by 12-15% of their original surface area.

Cyclic stretch of human pulmonary microvascular endothelial cells also results in production of IL-8, IL-6 and MCP-1 in a p38-dependent and Ca-channel

independent manner [343]. This finding is important because it suggests that high-stretch ventilation may result in an increase of systemic cytokine/chemokine levels without any physical damage to the alveolocapillary barrier, which may be highly relevant to the systemic inflammatory response associated with VILI (see below). Overall these findings indicate that a number of cell types of the lung parenchyma is capable to sense stretch (albeit by different mechanisms) and contribute to the inflammatory milieu associated with VILI. Although it remains contentious as to how much stretch each of these cell types is likely to be exposed to in vivo.

In addition to the aforementioned inflammatory cytokines/chemokines, recent experimental studies identified other molecules that may also mediate VILI within animal models, such as angiotensin-converting enzyme [344, 345], ROS [346], lipid derived mediators and adenosine receptors [347-349], however these have as yet unknown clinical significance.

Systemic inflammation and extra-pulmonary organ damage induced by mechanical ventilation

It has been appreciated for a long time that in addition to exacerbating the pre-existing lung injury, mechanical ventilation also has systemic effects such as compromising haemodynamics and thus leading to hypoperfusion of organs and consequent organ failure. More recent clinical and experimental observations, however also indicate that mechanical ventilation has other systemic sequelae unrelated to its effect on haemodynamics [350]. For example, Ranieri *et al.* showed that using a protective ventilator strategy in ARDS patients resulted in a significant reduction in the inflammatory response not only in the lungs but also in the systemic circulation, indicating that the systemic inflammatory response was triggered by

and/or originated from the lungs [330]. More importantly, after retrospective analysis the authors also found that protective ventilation strategy was associated with reduced prevalence of MSOF, particularly renal failure, after 72h of enrolment to the study, and there was a significant correlation between occurrence of organ failure at 72h and the plasma concentration of several inflammatory mediators at 36h [333]. Stuber *et al.* also found that a more conventional ventilatory strategy is associated with increased levels of cytokines both in BAL and the systemic circulation within 1 hour from the start of ventilation [331]. Interestingly they also showed that changing the ventilatory strategy to a less injurious type can reverse the increase in plasma cytokines, but the effect on organ failure was not investigated [331].

Experimental studies lent further support to the idea that lung-borne inflammatory mediators can become decompartmentalised and spill over to the systemic circulation [336, 351-355]. Studies have shown that hyperventilation in isolated perfused lung models leads to increases in TNF and IL-6 in the perfusate [336] [355]. In addition to inflammatory mediators, live bacteria and bacterial products were also shown to decompartmentalise into the circulation from the alveolar space, following injurious mechanical ventilation in dogs, rats and rabbits [356-358]. In a more chronic model Lin *et al.* exposed rats to either protective (V_T=7ml/kg, PEEP=5cmH_2O) or injurious (V_T=21ml/kg and ZEEP) ventilation protocol for 1 hour before intratracheal administration of *Pseudomonas aeruginosa*, after which the mortality was assessed up to 48 hours [359]. The authors found that injurious mechanical ventilation was associated with a non-significant trend of increased mortality (40% vs. 28%), significantly higher rate of positive blood cultures and absolute bacteria count in blood, and an impaired host defence in the surviving animals by means of cytokine response [359]. Therefore, these data suggest that

more injurious ventilatory strategies not only lead to dissemination of the inflammatory milieu of the lung to the systemic circulation but may also alter host defence predisposing the body to further infectious/inflammatory insults, which may be another contributing factor in the development of MSOF. Munford and Pugin suggested that the body's normal response to stress is to minimise systemic inflammation, and local inflammation is therefore accompanied by an anti-inflammatory response systemically [360]. This is to help the body to concentrate its forces at the local injury site while preventing the potentially deleterious inflammation in uninvolved sites. However, this is a double edged sword, as if the anti-inflammatory response is too intense, it may lead to immunosuppression. This theory is supported by the observations of Plötz *et al.* who found that ventilation of infants with healthy lungs (V_T=10ml/kg, PEEP=4cmH_2O) resulted in an increase of TNF and IL-6 in BALF after 2 hours, which was associated with a reduced capacity of cytokine production by their peripheral blood leukocytes and a decreased activity of natural killer cells [361]. This notion was later confirmed in experimental settings using healthy rats [362].

Our group demonstrated that injurious mechanical ventilation in healthy mice is associated with margination of inflammatory leukocytes (i.e. neutrophils and Gr-1^{high} monocytes) to distal organs, such as the liver, spleen and kidney [363]. However, whether this response is associated with organ injury was not investigated, and it is still unknown what stimulus or mediator directs leukocytes to these organs in response to mechanical ventilation. One possible mechanism, as demonstrated by Hegeman *et al.*, is the activation of endothelium in these organs. They showed that high-stretch ventilation in healthy mice led to an increased expression of adhesion molecules E-selectin, VCAM-1 and ICAM-1 on endothelial cells in the hepatic and

renal tissue but not in the brain, suggesting a specific susceptibility of different organs [364]. Activation of endothelium in these organs was also associated with increased tissue level of cytokines/chemokines (IL-1beta, TNF, KC) and margination of neutrophils (as measured by MPO assay), but again organ dysfunction was not measured, and it remains unclear what stimulus or mediator caused the increase in adhesion molecule expression [364].

Imai *et al.* were the first to demonstrate a direct link between VILI and MSOF. Using an acid aspiration rabbit model, the authors showed that injurious mechanical ventilation led to significantly increased apoptosis of epithelial cells in the kidneys and small bowels, which was associated with organ dysfunction as measured by serum creatinine, lactate dehydrogenase and aspartate transaminase [365]. In addition, plasma from animals ventilated with the injurious strategy was shown to induce apoptosis in kidney epithelial cell in vitro, which was prevented by blocking the pro-apoptotic mediator Fas ligand [365]. When the authors assessed samples from patients with ARDS they also found a significant correlation between changes in soluble Fas ligand and changes in creatinine. However, it remains unclear as to whether Fas ligand is produced in the lung and decompartmentalised to the systemic circulation in response to injurious ventilation.

There is also evidence that mechanical ventilation may result in increased permeability of the intestines which would predispose to translocation of bacteria to the systemic circulation. Guery *et al.* subjected rats to high or low tidal volume ventilation (30ml/kg vs. 10ml/kg) and found that high tidal volume ventilation led to pulmonary oedema and increased gut permeability, both of which could be abrogated by neutralising antibodies to TNF [366]. As Fas ligand and TNF share the same downstream signalling pathways, it is tempting to speculate that increased gut

permeability in this model was also secondary to epithelial apoptosis, such as that observed by Imai and colleagues [365]. Peripheral immunosuppression may also influence the gut immune system and predispose to bacterial translocation [367].

Overall these studies provide sufficient evidence to suggest that mechanical ventilation propagates a switch from localised pulmonary inflammatory response to a systemic inflammatory process, which also affects extra-pulmonary organs and thus may influence MSOF and mortality in ARDS patients.

1.3 Therapeutic approaches to Acute Lung Injury

Therapeutic approaches to ARDS can be grouped as ventilator and non-ventilatory approaches. While ventilator approaches are aimed at preventing or minimising the harmful effects associated with mechanical ventilation, non-ventilatory approaches are designed to restore the homeostasis of the lung by halting injury progression and facilitating resolution. Only those that have been tested clinically will be discussed in this section.

1.3 Ventilatory approaches

Low versus high tidal volume ventilation – avoiding baro-/volutrauma

The traditional approach to ventilating patients with ARDS in the 1970s and 1980s was to maintain normal arterial PCO_2 and PO_2. While adequate CO_2 clearance was achieved by using high pressure and volume ventilation (V_T often exceeding 20ml/kg, [368], despite a recommended standard 12-15ml/kg [369]), the key manoeuvre to improve P_aO_2 was to apply PEEP [3, 368]. However, it was soon realised that this approach is not without deleterious consequences to the lungs as pneumothorax and pulmonary hyperinflation were common complications [370, 371].

The milestone experimental studies by Webb and Tierney and then by Dreyfuss *et al.* demonstrating that high end-inspiratory lung volumes lead to increased permeability oedema (see in previous section) [286, 292, 372], greatly influenced future ventilatory care. Hickling *et al.* were the first to demonstrate that lowering tidal volume (V_T= 4-7ml/kg) and peak airway pressures (PIP=30-40cmH_2O) and allowing hypercapnia resulted in significantly reduced mortality in ARDS patients than predicted by APACHE II scores [373, 374]. Subsequently to these observations, five multicentre, randomised trials were conducted to investigate the effects of low

vs. high tidal volume ventilation on mortality in ARDS patients. Stewart *et al.* compared the effect of a volume- and pressure-limited ventilation strategy (V_T<8ml/kg, PIP<30cmH_2O) with conventional ventilation [375], and found no difference in mortality or organ failure and the incidence of renal failure was significantly higher in the 'protective' ventilation group [375]. The study by Brochard *et al.*, limited end-inspiratory plateau pressure of up to 25cmH_2O and a V_T<10ml/kg [376]. There was no significant difference between the two groups in 60-day mortality or MSOF. The same year, however, Amato *et al.* reported beneficial effects with protective ventilation strategy (V_T<6ml/kg and PEEP above the LIP) in 28-day mortality [377]. In a subsequent study Brower *et al.* also assessed the impact of low V_T by limiting P_{plat} (<30cmH_2O); however there was no difference in mortality between the groups [378].

To address the discrepancy in the findings of these studies, which was likely secondary to small sample size, study design and lack of power to detect differences between treatment arms in most of the studies, in 2000 the National Heart, Lung and Blood Institute (NHLBI) Acute Respiratory Distress Network (ARDS Network) conducted a multicentre phase III clinical trial enrolling a total of 861 patients [329]. This study found a 22% reduction in mortality (from 39.8% to 31%) when V_T was reduced to 6ml/kg from 12ml/kg predicted body weight and plateau pressure was limited to less than 30cmH_2O. Patients in the protective arm also had more ventilator-free days (VFDs) and organ failure-free days (OFFDs) compared to controls. Interestingly, patients ventilated with lower V_T also showed a greater decrease in their plasma IL-6 levels from Day 0 to Day 3, indicating attenuation in the systemic inflammatory response, which may have contributed to the beneficial effects of low V_T on organ failure.

Although the ARDS Network trial is not without its critics [379, 380], most importantly, this was the first trial that was adequately powered to show a mortality benefit in ARDS and established a new standard of ventilatory management to which new treatment strategies should be compared.

Lowering tidal volume has a beneficial effect on but does not completely abrogate mortality in ARDS patients, indicating that although VILI is a significant contributing factor, it may not be the only determinant of the outcome. One can argue, however, that some of the residual mortality seen in the Amato and ARDS Network studies (38% and 31%, respectively) is still related to lung over-distension which may happen even at lower tidal volumes. When conducting quantitative CT imaging studies in patients with ARDS, Gattinoni *et al.* found that the amount of normally aerated lung tissue, measured at end-expiration, was about 200-500g, equivalent to the normally aerated lung tissue of a 5-6 years old healthy boy [381]. Based on these observations Gattinoni and Pesenti proposed that lung over-distension can occur even at lower tidal volumes, a phenomenon they termed the "baby lung" [368].

Another interesting finding of these studies was that the respiratory compliance correlated only with the size of the normally aerated lung tissue, i.e. compliance reflected the size of the "baby lung". The studies also revealed that the ARDS lung is not homogenously "stiff", as previously considered, but small, and the elasticity of the "baby lung" is nearly normal. Based on this and the CT imaging, the authors initially thought that the "baby lung" was a healthy anatomical structure, located in the non-dependent regions (ventral parts in the supine position) of the original lungs. This gave rationale to start using the prone position in order to increase perfusion of the anatomical "baby lung" and thus improve oxygenation.

Although oxygenation did indeed improve [382], CT imaging soon revealed that gravity-dependent densities shifted from dorsal to ventral regions within minutes when the patient was turned to prone position, indicating that the non-aerated regions represented collapsed alveoli and the “baby lung” is not a healthy anatomical but rather a functional structure, consisting of non-healthy aerated lung units [382].

The “baby lung” model therefore means that during positive pressure ventilation, even at low tidal volumes, the aerated lung regions receive disproportionally greater volume of gas in ARDS patients, which can ultimately lead to volutrauma and consequent biotrauma. This would also suggest that reducing tidal volume even further may be beneficial and could potentially further reduce VILI associated mortality. On the other hand, using significantly lower tidal volumes than 6ml/kg could potentially be harmful, as it could lead to hypoxaemia, hypercapnia, reduced cardiac output and consequent organ hypoperfusion [383]. Some of these limitations can be resolved by utilising extracorporeal CO_2 removal and membrane oxygenation (ECMO), which would allow “lung rest” using low tidal volumes whilst maintaining gas exchange. The first multicentre, prospective, randomised trial of ECMO versus conventional support in ARDS patients was conducted in the late 1970s and published by Zapol *et al.* [255]. This study was stopped early because of high mortality (90%) in both groups. Bleeding was the main complication associated with ECMO, which led to several blood transfusions which may have contributed to lung injury. Importantly, this study was performed 25 years before the ARDS Network trial and used high tidal volume ventilation in both groups. Subsequently, ECMO was used successfully with high survival rates in new-born and paediatric patients with respiratory failure [384-386], and there have been encouraging results in adult patients [387-393] [394]. However, due to its associated high bleeding risk

(54%), ECMO is contraindicated in patients who are not suitable for anticoagulation due to other reasons (e.g. acute stroke, known bleeding disorder, etc.). Furthermore the benefits of ECMO decline after ARDS duration of more than 7 days [395]. Overall, ECMO may be a useful salvage therapy for patients with early and severe ARDS.

Another possible way of reducing tidal volume to a minimum and thus avoid volutrauma is by the use of high frequency ventilation (HFV) strategies. The concept of HFV is to ventilate the lungs with very low tidal volumes in conjunction with high frequencies (3-7Hz) and high mean airway pressures to keep the lungs open. By this approach both overdistension and shear stress generated by repetitive opening closing of lung units can be potentially avoided. There is experimental evidence that HFV can attenuate VILI [319-322], and it has been commonly and safely used in premature infants with surfactant deficiency, although a Cochrane Database review showed no evidence for a benefit over conventional ventilation strategies in this patient population [396]. In ARDS patients there were two small randomised trials that compared HFV with conventional ventilation, each of which showed that this approach was safe and improved oxygenation, but were not powered to show a survival benefit [397, 398]. Outcome benefits of HFV are currently being evaluated by the randomised multicentre Oscillation for ARDS Treated Early (OSCILLATE) trial, until then HFV remains an experimental approach.

Optimising PEEP - avoiding atelectrauma

Another strategy to reduce the lung injury during mechanical ventilation is by the application of PEEP, which is used to improve oxygenation and reduce the chance of alveolar collapse at end-expiration and resultant atelectrauma [3, 286, 292].

Optimising PEEP, however, might be challenging as too high levels might be more injurious than beneficial as discussed earlier. One major difficulty is that the lung is heterogeneously injured/atelectatic in ARDS, and a set PEEP that is optimal to keep less aerated parts open may cause overdistention in better aerated units (see "baby lung" theory above). Moreover, high PEEP levels may have detrimental effects on haemodynamics by increasing intra-thoracic pressure consequently reducing venous return and cardiac output, which could ultimately lead to reduced organ perfusion. Amato *et al.* demonstrated reduced mortality in a small trial with a combination of low tidal volume and titrating PEEP above the LIP on the P/V curve [377]. Subsequently, the ARDS Network conducted the ALVEOLI study, a prospective, randomised, multicentre trial comparing the effect of higher PEEP (13cmH_2O) versus lower PEEP (8 cmH_2O) in conjunction with lung-protective tidal volume (6ml/kg) on hospital or 60-day mortality [399]. The study was discontinued prematurely after enrolling 549 patients, due to lack of efficacy. One can argue, however, that the beneficial effects of lung protective tidal volume may have masked those from higher PEEP. In some contrast, a more recent meta-analysis of three multicentre trials comparing the effect of higher (15±3cmH_2O) versus lower (8±3cmH_2O) PEEP showed a survival benefit in patients with moderate-severe ARDS (P_aO_2/F_iO_2 <200), whereas a trend towards increased mortality in mild ARDS (P_aO_2/F_iO_2 =200-300) with the use of higher PEEP [400]. These findings highlight the "double-edged sword" nature of PEEP, but also indicate that using high levels of PEEP may have a benefit in patients with the more severe forms of ARDS.

Prone positioning

Prone positioning was tangentially discussed above in the context of the “baby lung” phenomenon. The idea behind prone positioning is to alleviate the compressive gravitational forces expressed on the dorsal dependent regions thus facilitating more evenly distributed ventilation throughout the lung, which ultimately manifests in improved oxygenation (by a reduction in ventilation/perfusion mismatch and shunt) [401]. The more uniform distribution of tidal volume may also help to reduce excessive stretch at margins between atelectatic and aerated lung units in the dorsal regions, and attenuate atelectrauma by preventing repeated opening closing of small airways [315]. However, prone positioning may result in adverse events, such as dislodgement of endotracheal tube, intravascular lines, facial oedema, eye damage and increased intra-abdominal pressure [315]. A large, multicentre, randomised trial of prone positioning for patients with ARDS was published in 2001 by Gattinoni *et al.* [402]. The P_aO_2/F_iO_2 ratio was significantly higher in the prone group compared to the supine group (N=152), but prone positioning did not improve survival. Importantly, however, there was no difference between the groups in the complications related to positioning, such as pressure sores and accidental extubation.

A meta-analysis and systemic review of ten clinical trials (total of 1867 patients) published in 2010 demonstrated a significant reduction in mortality with prone positioning in patients with severe ARDS (P_aO_2/F_iO_2 <100) [403]. The meta-analysis also revealed a significant increase in the risk of pressure ulcers, endotracheal tube blockade and chest tube dislodgement associated with prone positioning. More recently, Guerin *et al.* published the results of the PROSEVA trial (Effect of Prone Positioning on Mortality in Patients With Severe and Persistent Acute Respiratory Distress Syndrome) in the New England Journal of Medicine

[404].This this multicentre, prospective, randomised, controlled trial, randomly assigned 466 patients with severe ARDS (defined as PaO_2/FiO_2 <150mmHg, at a minimum FiO_2 of 0.6 and a minimum PEEP of 5cmH_2O, V_T~6ml/kg predicted body weight) to undergo prone-positioning sessions of at least 16 hours or to be ventilated in the supine position. The study demonstrated an impressive more than 50% relative reduction in 28-day hospital mortality (primary outcome, 16% in prone positioning vs. 32.8% in supine), and a similar effect in unadjusted 90-day mortality (23.6% vs. 41%). Also, the incidence of complications did not differ significantly between the groups, except for the incidence of cardiac arrests, which was higher in the supine group [404]. Therefore, while prone positioning should not be used routinely for all ARDS patients, but it is reasonable to utilise this approach in patients with severe ARDS.

Recruitment manoeuvres

Low tidal volume ventilation may result in loss of lung volume and derecruitment of lung units, ultimately leading to impaired gas exchange. This can be prevented by the delivery of intermittent breaths of large tidal volumes (sigh) [405]. In a clinical study by Pelosi *et al.* [406], application of intermittent sighs by increasing the plateau pressure by 10cmH_2O every 20 seconds over a period of 1 hour resulted in a 26% reduction in shunting with a 50% increase in oxygenation. Two more recent meta-analyses, including up to 40 clinical studies comparing recruitment manoeuvres (RM) to conventional ventilation strategy, found that RM resulted in a significant but transient increase in oxygenation with very few non-significant adverse events (barotrauma, hypotension, desaturation, arrhythmias), but there was no significant difference in 28-day mortality, and there was not enough data on the length of

ventilation or hospital stay to pool the results [407, 408]. Therefore, given the uncertain benefit of transient oxygenation improvements in patients with ALI/ARDS, the routine use of RMs cannot be recommended, but should be considered for use in patients with ARDS who have life-threatening hypoxemia on an individually judged basis.

Strategies reducing patient-ventilator asynchrony (PVA)

A ventilated patient's own respiratory effort driven by their neural respiratory cycle is often only partially suppressed and asynchronous to the ventilator, which can result in increased transpulmonary pressure, with a risk of barotrauma and VILI, and prolonged duration of ventilation [409]. This even happens with ventilators designed to detect the patients' own respiratory effort, such as pressure support ventilation (PSV) and assisted spontaneous breathing.

In a large clinical trial with ARDS patients, suppression of the patients' own breathing effort using short-term, early neuromuscular blockade was shown to improve PVA and reduce 90-day adjusted mortality without significant complications [410].

Another way of improving PVA is by the use of proportional assist ventilation (PAV). It provides assisted pressure support ventilation by means of a complex algorithm based on the compliance and resistance of the airways [409]. However estimation of these parameters may prove to be difficult in clinical practice and PVA is only available on a limited number of ventilators, therefore it's use is not widely adopted [409].

A more recent development to this field is the use of neurally adjusted ventilatory assist (NAVA), in which the electrical activity of the patient's diaphragm is

measured by electrodes attached to a nasogastric tube, serving as a surrogate of the patient's neural respiratory effort resulting in a high level of patient-ventilator synchrony [409]. A small clinical trial (N=14) by Colombo *et al.* comparing NAVA and PSV [411] showed that tidal volume and the incidence of patient-ventilator asynchrony was significantly higher in the PSV group, indicating that NAVA may improve patient-ventilator interaction and avoid overassistance. In a rabbit model of ALI induced by intratracheal HCl, NAVA provided the same level of lung protection as low-tidal volume ventilation when compared to high-tidal volume ventilation and was just as efficient in attenuating extra-pulmonary organ injury [412]. These findings suggest that NAVA may be a safe and effective tool to reduce VILI and MSOF, but large clinical trials are needed to establish its role in ARDS patients.

1.3 Non-ventilatory approaches

Haemodynamic management

Pulmonary oedema formation is greatly influenced by Starling forces, even if not cardiogenic in origin, and experimental data suggest that even a small decrease in hydrostatic pressure in the pulmonary vasculature can attenuate it [413]. Also, reduction in pulmonary artery wedge pressure is associated with improved survival in ARDS patients [414], likely secondary to a decrease in extravascular lung water [415]. This would suggest that a more conservative fluid management may confer benefits in this patient population, although one has to take into account that maintenance of organ perfusion should not be compromised to achieve this. To investigate this further, the ARDS Network conducted the Fluid and Catheter Treatment Trial (FACTT), a large, multi-centre, randomized trial comparing liberal to conservative fluid management strategy in patients with ARDS [416]. The study

found that compared to the liberal fluid management arm, those patients in the conservative fluid management group had significantly more ventilator-free days (VFDs), improvement in lung function and more ICU-free days without an increase in the incidence of non-pulmonary organ failures. However, 60-day mortality was not improved significantly.

ARDS is associated with increased dead space fraction due to pulmonary microvascular vasoconstriction leading to shunt and ventilation-perfusion mismatch [35]. Restoration of pulmonary microvascular circulation therefore would theoretically improve gas exchange thus decrease ventilator-dependency and improve survival. Nitric oxide (NO) is a potent endogenous vasodilator predominantly produced by endothelial cells exogenous forms of which can be also delivered by inhalation to achieve selective vasodilation in the pulmonary vasculature without any systemic effect (due to rapid inactivation by haemoglobin [417]) [418, 419]. Indeed, when ARDS patients were treated with inhaled NO (iNO) in a small observational pilot, they showed a significantly decreased pulmonary artery pressure without changes in systemic arterial pressure, and this was associated with a significant improvement in oxygenation and a decrease in pulmonary shunt [420]. Two subsequent, small (30 and 40 patients), randomized trials confirmed these positive findings, although these were found to be transient and did not manifest in improved survival [421, 422]. Unfortunately, this did not seem to be related to small sample size, as three subsequent large, multi-centre, randomised, double-blinded, placebo-controlled trials also failed to show a benefit in mortality or VFDs of using iNO in ARDS patients, despite the short-term improvement in oxygenation [423-425]. Moreover, Ader et al demonstrated in a mouse model of Pseudomonas aeruginosa pneumonia that iNO can increase pulmonary endothelial permeability [426]. Therefore, apart from using

as a rescue therapy for refractory hypoxaemia, iNO is generally not recommended for the treatment of ARDS patients.

Prostaglandins (PGs, e.g. PGI_2, PGE_1, PGE_2), similarly to NO, are important endogenous vasodilators, which also inhibit platelet aggregation and activation of leukocytes [427], therefore seem attractive as a potential treatment tool for ARDS. When aerosolised PGI_2 was given to ARDS patients [428], it conferred selective pulmonary vasodilation and showed similar efficacy to iNO. However, no large randomised trial has been conducted with inhaled PGs. Systemic delivery of PGs for the treatment of ARDS was also tested. In animal models of ALI, such as that induced by bleomycin or pancreatitis [429, 430], intravenous infusion of PGE_1 reduced pulmonary neutrophil margination and vascular leak. The first clinical trial of PGE_1 in 41 ARDS patients showed a trend toward mortality benefit and triggered larger studies [431]; however these could not show a significant improvement in survival [432, 433]. Interestingly, there was no significant effect on neutrophil activity either. In a subsequent study by Abraham *et al.* where neutrophils were targeted more specifically by the use of liposomal PGE_1 in 25 patients with ARDS, treatment led to a significant improvement in oxygenation, lung compliance and ventilator dependency [434]. However, large clinical trials conducted in North-America and Europe failed to confirm these positive findings [435, 436], and therefore systemic PGs are not recommended for treatment of ARDS patients.

Enhancement of alveolar fluid clearance

Resolution of alveolar fluid is critical for the recovery from ARDS [22, 35], and early resolution is associated with improved survival [270]. Experimental studies on *ex vivo* human lungs indicate that beta-agonists can accelerate alveolar fluid clearance

by cAMP-mediated up-regulation of ENaC and Na-K-ATPase with subsequent increase in sodium transport in type II AECs [437, 438]. There is also data to support that beta-agonists may attenuate alveolar-capillary permeability [439]. These effects are mediated through the beta2-adrenoceptor [440, 441], and a placebo-controlled clinical trial in healthy volunteers who were at known risk of developing high-altitude pulmonary oedema (HAPO) showed that inhaled beta2-agonist salmeterol reduced the incidence of high-altitude pulmonary oedema [442].

These experimental data paved the way for a clinical trial using beta2-agonist salbutamol intravenously (15ug/kg/h for 7 days) in ARDS patients, the beta-agonist lung injury trial (BALTI) with an attempt to accelerate the resolution of pulmonary oedema [443]. This single-centre, randomised, double-blind, placebo-controlled phase 2 trial enrolled a total of 40 patients, and found a significant reduction in extravascular lung water (EVLW) and P_{plat} at Day7 in the treatment group, and a non-significant trend toward a reduced lung injury score (LIS), but no difference in VFD or survival. However, the positive results in the primary outcome called for large, multi-centre, randomised trials. One was designed and performed in North-America by the ARDS Network and named ALTA [14]. This study used aerosolised salbutamol (5mg every 4h for 10 days) partly because intrapulmonary delivery of beta-2-agonist reduced pulmonary oedema in a preclinical model of lung injury [444] and the amount of salbutamol achieved in the pulmonary oedema fluid by this method is sufficient to stimulate AFC in *ex vivo* human lungs [445], and partly because of safety concerns (i.e. potentially less systemic side effects, e.g. arrhythmias). Unfortunately, the study was terminated for futility after 282 patients had been enrolled. The reasons behind this lack of benefit are unclear, but the possibility of a sub-optimal drug delivery to the injury site was raised as an

explanation. Another possible explanation is that the ALTA study enrolled patients with less severe ARDS with an overall 60-day mortality of 20% when compared to the BALTI trial (28-day mortality of 60%), and more severely ill patients may have more significantly impaired AFC, and so are more likely to benefit from interventions that accelerate~~s~~ AFC [446].

Parallel to the ALTA study, the BALTI-2 trial was run in 46 intensive care units across the UK evaluating the effects of intravenous salbutamol in ARDS patients with 28-day mortality being the primary outcome [447]. Unfortunately, similarly to the ALTA trial, the BALTI-2 trial also had to be stopped early because of safety concerns. Salbutamol treatment was associated with a significantly increased 28-day mortality compared to placebo (34% vs 23%, respectively), and other adverse effects such as tachycardia, arrhythmias and lactic acidosis, and also reduced VFDs and OFFDs. Therefore intravenous beta-2 agonist therapy cannot be recommended for ARDS patients.

Surfactant therapy

The role of surfactant in lung homeostasis, alterations to the surfactant system in patients with ARDS and the success of exogenous surfactant therapy in pre-mature babies with neonatal respiratory distress syndrome (NRDS) [448-452] give a rationale for surfactant therapy in ARDS. However, whilst in NRDS the main pathology is surfactant deficiency, in the case of ARDS the main problem is surfactant dysfunction associated with a much more complex pathology as described above, which is complicated only further by the heterogeneity arising from different aetiologies of the syndrome [453].

Several animal studies demonstrated the efficacy of exogenous surfactant in different models of lung injury, such as acid-aspiration [454-456], bacterial and endotoxin-induced lung injury [457-462], hyperoxia [463-465] and *in vivo* lavage [466-470]. However, in adult patients with ARDS, despite the initial encouraging results in small clinical studies, large controlled clinical trials were unsuccessful, apart from a temporary improvement in oxygenation in some but not all studies [471] [472] [473, 474], [475]. This apparent lack of benefit can be partly explained in some of these trials by the low efficacy (secondary to low or no SP-B content) [476-480] of the material used and the delivery method (aerosolised vs. instilled) that may have also influenced the bioavailability of the drug [453].

In summary, the treatment of ARDS in adult patients with exogenous surfactant has been unsuccessful. Future success of this approach may simply lie in optimising the drug components, the delivery method, and/or in using it in conjunction with other therapeutic methods [453].

Prevention/reversion of the overwhelming pro-coagulant state

Alteration to the coagulation pathway and evolvement of a pro-coagulant state is characteristic to ALI/ARDS and is a significant contributor to the physiologic changes seen during the pathogenesis, such as increased vascular permeability and inflammation [22, 35].

Genetic and pharmacological targeting of tissue factor (TF) was shown to attenuate endotoxin/sepsis-induced lung injury, extra-pulmonary organ injury and mortality in several animal models [481-485]. Despite these encouraging results, when TF pathway was blocked using a recombinant human tissue factor pathway inhibitor TFPI in a large multicentre randomised clinical trial in patient with sepsis, it

conferred no benefits in mortality [486]. The reasons behind this lack of benefit are not known, but it highlights the complexity of the role of TF and the coagulation pathway in the pathogenesis of sepsis and ALI/ARDS.

In vitro studies indicate that activated protein C (APC) has anti-inflammatory and anti-apoptotic effects on PECs [487, 488]. It has been demonstrated *in vitro* that APC can inhibit thrombin-induced permeability in PECs [489]. Also, systemic administration of APC prevents LPS-induced pulmonary vascular injury *in vivo* by inhibition of TNF production [490]. Conversely, during sepsis PECs shed thrombomodulin (TM) and endothelial protein C receptor (EPCR) from their surface [491, 492], which results in decreased ability to activate protein C and consequently propagates lung injury. These findings coupled with the clinical observations that sepsis/ARDS are associated with reduced APC levels, prompted clinical trials using recombinant human APC (rhAPC) with an attempt to improve clinical outcome. The first large, multicentre placebo-controlled trial, the Prospective Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study demonstrated a 6.1% absolute risk reduction in 28-day mortality in the treatment group [493]. More than 70% of the patients in both study arms had respiratory dysfunction, and the resolution of this was significantly quicker in the rhAPC-treated group [494]. A subgroup analysis suggested that the mortality benefit was limited to patients with an increased risk of death, therefore the FDA requested additional trials involving less severely ill adults and children. These trials, the Administration of Drotrecogin Alfa (Activated) in Early Stage Severe Sepsis (ADDRESS) [495] and the Resolution of Organ Failure in Pediatric Patients with Severe Sepsis (RESOLVE) [496], were terminated early for futility. Subsequent trials and analyses also called into question the results of the PROWESS study [497], which led to the PROWESS-

SHOCK study to test whether rhAPC would reduce mortality in patients with septic shock. This showed no benefit from the use of rhAPC in patients with sepsis, and therefore it is currently not recommended for clinical use and the manufacturer has withdrawn the product from the market [498].

Several small human studies indicated the effectiveness of AT III in reducing the mortality and organ dysfunction in patients with sepsis [499-501], which led to a large, randomised, double-blind, placebo-controlled trial [502]. Disappointingly, this trial did not show any beneficial effect on mortality [502], however a subgroup analysis revealed that AT III treatment resulted in a 6.8% absolute risk reduction in 56-day all-cause mortality in patients with severe sepsis and high risk of death [503]. Interestingly, this benefit was more prominent in patients who did not receive concomitant heparin, which warrants for further prospective validation.

In summary, although the role of coagulation pathway in the pathogenesis of sepsis/ALI/ARDS is well established in experimental models and clinical studies, therapeutic interventions aiming to restore the altered haemostasis showed very limited benefits and only to a very specific subpopulation, which only highlights the complexity of these pathways.

Counteracting oxidative stress

As the pathogenesis of ARDS is associated with excessive production of free radicals and depletion of antioxidant systems, it clearly delineates the two main therapeutic approaches to counteract oxidative stress during ARDS: 1) inhibit the production of free radicals, and 2) replenish antioxidant systems.

While NO is important in maintaining pulmonary vascular function, its derivatives (RNS) can also promote injury due to their high reactivity with other

molecules. Excessive production of NO during lung injury is a result of induced activity of iNOS in inflammatory cells, such as AMs [504]. Genetic inhibition of iNOS confers protection from lung injury of different aetiologies in animal models [505, 506]. On the contrary, overexpression of eNOS attenuates VILI [507] and LPS-induced [508] lung injury. This suggests that while excessive production of NO by iNOS may act as a free radical, maintenance of a baseline NO expression by eNOS is required for appropriate endothelial function, implicating that a specific blockade of iNOS may confer more benefits than total NOS inhibition. Indeed, selective targeting of iNOS with the inhibitor ONO-1714, has been demonstrated to attenuate hyperoxic [509], Candida- [510, 511], LPS- [512] and acid aspiration-induced [513] lung injury in animal models, but clinical trials using this agent are lacking.

ARDS is associated with reduced GSH and increased GSSG levels clinically [514]. Systemic administration of the GSH precursor N-acetyl cysteine (NAC) attenuated LPS-induced alteration of pulmonary circulation, lung compliance, and production of inflammatory cytokines in sheep [515]. However, when trialled in human ARDS, even though it successfully increased GSH levels [516], NAC failed to show any mortality benefit [517-520].

Haem oxygenase (HO)-1, is a ubiquitous inducible enzyme catalysing the oxidative degradation of haem into bilirubin and biliverdin with the release of by-products carbon monoxide (CO) and iron [521, 522]. Both bilirubin and biliverdin have antioxidant properties [523]. Moreover, overexpression of HO-1 confers increased resistance to hyperoxia *in vitro* and *in vivo* [524], [525] and LPS-induced lung injury [526] *in vivo*, partly via enhanced expression of IL-10 by AMs [526]. Exogenous administration of biliverdin has been also shown to provide protection from LPS- [527] and ischaemia-reperfusion [528, 529] induced lung injury. Although

clinical evaluation of HO-1 gene transfer and delivery of exogenous biliverdin is still awaited, these may offer an alternative therapeutic approach. CO also has anti-inflammatory properties and exposure to low concentrations attenuated several models of lung injury including VILI [530-532], acid aspiration [533], ischaemia-reperfusion injury [529, 534], injury associated with cardiopulmonary bypass [535-538], mainly by attenuating the associated inflammatory response. However in other models of lung injury, such as LPS- and oleic acid-induced [539], it failed to show protection, or required doses that increased lung permeability and produced potentially toxic levels of carboxyhaemoglobin [540]. With regards to clinical application, inhaled CO failed to exhibit anti-inflammatory effects during endotoxaemia in healthy volunteers [541], but was effective in reducing sputum neutrophils in COPD patients and improved bronchial responsiveness [542]. Therefore, further clinical trials are needed to establish the potential benefits of inhaled CO for the treatment of ARDS [543].

Albumin has antioxidant properties through its thiol groups and its administration to patients with sepsis or lung injury replenished plasma antioxidant capacity [544, 545] and improved gas exchange [546]. However it remains to be elucidated whether albumin administration to patients with ARDS could also improve survival. Supplementation of dietary antioxidants such as vitamin C- and E and carotenoids were also shown to be beneficial in preventing ozone-induced respiratory dysfunction and inflammation in healthy human subjects [547], and may be favourable to use in patients with acute lung injury.

Anti-inflammatory drugs

Inflammation is a key to the pathogenesis of ARDS, and halting inflammatory processes is a prerequisite of successful resolution of injury. Therefore, anti-inflammatory drugs as potential therapy have been widely tested.

Glucocorticoids seemed to be an ideal candidate not only because they are widely used in clinical practice, but also because they can inhibit or suppress many inflammatory mechanisms that are well known to be involved in the development of ARDS. For example, glucocorticoids inhibit the expression of inflammatory cytokines IL-1, IL-6, IL-8, TNF and GM-CSF [548, 549] through the inhibition of transcription factors NFkB and AP-1 [550-552], suppress expression of adhesion molecules ICAM-1 and E-selectin [553, 554] and the synthesis of enzymes (e.g. PLA_2, COX, iNOS) that are responsible for the production of other mediators such as prostanoids, PAF and NO [555]. Glucocorticoids can also induce the breakdown of collagen, thus preventing excessive collagen deposition in the lung that would lead to fibrosis. However, when clinical trials were conducted with methylprednisolone for the prevention [556-559] or treatment of either early [560] or late [561-566] phases of ARDS, these showed little clinical benefits [565] and in one study even increased mortality [556].

Ketoconazole is an antifungal agent with anti-inflammatory properties. It can inhibit synthesis and release of leukotrienes and TXA_2 by AMs, which are important mediators of vasoconstriction, platelet aggregation and neutrophil activation in the pathogenesis of ARDS [567, 568]. While oral ketoconazole successfully reduced the incidence of ARDS in surgical patients who were at high risk [569, 570], it failed to show a mortality benefit or a positive impact on VFDs in patients who already developed the syndrome [571]. Therefore ketoconazole may be useful

prophylactically in some patient populations at risk of ARDS, but should not be used for ARDS therapy.

The xanthine derivative pentoxifylline is a competitive non-selective phosphodiesterase inhibitor, commonly used in vascular surgery to improve peripheral circulation [572]. Besides its haemorrheologic effects, pentoxifylline and its derivative lisofylline have potent anti-inflammatory properties. For example, pentoxifylline has been shown to inhibit neutrophil chemotaxis and TNF release by macrophages in animal models of ARDS [573-575], and pre-treatment with pentoxifylline successfully attenuated pulmonary oedema in saline lavage and acid-aspiration models of ARDS [574, 576]. However, there is only one human pilot study in ARDS patients, in which pentoxifylline showed no positive effect on respiratory physiology [577]. Lisofylline can also attenuate production of pro-inflammatory cytokines, reduce neutrophil accumulation and pulmonary oedema formation and improve survival in animal models of sepsis [578-580]. However, similarly to pentoxifylline, i.v. lisofylline failed to confer benefits in ARDS patients [581].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine which plays an important role in pulmonary homeostasis by regulating AM function in innate host defence, preventing apoptosis in AECs in response to noxious stimuli and through its involvement in surfactant homeostasis [582, 583]. Animal models studying either administration or overexpression of GM-CSF indicated attenuation of lung injury [582, 584]. Similarly, beneficial effects of intravenously administered recombinant human (rh)GM-CSF were observed in a small scale trial amongst septic patients [585], although a subsequent larger trial in patients with ARDS failed to confirm benefits [586], although showed a very subtle positive trend in 28-day

mortality and OFFDs. Thus larger trials are needed to establish the benefits, if any, of rhGM-CSF therapy in ARDS.

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitors, or statins, have been used in primary and secondary prevention of cardiovascular disease for a long time [587-589], not only for their beneficial effect on lipid profile but also because of their immunomodulatory and anti-inflammatory effects [590, 591]. This pleiotropic effect has been utilised in different inflammatory conditions, such as rheumatoid arthritis and multiple sclerosis with good results [592, 593], and thus has also been the interest of ALI research. For example, in a mouse model of sepsis statin pre-treatment reduced plasma TNF and IL-1 that was associated with increased survival [594]. Also, mice pre-treated with statin had significantly attenuated pulmonary inflammation and injury following intratracheal LPS administration [595, 596], and in a model of VILI [597]. In healthy volunteers, pre-treatment with 80mg simvastatin for 4 days significantly attenuated systemic and pulmonary inflammatory response following intravenous and inhaled LPS, respectively [598, 599]. Observational cohorts of patient with sepsis and ICU admission also demonstrated a mortality benefit in those patients who were on statins prior to their illness [600-603], and patients on pre-hospital statin treatment who were admitted to ICU were less likely to develop sepsis or ALI/ARDS than those who were not on statins [604]. These positive observations led to the randomised, double-blind, placebo controlled trial of Hydroxymethylglutaryl-Coenzyme A Reductase inhibition for Acute Lung Injury (The HARP study) recruiting 60 patients with ALI, who either received 80mg of simvastatin or placebo daily until cessation of mechanical ventilation or up to 14 days [605]. By day 14 the statin group showed improvement in non-pulmonary organ dysfunction, significant reduction in pulmonary

and systemic inflammation and a non-significant trend for better pulmonary function, but no difference in mortality although this was not the primary end-point and the study was not powered to detect difference in mortality (the HARP-2 study is currently recruiting a total of 540 patients to better assess the potential benefits of simvastatin use in ALI patients [606]). In a more recent, randomised, double-blind, multicentre, placebo-controlled trail enrolling 250 critically ill patients, daily administration of 20mg atorvastatin did not show any significant reduction in plasma IL-6 (primary end-point), organ failure or mortality [607]. Interestingly, however, pre-hospital statin users randomised to the placebo group had a greater 28-day mortality (28% vs. 5%) compared to those receiving atorvastatin, suggesting an important role for statins in regulating the inflammatory response in severe sepsis.

IL-1-beta, similarly to TNF is an early expressed cytokine, closely linked with the pathogenesis of ARDS as discussed earlier. IL-1-beta has two receptors, IL-1R1 and IL-1R2, but exerts its biological effects only through IL-1R1 [608, 609]. Both IL-1-beta and its natural antagonist IL-1Ra are elevated in the BALF of ARDS patients, and lung protective ventilation decreases BALF IL-1-beta levels, without affecting that of IL-1Ra, suggesting a contributory role for IL-1-beta in the pathogenesis of VILI [330]. Indeed, animal models confirmed these observations [335, 610], and genetic or pharmacological inhibition of IL-1-beta signalling successfully attenuated VILI [338, 339, 611]. It has been demonstrated that deleterious effects of IL-1 on pulmonary permeability in VILI can be attributed to down-regulation of epithelial ENaC expression [612], neutrophil recruitment through up-regulation of KC, ICAM-1 and iNOS, and associated epithelial cell injury [338]. However, when i.v. recombinant human (rh)IL-1Ra was given to sepsis patients in a large multi-centre trial, it proved unsuccessful to reduce mortality [613]. This lack of efficacy could be related to the

route of delivery (i.e. systemic instead of intrapulmonary), or the multifaceted role of IL-1 signalling in the pathogenesis of ARDS. For example, IL-1-beta is also involved in alveolar repair after injury [614]-[615], thus its blockade may even be deleterious. Overall these studies suggest that while anti-IL-1-beta treatment might be beneficial in the acute phase of ARDS, it might be harmful in the resolution phase. These important aspects need to be considered if clinical trials targeting IL-1-beta will be attempted again in ARDS patients in the future.

Similarly to targeting IL-1-beta, clinical trials with i.v. anti-TNF therapies including anti-TNF monoclonal antibodies [616-622], soluble TNF receptor (sTNFR)-immunoglobulin fusion proteins [623-625] and polyclonal Fab IgG fragments targeting TNF [626] in septic patients with ARDS showed little or no benefit in improving the clinical outcome. The possible reasons behind the lack of success with this therapy and the role of TNF signalling in ARDS/VILI will be discussed in more detail later in this chapter.

In summary, while ARDS is clearly an inflammatory condition, anti-inflammatory therapeutic approaches were so far unsuccessful. This only highlights the complex interplay of inflammatory mechanisms in the pathogenesis and indicates that better understanding is an absolute necessity if one wants to achieve success.

It is clear that the only therapy so far that has reduced the mortality associated with ARDS has been altering the approach to mechanical ventilation. However, the mortality remains unexpectedly high indicating that new therapies are required. The recognition of ARDS as an inflammatory condition directed researchers to explore potential therapeutic targets that regulate the inflammatory response. Despite extensive investigations there has been no significant breakthrough in treating ARDS. More recently new potential therapeutic approaches were suggested (e.g.

angiopoietin-1 [627-629], KGF [630-633], sphingosine-1 phosphate receptor 1 [129, 634, 635], mesenchymal stem cells [273, 636-638]) based on experimental studies, however these were not yet tested clinically.

One mediator that has been consistently implicated in ARDS/VILI in clinical and experimental studies is TNF [109, 322, 330, 334-337, 344, 347, 348, 639, 640]. TNF is an early response cytokine, upstream of many implicated mediators such as IL-1, IL-10, ROS and lipid mediators and influences the coagulation and apoptotic pathways and the surfactant system [641-648]. Thus, TNF signalling may represent an important common pathway and therapeutic target. Its roles in ARDS will now be discussed in more detail.

1.4 Tumour necrosis factor-α signalling in Acute Lung Injury and Ventilator-Induced Lung Injury

TNF

TNF is the prototypic member of the TNF ligand family, comprising of 17 similar proteins with different functions, including: TNF, lymphotoxin-alpha (TNF-beta), CD40 ligand, receptor activator for NF-kappaB ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL) [644]. TNF is mainly produced by activated macrophages and monocytes, but also by many other cells including lymphocytes, fibroblasts, mast cells, cardiomyocytes, adipose tissue, neuronal tissue and endothelial cells [644, 649]. It is expressed as a 26kDa type II transmembrane protein arranged in a non-covalently bound homotrimer form [650]. This transmembrane form (mTNF) can be cleaved primarily by the metalloprotease TNF-alpha converting enzyme (TACE or ADAM-17), but also by proteinase-3 to release the soluble TNF form (sTNF) [650-652]. The 51kDa trimeric sTNF tends to dissociate below the nanomolar range (forming three 17kDa protomers), thereby losing its bioactivity [649]. Historically, mTNF was only considered as a precursor to the bioactive sTNF with no active function, however it was later recognised that mTNF can also elicit cellular responses independently of sTNF [653-656]. Large amounts of TNF is released in response to LPS and other bacterial products and IL-1[649], whereas IL-4, IL-10 and glucocorticoids are potent inhibitors of TNF release [657, 658]. IL-10 inhibition of TNF release represents a negative feedback loop as its production is induced by TNF in monocytes [648].

TNF is a pleiotropic cytokine that mediates a vast spectrum of biological responses, some of which often seem conflicting. For example, in addition to its firstly recognised necrotising effect and cytotoxicity in some tumours and tumour cell

lines, TNF is also involved in cell proliferation and cell differentiation [644, 649]. In concert with other cytokines, such as IL-1 and IL-6, TNF is a key player in the systemic inflammatory response syndrome [659]. It acts as a potent early-response pro-inflammatory mediator by inducing the expression of the chemokines MCP-1 and IL-8 (or its murine homologs MIP-2 and KC) and endothelial adhesion molecules E-selectin, VCAM-1 and ICAM-1, thereby facilitating leukocyte migration to sites of inflammation [660-663]. It also augments the phagocytic function of macrophages, expression of other cytokines such as IL-1, IL-6 and IL-10, oxidants and the pyrogen prostaglandin-E_2 [642, 648, 664-666]. TNF can also induce apoptotic cell death, tumourigenesis and viral replication [644].

Since its discovery, TNF has been shown to be a primary mediator in several pathophysiological processes and conditions. For example, while high amounts of TNF are produced in septic shock, more prolonged exposure to TNF at low concentrations leads to wasting or cachexia such as that observed in cancer and chronic infection, hence its other name cachectin [667]. TNF has been also linked with atherosclerosis [668], and autoimmune conditions such as ankylosing spondylitis [669], rheumatoid arthritis [670], psoriasis [671] and inflammatory bowel disease [672], Alzheimer's disease [673], major depression [674] and various pulmonary disorders [675], but will be primarily discussed here in relation to ARDS and VILI.

TNF in ALI/ARDS and VILI pathogenesis

Role of TNF in clinical ARDS and VILI models

Multiple clinical studies investigated the relevance of TNF in ARDS pathology and demonstrated changes in its levels both in the alveolar compartment and the systemic circulation, although some of these are not consistent in their findings.

In a small study by Armstrong *et al.* enrolling a total of 26 patients the authors found a tendency toward increased sTNF levels in BALF and AM supernatant of patients with ARDS (N=10) compared to at risk patients, without any difference in plasma levels [676]. The same authors later also demonstrated an increased expression of mTNF on AMs derived from ARDS patients compared to those isolated from at risk patients, and this mTNF was functionally active in a cytotoxicity assay [677]. Despite increased mTNF expression the authors failed to show an increase in sTNF in BALF or AM supernatant. Pugin *et al.* showed up-regulation of sTNF in BALF but not in plasma obtained from ARDS patients, and this TNF was bioactive mainly in concert with IL-1-beta [102, 259].

In agreement with the findings of Pugin *et al.*, Parsons and co-workers also failed to show an increase in plasma levels of sTNF during the course of ARDS, and plasma TNF levels had no value in predicting the development or clinical outcome of ARDS [678, 679]. In direct contrast, in the study by Meduri *et al.* plasma TNF levels were found to be significantly higher on day 1 of ARDS and remained persistently elevated in non-survivors compared to survivors, thus predicting the clinical outcome [680]. BALF TNF levels were also high and showed the same trend as plasma TNF levels, but more importantly, BALF:plasma ratio was also elevated, hinting toward a pulmonary origin of TNF. There is also clinical evidence that ventilation of ARDS patients with more conventional settings (i.e. higher V_T) results in elevated BALF and plasma TNF levels and is associated with higher mortality [330].

The role of TNF is also subject of debate when it comes to VILI, especially in experimental studies. In *in vivo* saline-lavaged rabbit models conventional ventilation strategy up-regulates intra-alveolar TNF both at messenger and protein level as compared to protective HFV [322, 681], and administration of i.t. anti-TNF monoclonal antibodies attenuates VILI [334]. Also, Tremblay *et al.* demonstrated in an *ex vivo* isolated non-perfused rat lung model, that injurious ventilation up-regulates intra-alveolar levels of TNF [335, 682]. Using an isolated-perfused mouse lung system, von Bethman *et al.* and Held *et al.* showed that over-inflation of the lungs results in increased perfusate levels of TNF, but they did not investigate BALF levels [336, 683]. In direct contrast, Verbrugge *et al.* showed no increase in BALF or serum levels of TNF in surfactant-depleted rats subjected to high-pressure ventilation [684]. Similarly, Ricard *et al.* found no TNF in BALF obtained from rats or isolated rat lungs ventilated with high V_T [610].

Some of these inconsistencies may arise from the use of two-hit (i.e. pre-injured lungs subjected to high stretch) vs. one-hit VILI models ('pure' VILI), differences in the species used or in the timing of TNF measurements and kinetics. This was clarified, at least in part, by a study from our group using an *in vivo* mouse model of VILI without pre-existing lung injury [337]. We found that high-stretch mechanical ventilation resulted in a transient up-regulation of intra-alveolar TNF with an early-peak during the course of VILI with undetectable levels at later stages. This could therefore explain the previous conflicting of TNF up-regulation in one-hit pure VILI models. Bioactivity of TNF was however still present at the end stages of injury progression as demonstrated by WEHI bioassay [337]. Our group later also demonstrated that this intra-alveolar TNF played a role in the pathogenesis of VILI by using mice deficient in TNF receptors and treating wild-type mice with anti-TNF

antibodies [109]. The authors found that blockade of total TNF signalling in receptor deficient mice and by i.t. administration of anti-TNF mAb significantly reduced the influx of neutrophils to the alveolar space. Importantly, intravenous administration of the same antibody was without effect, hinting towards the importance of intra-alveolar TNF signalling at least in this aspect of VILI.

TNF was also implicated in other experimental models of ARDS and even MSOF. For example, acid aspiration-induced lung and systemic organ injury can be attenuated by pharmacological blockade of systemic TNF signalling as demonstrated by Goldman *et al.* [685]. Davidson and colleagues demonstrated similar protective effect of i.t. administered anti-TNF antibody on lung injury induced by acid aspiration [686].

TNF-mediated cellular and molecular mechanisms in the pathogenesis of ARDS/VILI

As discussed before, the pathogenesis of ARDS of all aetiologies involves a complex interplay of molecular and cellular mechanisms, and TNF has been demonstrated to play a role in most, if not all of these processes.

Increased permeability oedema is a key feature of ARDS [22], and TNF mediates alveolar-capillary barrier dysfunction in different ways, but mainly involving cytoskeletal re-arrangements and apoptosis of the cells. For instance, TNF can induce pulmonary vascular permeability *in vivo* and *in vitro* [687]. TNF treatment in pulmonary endothelial cell culture results in a decrease in F-actin and an increase in G-actin, and this imbalance between the two manifests in increased permeability [688]. Similarly, Petrache *et al.* showed that TNF induces phosphorylation of the myosin light chain (MLC), resulting in stress fibre formation, apoptosis of pulmonary endothelial cells and increased permeability [689]. The same investigators later

demonstrated that TNF-induced endothelial cell permeability is mediated by the destabilisation of tubulin-based microtubule network in a p38 MAPK-dependent manner, leading to an increase in actin stress fibres, intercellular gaps and compromise of VE-cadherin based adherens junctions [690]. This latter is secondary to TNF-induced tyrosine phosphorylation of VE-cadherin leading to its redistribution on the endothelial cell surface which subsequently leads to intercellular gaps and increased permeability [691-693]. In addition to its role in regulating endothelial permeability, TNF also induces alveolar epithelial permeability *in vitro* by causing cytoskeletal re-arrangement and consequent gap formation [694]. This is important as alveolar epithelial integrity is the main determinant of alveolar flooding in ARDS [67].

Margination of inflammatory leukocytes to the lung is another hallmark of ARDS [22], and was shown to be mediated by TNF by at least three mechanisms. Firstly, TNF regulates the production of other inflammatory cytokines and chemokines such as MIP-2 and MCP-1 that leads to pulmonary sequestration of circulating inflammatory leukocytes [660, 661, 663]. Secondly, TNF induces the expression of adhesion molecules on both endothelial and epithelial cells, which could affect leukocyte margination and migration [695]. Thirdly, TNF triggers paracellular gap formation in the endothelial layer, thus facilitating leukocyte migration. This was elegantly demonstrated *in vitro* by Nottebaum *et al.*, who found that pre-treatment of endothelial cells with TNF was required for neutrophil- and leukocyte-induced dissociation of vascular endothelial receptor-type protein tyrosine phosphatase (VE-PTP) from VE-cadherin, which ultimately resulted in increased tyrosine phosphorylation of VE-cadherin, weakening of adherens junctions and enhanced leukocyte transmigration [48].

Surfactant dysfunction is also characteristic to the pathogenesis of ARDS [22], resulting in deterioration of respiratory mechanics and increasing the need for mechanical ventilation, which impairs further the surfactant layer and function thereby generating a vicious cycle [183-189]. Moreover, surfactant dysfunction has a detrimental effect on host defence from airborne as well as blood borne pathogens [696, 697]. TNF contributes to this pathology by suppressing the transcription of SP-A and SP-B [641, 645], the surfactant proteins playing a crucial role in host defence [152, 153] and biophysical properties [151] of the surfactant system, respectively. TNF-induced suppression of SP-A transcription in lung epithelial cells is apparently mediated by the p38 MAPK pathway [645].

Intravascular and intra-alveolar coagulation in ARDS results in increased shunt fraction leading to hypoxia and is also linked with the initiation and propagation of the inflammatory response [38, 41]. TNF facilitates this process by activating pro-coagulant factors and inhibiting those involved in fibrinolysis [646]. For example, TNF indirectly activates coagulation by inducing IL-6 production which then stimulates tissue factor (TF) expression [698]. Also, TNF down-regulates thrombomodulin (TM), a crucial mediator of thrombin-induced protein C activation, on the endothelial surface thus facilitating the coagulation cascade [699, 700]. TNF also exerts anti-fibrinolytic effects by stimulating the release of plasminogen activator inhibitor (PAI)-1 [701].

Production of reactive oxygen species by inflammatory cells and cells of the lung parenchyma facilitated by supplementary oxygen during mechanical ventilation, in conjunction with deprivation of endogenous antioxidants leads to a redox imbalance and oxidative injury to the lung during ARDS [206]. Glutathione (GSH) is one of the most important endogenous antioxidants [213], levels of which are greatly

reduced in the BALF and plasma of ARDS patients [215, 216]. Over-expression of TNF in transgenic mice is associated with decreased levels of GSH in their lungs, liver and kidney, suggesting that TNF can impair anti-oxidant capacity [702]. Exposure of human pulmonary artery endothelial cells to TNF *in vitro* also reduces intracellular GSH levels enhancing the susceptibility of cells to oxidative injury [703, 704]. TNF is also involved in propagating oxidative stress by inducing production of ROS. For example, TNF treatment induces mitochondrial ROS production by HUVECs and human pulmonary microvascular endothelial cells *in vitro* [664, 705]. TNF was also shown to stimulate ROS production by neutrophils which altered pulmonary vasoreactivity *in vivo* [665]. This effect is likely to be related to ROS-induced stiffening of pulmonary endothelial cells as demonstrated by Wang *et al.* [705].

TNF also plays an important role in the resolution phase of ARDS by regulating alveolar fluid clearance, although some of the reports are conflicting in their findings [706-710].

Clearly TNF is involved in most aspects of the pathogenesis of ARDS, and its effects are mainly detrimental. However, clinical trials with anti-TNF therapy in sepsis/ARDS patients using agents that were effective in other TNF-mediated pathologies such as rheumatoid arthritis and inflammatory bowel disease, proved to be largely unsuccessful [616, 617, 620, 623-625]. The reasons as to why anti-TNF therapy failed in these trials are not fully understood, but the answer may lie in the complexity of TNF biology and how its effects are mediated through its cell surface receptors. This will now be discussed in more detail.

TNF receptor subtype signalling

TNF receptors - overview

Similarly to TNF-like ligands, the TNF receptor (TNFR) superfamily is also extensive, counting at least 41 members that share different level of homology in their cysteine-rich extracellular sequence, including TNF receptors, CD40, Fas, nerve growth factor receptor (NGFR), TRAIL receptors, RANK and death receptors (DR) [644]. Most of the members are activated by one specific ligand, however TNF has the ability to bind to and exert its cellular effects via two distinct cell surface receptors: the 55kDa TNF receptor p55 (also known as TNFR 1, CD120a, TNFRSF1A) and the 75kDa TNF receptor p75 (TNFR 2, CD120b, TNFRSF1B), both of which are also activated by lymphotoxin-alpha [644]. Both TNFR p55 and p75 are single transmembrane glycoproteins sharing 28% homology, mainly in their extracellular domains [644]. They share similarity in that they both contain an extracellular pre-ligand-binding assembly domain (PLAD) that facilitates trimerisation of the receptors, particularly upon ligand binding [711]. In contrast, the differences in their extracellular domain lend them different ligand binding and activation characteristics. Moreover, their intracellular domains are largely unrelated, allowing for their diversity in signalling functions [712]. The main difference is that TNFR p55 contains a death domain (DD) in its intracellular portion, that plays a critical role in its cell-death inducing function [713], whereas TNFR p75 does not have a DD and recruits different adaptor molecules in order to exert its cellular effects [644].

TNFR p55 expression

TNFR p55 is considered to be expressed on all nucleated cells [649], including circulating monocytes [714-718], macrophages [719, 720], neutrophils [721, 722], T-

and B-lymphocytes [712, 716, 723], and parenchymal epithelial [656] and endothelial cells [53, 724, 725].

TNFR p55 can be cleaved from the cell surface by metalloproteases such as TACE [718, 721, 726]. Upon ligand binding it can also be internalised to initiate signalling pathways, but also to inhibit its long-term actions thus acting as a negative feed-back loop [726-728]. Surface expression of p55 receptor can also be up-regulated on some cells by certain stimuli [719, 729, 730], but not as readily and dynamically as p75 (see below).

In addition to its surface expression, there is also evidence that large amounts of TNFR p55 can be found localised in the perinuclear-Golgi complex [731, 732]. From this intracellular reservoir, full length p55 receptors can be released to the extracellular environment in exosome-like vesicles [733, 734], but there is no evidence that these Golgi-contained receptors would play a role in reconstitution of surface p55 expression.

There are only few studies investigating the expression of TNFR p55 in the lung, and some of these studies are conflicting in their findings. For example, Nakamura *et al.* showed expression of both TNF receptors in lung tissue at mRNA level, but did not investigate their expression at the protein level [730]. The same study also used type II-like A549 cells that were found to express only TNFR p55 both at gene and protein levels (the latter assessed indirectly by ligand binding studies). Islam *et al.* demonstrated surface expression of TNFR p55 on human small airway epithelial cells [734], whereas in another study by Liu *et al.* primary rat type II AEC and type II-like MLE-Kd cell line were found to express both receptors with some dominance of p55 [656]. In direct contrast, Ermert *et al.* using human and rat lung sections found no expression of p55 on the alveolar septum [735], while TNFR

p75 was expressed on nearly all cells, including the alveolar epithelium, and both receptors were found on pulmonary endothelial cells, consistent with Grau *et al.* [53]. Some of these discrepancies may lie in the different techniques employed. For example, while Liu *et al.* and Grau *et al.* used quantitative flow cytometry, Ermert *et al.* used immunohistochemistry, and with such a qualitative/semi-quantitative method, the relative expression of the two receptors may be difficult to determine. In addition, none of these studies validated their staining technique with TNFR knock-out animals. Therefore, the exact expression of TNFRs in lung tissue remains to be elucidated, and will form one focus of this project.

TNFR p55-associated signalling

TNFR p55 is considered to be the major mediator of TNF signalling [649]. Both soluble and membrane-bound forms of TNF can activate TNFR p55 equally well [644]. As TNFRs do not possess a catalytic domain, they exert their signalling effects through adaptor molecules that are recruited upon ligand binding. Differences in their cytoplasmic domain account for the difference in what adaptor molecules they recruit and what signalling pathways they activate, although considerable overlap exists between the receptors in this regard.

TNFR p55 is a death receptor in the classical term owing to its intracellular DD, but can also mediate cell proliferation through activation of the NF-kB pathway. How activation of the same receptor in the same cell can mediate such opposing cellular responses was elegantly explored and demonstrated by Micheau *et al.* [736]. According to their model, after binding of TNF to TNFR p55, a rapid assembly of TNFR p55-associated death domain (TRADD), receptor interacting protein-1 (RIP1) and TNFR-associated factor-2 (TRAF2) occurs forming Complex I. Complex I then

activates the NF-kB, promoting protein synthesis and cell survival. The formation of Complex I is however transient, as TRADD, RIP1 and TRAF2 dissociates from TNFR p55 within an hour while the receptor undergoes endocytosis. Following dissociation from TNFR p55, TRADD and RIP1 recruit Fas-associated death domain (FADD), caspase-8 and 10, forming Complex II that triggers apoptosis. According to a second model, upon ligand binding TNFR p55 will become internalised in a receptosome where it associates with TRADD, FADD and caspase-8 to trigger apoptosis, whereas the lack of receptor internalisation promotes NF-kB activation, thus cell survival [728].

TNFR p55 can also activate the MAP kinases (MAPKs) p38, extracellular signal regulated kinase (ERK or p44/p42) and c-Jun N-terminal kinase (JNK) via TRAF2 and RIP-mediated activation of their upstream kinases (MAPKKK, MAPKK) [644, 649, 737, 738]. Activation of the MAPK pathways can mediate cell death, survival and inflammation.

Activation of TNFR p55 can result in TRAF-2 mediated ROS production by the mitochondria [739]. Stimulation of TNFR p55 facilitates the interaction of apoptosis-regulating kinase 1 (ASK1) with TRAF2 leading to the activation of ASK1 in a redox sensitive manner, in which thioredoxin (Trx) plays an essential role [740]. Under reducing conditions, the reduced form of thioredoxin binds and inhibits ASK1. When ROS are produced, thioredoxin becomes oxidised and dissociates from ASK1 which then forms a JNK-inducing complex with TRAF-2 [741], leading to JNK-activation and apoptosis. At the same time, TNF-induced ROS can inhibit the NF-kB pathway thus further facilitating apoptotic cell death [647]. On the contrary, there is also evidence that ROS can activate NF-kB and promote cell survival [742], adding to the complexity of TNF biology. It has been demonstrated by Yazdanpanah *et al.* that

TNFR p55 can also recruit riboflavin kinase via TRADD that that activates membrane-associated NADPH oxidase, lading to ROS production and cell death [743]. Overall, these findings point toward a mainly pro-apoptotic function of TNFR p55-induced ROS.

In addition to the above mentioned signalling pathways, there is also evidence that TNFR p55 can bind other adaptor proteins to sites on its cytoplasmic domain that are distinct from its DD. For instance, it binds factor associated with neutral sphyngomyelinase activation (FAN), which facilitates the degradation of sphingolipids by neutral sphyngomyelinase into ceramide-containing signalling intermediates that are involved in a pro-apoptotic function [644, 649]. Another molecule that interacts with the juxtamembrane region of TNFR p55 is brain and reproductive organ-expressed (TNFRS1A modulator) (BRE), the role of which is to inhibit and dampen TNFR p55-mediated signalling [644].

Physiological roles of TNFR p55

In addition to its role in mediating cytotoxicity, TNFR p55 is also recognised as a major mediator of the pro-inflammatory response exerted by TNF and is involved in the pathogenesis of a number of pathological conditions. For example, TNF contributes to the insulin resistance seen in type II diabetes mellitus, and this effect is mediated by TNFR p55 [744], and so is mTNF-induced arthritis [653]. The pro-inflammatory role of TNFR p55 is best demonstrated in the autosomal dominant TNFR 1-associated periodic syndrome (TRAPS) where a mutation of TNFR p55 leads to impaired shedding of the receptor and increased cell-surface expression, and is characterised by recurrent high fevers, abdominal pain, rash and arthralgia [745].

TNFR p55 mediates the generation of pro-inflammatory cytokines/chemokines IL-6, IL-8 (and its murine homologues MIP-2 and KC), MCP-1 and GM-CSF [655, 662, 746-748], and up-regulation of endothelial adhesion molecules (E- and P-selectin, VCAM-1, ICAM-1) [662, 725, 749, 750], leading to leukocyte chemotaxis, entrapment and transmigration to sites of inflammation/infection, and also mediates the oxidative burst in neutrophils [722].

Transgenic mice deficient in TNFR p55 have greater sensitivity to infection by *Listeria monocytegenes* and *Legionella pneumophila* infection with associated high mortality, yet are resistant to TNF-, IL-1- and LPS-induced lethality *in vivo* [751, 752], indicating that while TNFR p55 is crucial in host defence against active bacterial infection, it can also be deleterious by activating an overwhelming inflammatory response. TNFR p55 has also been implicated in early acute graft-versus-host-disease [753].

TNFR p75 expression

Historically it was thought that while expression of TNFR p55 is ubiquitous, TNFR p75 is predominantly expressed on haemopoetic cells [754]. However, similarly to p55 it was found to be expressed on B- and T-lymphocytes [716, 755], monocytes [714-718], macrophages [719, 720], neutrophils [722], endothelial [662, 724, 725] and epithelial cells [656].

Similarly to TNFR p55, surface expression of TNFR p75 can be down-regulated to some extent by receptor internalisation [726, 727, 756], but it is more inclined to shedding by TACE and elastase [757, 758] compared to TNFR p55 [658, 714, 726, 727, 747]. It has been suggested that TNFR p75 shedding is in part mediated by TNFR p55 [727, 747]. On the other hand, expression of TNFR p75 can

also be up-regulated and is more dynamically controlled than that of p55 [754]. This suggestion is mainly based on *in vitro* experiments using haemopoietic cell lines, where cells exhibited increased p75 expression with unaffected p55 levels in response to a variety of inflammatory stimuli (e.g. LPS, IL-2, mitogens, PMA) [723, 759, 760], but whether this applies to the *in vivo* setting remains unclear. This readily changeable expression of TNFR p75 coupled to a largely unmodulated TNFR p55 expression can rapidly alter the TNFR p55:p75 ratio on the cell surface and is likely to play an important physiological role in determining the overall TNF response in that particular cell [761-763], and suggests an important functional role for this receptor.

TNFR p75-associated signalling

In vitro studies in the early 1990s indicated that antagonistic TNF receptor specific muteins against p55 as well as p75, at least to some extent, can inhibit sTNF-induced cell death [764] in tumour cells and sTNF-enhanced adhesion molecule expression on endothelial cells [725, 764], suggesting that both receptors are involved in mediating these cellular responses. However, when receptor agonistic muteins were employed, it revealed that only TNFR p55 activation is responsible for these TNF-induced bioactivities [724, 765-769]. The discrepancy between the findings of these receptor agonistic and antagonistic studies was historically explained by the ligand-passing theory [770]. According to this, TNFR p75 having a much higher affinity to sTNF than p55 (K_d 100pM and 500pM, respectively), as well as a much more rapid dissociation rate ($t_{½}$=10min and >3h, respectively), preferentially binds sTNF, especially at low ligand concentrations, and then passes it onto the p55 receptor in close proximity, thereby promoting its activity [770].

However, the TNFR p55:p75 ratio seems to be very important, as artificially high levels of p75 can reduce p55 activity by ligand depletion [770], whereas when the expression levels of p75 are very low, its contribution to TNF-induced cytotoxicity is negligible [765, 767, 768].

More recently, however, a growing body of evidence indicated that TNFR p75 can mediate distinct cellular pathways, partly or completely independently from TNFR p55 [644]. Moreover, it has been also found that mTNF is the prime activator of TNFR p75 and it cannot be efficiently activated by sTNF [655], thus the *in vitro* studies may not accurately reflect the *in vivo* function of TNFR p75, which therefore may have been underestimated in the past [644].

As TNFR p75 does not possess a DD, it mainly exerts its signalling via recruitment of TRAF adaptor proteins, more specifically TRAF2 [644]. TRAF2 allows the recruitment of other factors, such as RIP and FADD through which TNFR p75 can trigger apoptosis [755]. According to other studies employing sTNF and receptor agonistic antibodies, TNFR p75-induced apoptosis does not involve direct engagement with the apoptotic pathways, but relies on the induction of mTNF, which subsequently activates TNFR p55, as well as on the concomitant depletion of TRAF2 and TRAF2-associated protective factors [771, 772]. On the other hand, TNFR p75 activation can also lead to recruitment of cellular inhibitor of apoptosis (cIAP)-1 and -2 via the TRAF2-TRAF1 heterocomplex [773], and in the absence of RIP it can also activate the NF-kB pathway [755] [774] allowing the transcription of anti-apoptotic and pro-inflammatory genes. There is evidence that this activation of the NF-kB pathway by TNFR p75 is distinct from its p55-mediated activation (thereby called the alternative NF-kB pathway) and is restricted to TNFR p75 engagement with mTNF

[775]. Therefore it seems that while TNFR p75 activation by sTNF is mainly cytotoxic, mTNF mainly triggers protein synthesis and cell survival.

The above mentioned mechanisms indicate that TNFR p75 signalling significantly overlaps with that of TNFR p55, mainly because they recruit the same adaptor proteins, particularly TRAF2. However, in addition to these shared pathways TNFR p75 can also activate its own specific signalling molecules, similarly to BRE and FAN for TNFR p55. For example the endothelial/epithelial tyrosine kinase (Etk/Bmx) constitutively associates with the cytoplasmic domain of TNFR p75 in a ligand-independent manner and remains in its closed inactive form [776]. Upon ligand binding the receptor goes through trimerisation and conformational change leading to an open conformation of Etk which then becomes activated via phosphorylation [776]. The active form of Etk then activates downstream signalling molecules such as Akt (or protein kinase B - PKB) [777], resulting in cell-specific responses, such as endothelial cell migration and angiogenesis in endothelial cells [776], and strengthening of intercellular junctions thus increasing trans-epithelial resistance in epithelial cells [778].

Physiological roles of TNFR p75

Transgenic mice deficient in TNFR p75 show increased resistance to low dose TNF-induced lethality and tissue necrosis [779], indicating an important role for this receptor in these biological responses. TNFR p75 can also facilitate p55-mediated apoptotic cell death by at least two distinct mechanisms: via ligand passing [770], and by TRAF2 depletion with concomitant up-regulation of mTNF and subsequent paracrine/autocrine TNFR p55 activation [771, 780]. It can also promote cell-death via caspase activation independent of TNFR p55 [755]. Beside cell death, TNFR p75

also mediates proliferation of kidney tubular cells, thymocytes and the production of GM-CSF independent of TNFR p55 [777, 781]. It is also involved in endothelial cell migration and angiogenesis via its distinct Etk/Bmx signalling pathway [776].

In addition to mediating cell proliferation and cell death, TNFR p75 is also heavily involved in TNF-mediated inflammation and immune response. For example, TNFR p75 is required for the migration of activated Langerhans' cells from the epidermis to the lymph nodes [782] and plays a minor role in mycobacterial immunity [783]. It has been also demonstrated that TNFR p75 plays a role in mediating TNF-induced expression/production of E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, KC, tissue factor and GM-CSF [655, 662, 747], mainly through enhancing signalling through TNFR p55. However, a study using TNFR knock-out mice suggested that TNFR p75 is important for TNF-induced expression of E-selectin, VCAM-1 and ICAM-1 in aortic endothelial cells, and its expression on endothelial cells is essential for adequate leukocyte rolling, adhesion and transmigration [749]. TNFR p75 also plays a pivotal role in the pathogenesis of cerebral malaria, as receptor deficient mice exhibit an absence of ICAM-1 induction on brain microvessels which is associated with brain protection and reduced mortality [784]. There is also evidence that TNFR p75 plays a role in the pathogenesis of $CD4^+$-T-cell mediated hepatitis [785], glomerulonephritis [786], and multiorgan inflammation [787].

Soluble TNFRs (sTNFRs)

As mentioned earlier both TNFRs can be shed from the cell surface by the action of proteases that cleave the receptor ectodomains. The physiological sheddases responsible for sTNFR production are TACE and elastase [757, 758]. TACE can cleave both cell-surface TNFRs, whereas elastase can only cleave TNFR p75, which

could account for the apparent increased susceptibility of TNFR p75 to be shed and its higher levels in biological fluids as compared to p55 [658, 714, 727, 747]. These cleaved monomeric receptor fragments (28 kDa and 40-42 kDa for TNFR p55 and p75, respectively) [726, 788], retain their ability to bind TNF although with lower affinities [649]. In the case of TNFR p55, uncleaved, full-length receptor can be also released via exosome-like vesicles as discussed earlier [733, 734].

TNFRs are constitutively shed *in vivo* and *in vitro* [657, 658, 678, 716, 726, 730, 788] and hence sTNFRs are present in many biological fluids under baseline conditions including the alveolar lining fluid, blood and urine, with sTNFR p55 produced mainly by parenchymal cells and sTNFR p75 by leukocytes [746, 789]. Soluble TNFR release can also be enhanced by a range of inflammatory mediators including LPS, TNF, IL-1, IL-6, IL-10 and many others [658, 714-716, 718, 721, 726, 730, 734, 746, 788, 790] and be inhibited by steroids, IL-4 and PKC inhibition [657, 658, 714, 791]. The sTNFRs are mainly cleared from the circulation by renal excretion [792].

Soluble TNF receptors have been implicated in a variety of clinical conditions. For example, BALF sTNFR levels are elevated in patients with sarcoidosis [793, 794] and ALI [678], where their levels seem to correlate with mortality. Increased plasma levels are associated with sepsis/endotoxaemia [795-797], cancer [798] and autoimmune diseases such as inflammatory bowel disease [799], rheumatoid arthritis [800] and systemic lupus erythematosus [746], with as yet unknown significance.

As they are unable to transduce signals from TNF, sTNFRs were originally considered to have one biological role, namely to neutralise TNF actions as decoy receptors [200, 654, 788, 797, 798, 800-806], which mechanism was utilised in

sTNFR-based therapies. For example, sTNFR constructs such as p55-PEG (soluble p55 linked to polyethylene glycol) and etanercept (dimeric soluble p75:Fc fusion protein) attenuates arthritis in transgenic mice overexpressing mTNF [654]. Similarly, etanercept has been used as an anti-TNF drug with success in rheumatoid arthritis, ankylosing spondylitis, psoriasis, psoriatic arthritis and juvenile idiopathic arthritis [672, 807]. In contrast, it has been also proposed that sTNFRs may prolong the bioactivity of TNF via the formation of a sTNFR-sTNF complex that stabilises its structure and release bioactive sTNF over time, thus increasing its half-life and activity [808, 809].

The specific role of sTNFRs in a given biological scenario may depend on their relative levels to TNF. When the sTNFR:sTNF ratio is high, they could be antagonistic, but the net outcome depends on whether TNF plays a deleterious or a protective role in that particular biological process [746, 809]. When the level of sTNFRs is moderate they may serve as buffers by inhibiting the effects of high concentrations of TNF, but also facilitating TNF signalling in a controlled manner [746].

Role of TNFRs in ALI/ARDS

Both clinical and experimental studies implicated roles for each TNFR subtype in the pathogenesis of ALI/ARDS. Grau et al. showed that cultured pulmonary microvascular endothelial cells (MVEC) isolated from patients with ARDS expressed higher levels of TNFR p75 (but not TNFR p55), VCAM-1 and ICAM-1 as compared to controls (lung cancer patients undergoing lobectomy) [53]. These cells also exhibited increased production of IL-6 and IL-8 upon TNF treatment compared to controls, indicating a role for TNFR p75. Upregulation of ICAM-1 was weaker in cells

from ARDS patients (approximately 2-fold increase from baseline as compared to a 5-fold increase in controls), but this might have been secondary to an already high baseline expression and a saturated response to TNF. Nonetheless, the absolute level of TNF-induced ICAM-1 expression was still significantly higher in cells from ARDS patients. It is important to point out, however, that culturing cells over several weeks (2-3 weeks in this particular study) can potentially result in phenotypic and functional change of cells, and therefore the data may not reflect the *in vivo* expression of TNFRs and their function.

Xu *et al.* showed that alveolar TNFR p55 mediated lung injury induced by adoptive transfer of $CD8^+$ T-cells activated by intraalveolar viral antigens [748]. Liu *et al.* reported an important role for TNFR p75 in the same process [656]. The authors found that TNFR p75 deficient mice were nearly free from alveolar infiltration by inflammatory cells and had significantly reduced lung injury compared to wild-types. They suggested that this protection from lung injury might be due to a reduced MCP-1 production by type II AECs in response to $CD8^+$ T-cell derived sTNF, because primary type II AECs isolated from TNFR p75 knockout mice produced no MCP-1 in response to sTNF. On the other hand, lack of TNFR p55 had the same effect, in agreement with the finding by Xu *et al.* [748]. Lung injury secondary to *Pneomocystis carinii* infection is also mediated by $CD8^+$ T-cells and involves both TNFRs as demonstrated by Pryhuber *et al.* [789]. Using a chimera model in which bone marrow from wild-type mice was injected to mice lacking both TNFRs (TNFR double knock-outs (DKO)), the recipients had significantly lower number of inflammatory leukocytes in their BALF than wild-type → wild-type chimeras, indicating a role for parenchymal TNFRs in the development of lung injury via the recruitment of damaging inflammatory cells, in support of the findings of Liu *et al.* [656] and Xu *et*

al. [748]. However, the individual role of each TNFR was not investigated in this study.

Mizgerd et al. showed *in vivo* that TNFR-DKO mice inoculated with i.t. *E. coli* exhibit increased neutrophil infiltration to their lung and an impaired bacterial clearance from the alveolar space compared to their wild-type control with no difference in pulmonary oedema or NF-kB activation [810]. Later the same group using the same model but employing TNFR p55/IL-1R1 knock-out mice demonstrated, that these animals had significantly reduced pulmonary oedema, BALF neutrophils and KC levels compared to wild-types [811]. Interestingly, in mice lacking the IL1R1 alone, neither the number of alveolar neutrophils nor the amount of pulmonary oedema were reduced, indicating an important role for TNFR p55 in pulmonary neutrophil trafficking and oedema formation [811]. Although the authors did not investigate the role of TNFR p75 individually in this model, the seemingly contradicting findings of their two studies can potentially be reconciled by a protective role for this receptor in this particular biological scenario.

Neumann *et al.* reported a crucial role for TNFR p55 in mediating i.p. TNF-induced E-selectin, VCAM-1 and ICAM-1 expression and leukocyte migration to the lungs and other organs, but did not investigate the role of TNFR p75 [695]. Calkins et al. investigated the role of individual TNFRs in pulmonary chemokine production and neutrophil sequestration in response to intraperitoneal LPS [812], and found that p55, but not p75, was responsible for LPS-induced KC and MIP-2 production and neutrophil infiltration to the lungs.

Ferrero *et al.* investigated the role of individual TNFRs on endothelial permeability *in vitro* and *in vivo* [813]. They found that TNFR p55 was responsible for TNF–mediated cytoskeletal re-arrangement of endothelial monolayers associated

with increased permeability, mediated by the p38 MAPK [813]. This suggests that p55 promotes endothelial barrier dysfunction and potentially pulmonary oedema formation in ARDS. However, *in vivo* blockade of TNFR p75 by antagonist antibodies partially inhibited TNF-induced vascular leak in the liver, indicating some role for this receptor [813].

Differential TNFR signalling

Although the biological functions of the TNFRs often overlap despite their ability to activate distinct signalling pathways, there is a growing body of evidence which indicates that they can also act in direct opposition in a variety of physiological scenarios including acute lung injury of different aetiologies.

In a model of 'pure' VILI using TNFR single and double knockout (KO) mice, our group has previously shown that while p55-KO mice were completely protected, p75-KO mice were more susceptible to develop pulmonary oedema [639]. Importantly, absence of all TNF receptor mediated signalling in receptor double knock-out animals led to a similar amount of injury compared to wild-types [639]. Moreover, protection from pulmonary oedema in p55-KO mice was not associated with decreased pulmonary neutrophil infiltration, as demonstrated by similar MPO activity compared to wild-types.

We then investigated whether such phenomenon also applies to ALI of different aetiology, and tested in a mouse model of acid aspiration-induced lung injury [814]. We found that TNFRs played a very similar role to that in the VILI model, i.e. p55-KO mice were protected from pulmonary oedema, whereas lack of p75 receptor may have exacerbated it. Further studies investigating the individual roles of TNFRs in this model indicated that acid aspiration-induced lung injury in wild-type

mice was associated with pulmonary inflammation (i.e. increased alveolar neutrophils, IL-6, KC, MIP-2) and markers of epithelial cell apoptosis (i.e. increased caspase-8, TUNEL staining) and dysfunction (i.e. reduced alveolar fluid clearance) [815]. Pulmonary inflammation was exacerbated by absence of the p75 receptor, while absence of p55 was without effect. In contrast, the p55 receptor contributed to epithelial apoptosis by activating caspase-8. Moreover, specific pharmacological inhibition of caspase-8 led to significant improvements in alveolar fluid clearance and oxygenation. These data suggest that p55 receptor is the main mediator of epithelial cell death and dysfunction contributing to lung injury, whereas p75 receptor may be protective via suppressing the inflammatory response.

Similar studies have been done in other areas, such as retinal ischaemia, heart failure, sepsis and colitis models, suggesting that such differential effects may be a universal phenomenon taking place in other organs and disease processes. For example, Fontaine *et al.* investigated the roles of TNFRs in a model of retina ischaemia-reperfusion damage using TNFR knock-out mice [729]. The authors found that while TNF deficiency (i.e. total TNF-signalling blockade) did not affect overall cell loss, TNFR p55-KO exhibited a significant reduction in neurodegeneration, whereas lack of p75 enhanced this process [729]. Higuchi *et al.* showed that in mice that overexpress myocardial TNF, and thus develop heart failure, the absence of TNFR p55 improved survival significantly, whereas TNFR p75-KO animals were more prone to develop heart failure and had reduced survival compared to mice expressing both TNFRs [816]. Ebach and co-workers demonstrated in a caecal ligation and puncture (CLP) model of sepsis that mice lacking TNFR p55 had prolonged survival and less hypothermia compared to wild-types [817]. In direct contrast, TNFR p75 deficiency led to significantly shortened survival and more

severe hypothermia compared to wild-types [817]. Furthermore, the level of free sTNF in the serum and peritoneal fluid was markedly elevated in TNFR p75-KO mice [817], indicating a role for this receptor in controlling sTNF levels. In a mouse model of inflammatory bowel disease the same group has demonstrated a protective role for TNFR p55, whereby mice lacking this receptor lost more weight, became hypothermic and had reduced survival compared to their wild-type control [818]. TNFR p75 deficiency on the other hand resulted in less weight loss, normal temperature and improved survival [818]. Similarly to their other model, TNFR p75-KO mice had increased levels of TNF [818].

With the exception of the colitis model, all these experiments demonstrate a deleterious effect for p55 and a protective role for p75. This differential, opposing signalling mechanism may explain why total TNF blockade has been unsuccessful in the treatment of ARDS, and indicates that specific targeting of TNFR p55 signalling is necessary to provide protection in these clinical entities. However, we have to be cautious when interpreting these findings for a couple of reasons. Firstly, genetic modifications in knock-out animals may cause chronic compensation as well as non-predictable changes in the expression of other, not directly targeted genes. Secondly, we don't know precisely where the locus of TNF signalling during VILI or acid aspiration occurs, be it intrapulmonary (alveolar) - or extrapulmonary (vascular), and what is the relative role of the receptor subtype signalling in different compartments of the lung. These cannot be elucidated solely by using knock-out animals that lack both alveolar and vascular expression of TNF receptors. In order to achieve success in the treatment of ARDS by targeting specific TNF receptor subtype signalling, the precise location where the key TNF-mediated signalling takes place *in vivo* has to be fully uncovered. Moreover, as the function of the individual

TNFRs shows correlation with their surface expression that may change dynamically leading to a varying p55:p75 ratio and net physiological outcome, it is an absolute necessity to explore the expression profile of TNFRs on each side of the alveolocapillary barrier in the context of ALI/ARDS. Previous work in our group has indicated that alveolar expression of TNFR p55 is well preserved during lung inflammation, whereas TNFR p75 is prone to shedding [819]. However, characterisation of TNFR expression and signalling on the endothelial side remains to be fully elucidated as previous studies are contradicting in their findings and rely on semi-quantitative methods and extrapolation of findings in phenotypically different endothelial cells. Therefore this will form one major aim of the current study.

Domain antibody technology

Although differential TNFR signalling in animal models of acute lung injury suggests that specific targeting of p55 receptor signalling may confer more benefits than total TNF blockade, monoclonal antibodies may not be optimal for this purpose for a couple of reasons. Firstly, monoclonal antibodies have two antigen binding fragments (F_{ab}) which can result in cross-linking of the target antigens, leading to receptor activation instead of the desired neutralising effect, which has been demonstrated in a cell-lysis assay and neutrophil respiratory burst *in vitro* [820, 821]. Additionally, monoclonal antibodies may bind non-specifically through their crystallisable domain ($F_{c)}$ to cellular F_c receptors, leading to their endo- or phagocytosis followed by recycling to the cell surface or extracellular space [822, 823]. This may, on one hand, limit their biodistribution (i.e. may not get to their target antigen), while on the other hand leads to a long half-life, which may not be practical in the clinical setting.

A novel development from GlaxoSmithKline, called domain antibody technology may overcome these limitations. Domain antibodies (dAbs) comprise the variable domains of either the heavy (V_H) or light chain (V_L), and are thus the smallest functional antigen-binding portions of the IgG molecule [824, 825]. Their small size (~12kDa as opposed to 150kDa for IgG) means they have high tissue penetration and can be delivered at much higher concentrations per unit mass than monoclonal antibodies or conventionally produced antigen-binding fragments (F_{ab}); their lack of F_c portions means they are less likely to be recycled in cells (therefore have shorter half-life) or to induce F_c effector responses such as complement-dependent cytotoxicity (CDC) or antibody-dependent cytotoxicity (ADCC). They have monovalent antigen binding characteristics, thus avoiding the potential for receptor cross-linking and activation.

1.5 Hypothesis and Aims

This project had the main hypothesis that the two TNF receptor subtypes have different expression kinetics and roles on the two sides of the alveolar-capillary membrane and can mediate opposing effects in the pathogenesis of ALI; specifically p55 TNF receptor mediates lung injury whereas p75 signalling has a protective role, therefore specific blockade of p55 signalling is necessary to attenuate ALI. This PhD thesis has been divided into two main parts. Firstly, this thesis will investigate the expression profile and roles of TNF receptor subtypes on the pulmonary endothelium. Secondly, this thesis will aim to modulate ALI progression by specifically targeting individual TNF receptors on the epithelial and/or endothelial side of the alveolar-capillary membrane in mouse models of ALI. These 2 main aims have the following sub-divisions and specific hypotheses:

1. Characterisation of TNF receptor expression and signalling on pulmonary endothelial cells:
    - Investigate the expression levels of individual TNF receptors on pulmonary endothelial cells under control and inflammatory conditions. We hypothesise that the expression level of the two TNFRs is differentially regulated on the pulmonary endothelium under control and/or inflammatory conditions.

    - Investigate the roles of individual TNF receptor subtype signalling in TNF-mediated pulmonary endothelial cell activation using TNF receptor knock-out animals and pharmacological inhibition of TNF receptor signalling *in vivo* and *in vitro*. I hypothesise that p55 and p75 TNF receptors have

differential or even opposing roles in TNF-induced pulmonary endothelial cell activation.

2. Differential TNFR signalling in ALI:

    - Investigate the effects of pharmacological TNFR p55 signalling blockade on the epithelial and/or endothelial side of the alveolar-capillary membrane on the progression of lung injury, using *in vivo* mouse models of VILI and acid-aspiration induced lung injury. I hypothesise that TNFR p55 promotes pulmonary oedema and inflammation during the pathogenesis of ALI and its blockade therefore attenuates lung injury.

2. Methods

This chapter will describe the various methodologies applied in this project, including different animal models of ALI, lung histology, flow cytometry and *in vitro* cell culture.

The majority of this project has been based upon the use of *in vivo* mouse models. A custom-made mouse mechanical ventilation system is described that has been used to model ALI induced by use of injurious high VT ventilation or intratracheal instillation of hydrochloric acid (in collaboration with Dr Kenji Wakabayashi and Dr Brijesh Patel). I have also used *in vivo* mouse models of systemic inflammation induced by i.v. administration of bacterial toxins or TNF in non-ventilated mice. Protocols for each of these models are described below. Sampling/harvesting of BALF, plasma and lung tissues from animal models is described with specific assays, and *in vitro* culture of primary mouse lung endothelial cells (in collaboration with Dr Justina O Dokpesi) to investigate TNF receptor subtype-mediated adhesion molecule expressions is also described.

A flow cytometry method for quantifying the expression of cell-surface TNFRs in normal lung tissue was utilised (in collaboration with Dr Anthony D Dorr) to study TNFR expression on pulmonary endothelial cells under baseline and inflammatory conditions. The principles of this technique will be discussed in detail below, including protocols for identification of pulmonary endothelial cells, and validation of specific TNFR staining. In addition, a lung histology protocol used for qualitative assessment and demonstration of lung injury is described. Beside the methods described in this chapter, specific protocols will be outlined where appropriate in subsequent chapters.

2.1 Animal models

Animals

All experimental protocols were approved by the Ethical Review Board of Imperial College London as well as the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals (where appropriate), and carried out under the authority of the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986. Experiments were performed using male wild-type C57BL6 mice aged 9-12 weeks, or age and sex-matched mice lacking p55 (p55KO) or p75 (p75KO) or both (DKO) TNF receptors (generous gifts from Amgen, Thousand Oaks, CA) [826]. DKO mice were bred by crossing mice heterozygous for p55 and p75 ($p55^{+/-}$ and $p75^{+/-}$, respectively). p55KO mice were C57BL6 inbred, whereas the p75KO and DKO mice were backcrossed onto their wild-type C57BL6 strain for five generations. Genotyping of knock-out mice was regularly performed by Surrey Diagnostics to confirm TNFR deficiencies.

In vivo systemic inflammation models

Intravenous instillation method was used to deliver inflammatory agents systemically targeting pulmonary endothelial cells and to monitor their specific response to these insults.

Mice were transferred from their cage into a plastic chamber and placed under direct heat from a light bulb for approximately 1 minute in order to facilitate vasodilation in the tail, but not to an extent to cause harm or excessive stress to the animals. Then mice were transferred to a cone mouse restrainer, the tail disinfected with 70% ethyl alcohol, and then the agent of interest was injected via the tail vein.

Mice were then marked appropriately and transferred back to their cage allowing access to food and water *ad libitum*. At desired time points, mice were euthanised by isoflurane overdose for sampling and harvesting.

TNF-induced pulmonary microvascular inflammation

In order to trigger TNF-mediated cellular response in pulmonary endothelial cells and to investigate the specific roles of individual TNF receptor subtypes, wild-type mice and mice lacking either one (p55KO and p75KO) or both (p55/p75 double KO) TNF receptors were injected intravenously with 500ng recombinant mouse TNF (R&D Systems Europe Ltd., Abingdon, UK) via the tail vein. Untreated mice served as controls. This produced distinct cellular response by pulmonary endothelial cells in terms of adhesion molecule expression at the defined time points, depending on the TNFR genotype.

Lipopolysaccharide-induced pulmonary microvascular inflammation

To investigate the effect of systemic inflammation on the TNF receptor expression profile on pulmonary endothelial cells, wild-type mice were injected intravenously with 20µg lipopolysaccharide (Ultra-pure LPS from E. coli O111:B4, Source BioScience UK Ltd, Nottingham, UK) and incubated for selected periods of time, after which the expression level of the individual TNF receptors was quantitatively assessed by flow cytometry and compared to control values. Systemic administration of LPS had diverse effects on the receptor subtypes that changed dynamically over time.

In vivo mechanical ventilation in mice

The mechanical ventilation system used in this project has previously been described in great detail [106, 109, 116, 337, 639]. In this study, mechanical ventilation was used to either deliberately produce lung injury by applying high V_T ventilation (VILI) with or without co-administration of i.t. LPS, or in a non-injurious manner either to serve as control in these models or to maintain animals after i.t. administration of acid during acid-aspiration induced ALI.

The ventilation system

This is a positive pressure ventilation system utilising a custom-made flow-controlled ventilator [827] supplied by dry gas from a cylinder. The gas enters the system through a flow regulator enabling the investigator to control the flow and thus the actual V_T (Figure 2.1.). 'Inspiration' and 'expiration' are regulated by two electronically controlled valves, the solenoids. When the inspiratory valve opens, it allows gas to enter the lungs. Then it closes whilst the expiratory valve opens simultaneously to facilitate gas flow out of the lungs. This 'expiration' is passive and entirely relies on the elastic properties of the chest wall and the lungs. The gas leaving the system through the expiratory pipe can either enter the atmosphere freely or can be led through an adjustable water column to apply positive end-expiratory pressure (PEEP). Then the expiratory valve closes and the respiratory cycle starts again. The respiratory rate is controlled electronically with a fixed inspiratory to expiratory time ratio of 1:2.

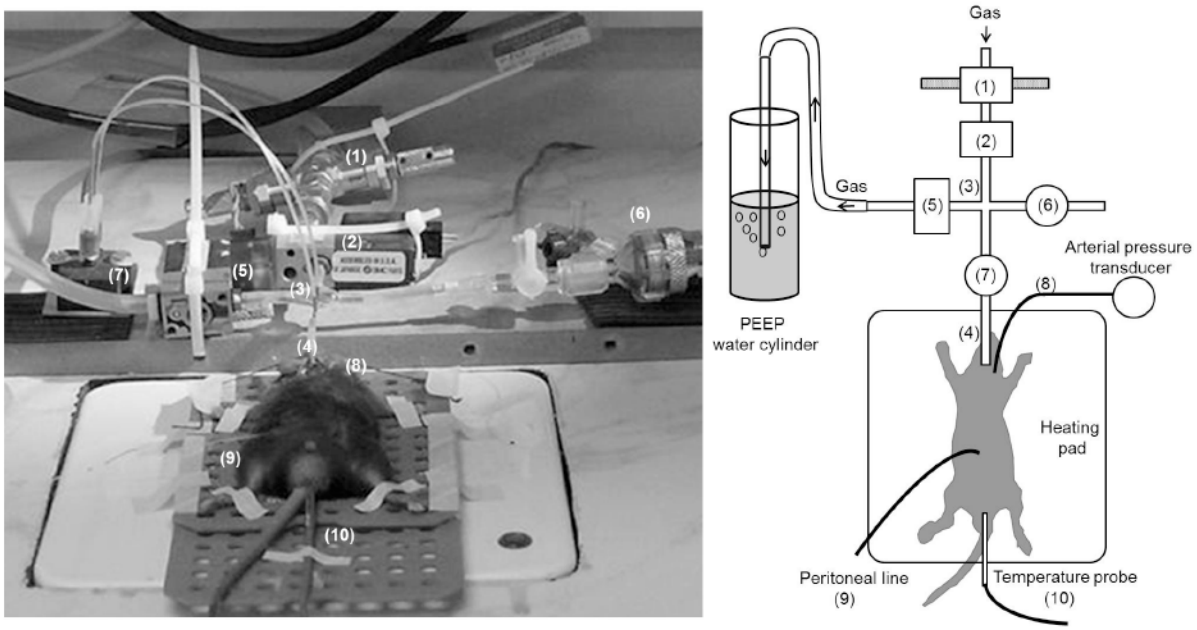


Figure 2.1 - Photograph and schematic diagram of the *in vivo* mouse ventilation system. High-pressure gas from the gas cylinder passes through a flow regulator **(1)**, which can be adjusted to give a desired constant V_T throughout the experiment. When open, gas flows through the inspiratory solenoid **(2)**, through the pneumotachograph **(3)** and into the mouse via the endotracheal tube **(4)**. After inspiration, the inspiratory solenoid closes, the expiratory solenoid **(5)** opens and the mouse exhales passively against either atmospheric pressure or a set positive end-expiratory pressure (PEEP) regulated by a water column. A pressure transducer **(6)** for measurement of airway pressure and a differential pressure transducer **(7)** for monitoring of airway flow, enable determination of respiratory mechanics. There is also an arterial line **(8)** for blood pressure and blood gas analyses and for maintenance fluid infusion, and an i.p. line for maintenance of anaesthesia **(9)**. The mouse is positioned supine on a heating pad to maintain physiological temperature (36-37^{o}C) monitored by a rectal probe. All information from the transducers is collected by a PowerLab data acquisition system and analysed by Chart software (AD Instruments, Chalgrove, UK).

Animal preparation

Mice were anaesthetised by intraperitoneal (i.p.) injection of ketamine (60mg/kg) and xylazine (6mg/kg). Anaesthesia was maintained throughout experiments via an i.p. line, and body temperature maintained between 36-37ºC using a heating pad and monitored with a rectal temperature probe (Figure 2.1). After induction of anaesthesia, mice were laid supine on a plastic perforated tray and fixed in this position with tapes applied on the limbs. Then the neck region was disinfected with 70% ethyl alcohol and a tracheostomy was performed to allow insertion of an endotracheal tube. Mice were connected to the ventilator via the endotracheal tube and ventilated using low V_T of 8-10 ml/kg, a PEEP of 3 cmH_2O and a respiratory rate of 120/min using 100% oxygen.

The left carotid artery was exposed and cannulated for constant monitoring of arterial blood pressure and administration of normal saline with 10U/ml heparin (to maintain patency of cannula) at a rate of 0.3-0.4 ml/h to compensate for increased fluid loss through the airways secondary to dry gas ventilation. The arterial line was also used for arterial blood gas monitoring at predetermined time points (~70µl/sample, followed by bolus saline to replace fluid loss). In some experiments the right internal jugular vein was also cannulated for continuous infusion of antibodies.

Airway pressure and gas flow were monitored real-time via transducers connected to a PowerLab data acquisition system (AD Instruments, Chalgrove, UK), and the data analysed using Chart software (version 5.5.6, AD Instruments). Plateau pressure (P_{plat}), respiratory system elastance (E_{rs}) and resistance (R_{rs}) were determined at 20 min intervals by the end-inflation occlusion technique [827], which is incorporated in this custom-made ventilator system. During this manoeuvre an

inspiratory pause is generated by closing the inspiratory solenoid with the expiratory solenoid still being in a closed state. Therefore, zero flow is achieved in the pneumatograph while the lungs remain inflated with a given V_T. Tidal volume is calculated by calculating the area under the curve on the flow chart (Figure 2.2) after correction for the frequent zero drift in the flow transducer.

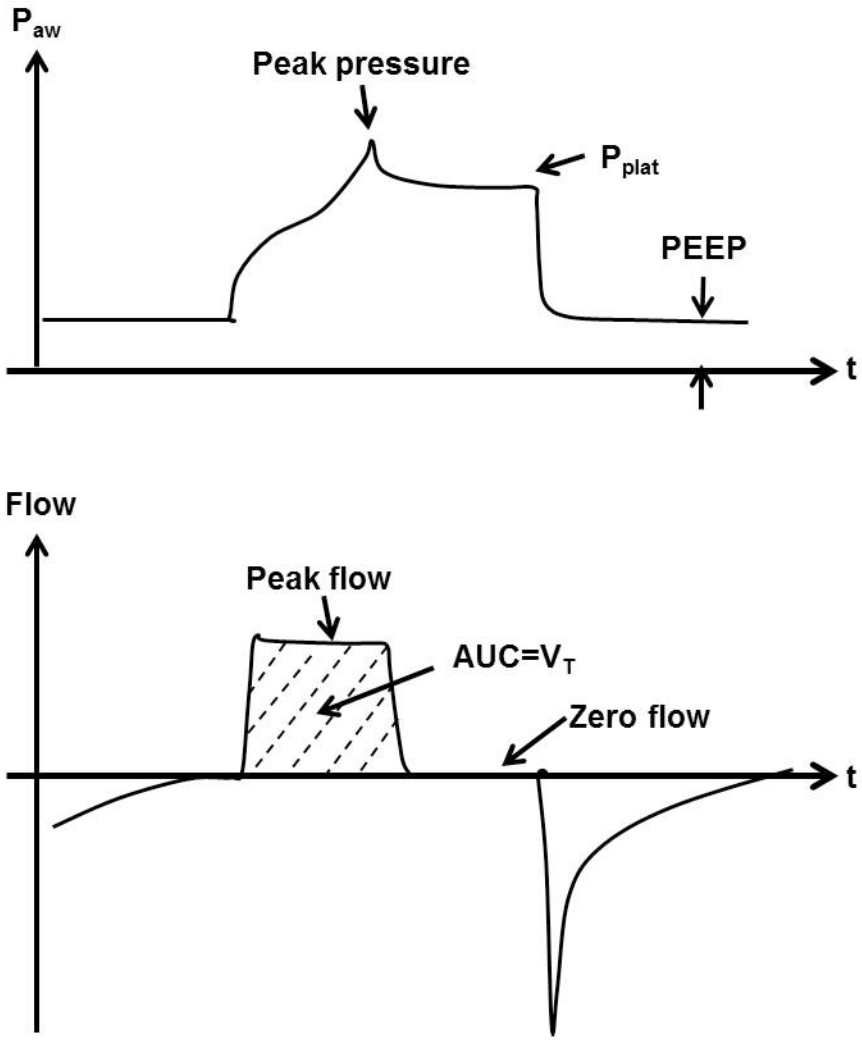


Figure 2.2 - Measurement of respiratory mechanics *in vivo* using end-inflation occlusion technique. Using pressure transducers, pressure and flow traces are obtained similar to those shown above. Using end-inflation occlusion and the gas flow trace, zero drift can be corrected and VT accurately measured by integrating the area under the curve (AUC). Using the pressure trace, peak, plateau (Pplat) and positive end-expiratory pressures (PEEP) can be accurately measured. Lung mechanics, such as respiratory system resistance (R_{rs}) and elastance (E_{rs}) can thus be calculated and monitored throughout experiments.

All of the ventilation models used in this project involved bolus intratracheal fluid administration at some point during the protocol. For this, mice were disconnected from the ventilator and the agent of interest was instilled through a fine cannula inserted in the endotracheal tube. In some experiments, bolus technique was substituted to a MicroSprayer delivery method (Penn-Century, Inc., PA, US). Then mice were reconnected to the ventilator, and to help the fluid distribute in the lungs, 4 subsequent sustained inflations with a pressure of 35cmH_2O for 5sec were given in 30sec intervals. Further single sustained inflations were also applied in every 20min to avoid development of atelectasis.

Upon termination of experiments, mice were overdosed with anaesthetic administered via the arterial line and exsanguinated by cardiac puncture prior to tissue sampling.

In vivo mouse model of ventilator-induced lung injury

Our in vivo mouse model of VILI is based upon previously published models [106, 109, 116, 337, 639]. In these, lung injury is produced within 2 hours solely by over-stretching healthy lungs with excessive V_T. However, as our model involves intratracheal administration of agents to the lungs in a fluid phase it has an intrinsic effect on respiratory mechanics. It is likely that the fluid interferes with the surfactant layer, hence changing the elastic properties of the lung and introducing significant heterogeneity into the system. As one of our main interests was to investigate the effect of p55 receptor blockade on the respiratory mechanics, we had to establish a model which can be well standardised and therefore has reasonably small variability in respiratory mechanics. We also had to take into account that if the p55 receptor blocking antibody used has any protective effect on VILI in terms of respiratory

physiology, it could be moderate to mild; therefore a model which causes a too harsh injury may not be feasible to reveal these effects. Therefore, a couple of modifications had to be introduced to the previously established VILI models. This will be discussed in more detail as part of a model development section in the relevant chapter, whereas the main steps of the final protocol are outlined here.

Mice receiving low V_T (low-stretch) were ventilated with 100% oxygen, whilst those receiving high V_T (high-stretch) were ventilated with oxygen supplemented with 4% CO_2 to avoid hypocapnia.

Surgery was followed by a 5 min equilibration period, after which baseline readings were taken and high-stretch ventilation started or low-stretch settings maintained (in some control animals). Immediately after setting high-stretch parameters, mice received an intratracheal bolus of either anti-p55 or anti-TNF antibody or their appropriate controls with or without a subclinical dose of LPS, and ventilated for 240 min or until BP dropped below 45 mmHg (indicative of cardio-respiratory collapse [337]), whichever occurred earlier. The dose of anti-TNF monoclonal antibody (50µg) derived from a previously published work from our group where it efficiently reduced high stretch induced neutrophil influx to the lungs [109], whereas the anti-p55 dAb dose (25µg) was selected based on previous mouse models of smoke inhalation and LPS-induced lung injury, where it significantly reduced the inflammatory response (interpersonal communication with GSK). In some experiments, mice also received a continuous intravenous infusion of either control or p55 specific blocking antibody via the jugular vein during high-stretch ventilation. Mice received sustained inflations in every 20 min to avoid atelectasis.

Development of lung injury was assessed by an increase in P_{plat}, R_{rs} and E_{rs} compared to their value at the start of high-stretch (expressed as % change in the

case of R_{rs} and E_{rs}). Deterioration in gas exchange, i.e. changes in PO_2 and PCO_2, was also monitored at predetermined time points as indicator of lung injury.

After termination, BALF, plasma and lungs were harvested and either used for immediate analysis or stored at -80ºC.

In vivo mouse model of acid aspiration

Experiments with the *in vivo* VILI model demonstrated that blockade of intrapulmonary p55 TNF receptor signalling by a domain antibody can attenuate VILI as will be described later. Therefore, we went on to investigate whether this approach would be also beneficial in another model of ALI, namely in acid induced lung injury. This work was done in collaboration with Dr Brijesh Patel and Dr Kenji Wakabayashi. The model is based on previously published models [365, 814, 828] and uses *in vivo* mechanical ventilation system for the maintenance of treated mice and the assessment of injury development. As with the VILI model this model also required fine-tuning secondary to the effects of intratracheal fluid instillation on respiratory mechanics and lung homeostasis. Also, as the antibody bolus could not be co-administered with the injurious insult (i.e. hydrochloric acid); this model involved two separate i.t. fluid instillations. A detailed model development will be described in Chapter 6, while here we outline the main steps of the final protocol.

After surgery and a 5 min equilibration period, mice received an i.t. bolus of 25µg of either dummy or p55 receptor specific domain antibody in a volume of 50µl as described earlier and ventilated with low-stretch settings (i.e. V_T= 8-9ml/kg). After 60min, mice also received an i.t. bolus of 65µl of 0.075M hydrochloric acid or normal saline and ventilated for further 180min or until BP dropped below 45mmHg, whichever happened earlier. Mice received a series of sustained inflations

(35cmH_2O for 5sec, 4 times with 30sec intervals) after each bolus instillation, and single sustained inflations every 20min thereafter to prevent atelectasis. All animals were ventilated with 100% oxygen to avoid hypoxia.

In this model, acid instillation produced significant lung injury by 3 hours, characterised by pulmonary oedema and recruitment of leukocytes to the pulmonary microvasculature and alveolar space. Upon termination, saline lavage was performed, plasma and lungs were harvested and either used for immediate analysis or stored at -80ºC.

2.2 *In vivo*/*Ex vivo* sampling

Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was obtained as described previously [337]. Sterile saline (750µl) was flushed into the lungs via the endotracheal tube and gently sucked out. This was repeated twice with the same saline. Samples were immediately centrifuged at 1500rpm for 5min at 4°C. Supernatants were aliquotted and stored at -80°C for analysis by ELISA or protein assay, and cell pellets resuspended in 350µl sterile saline. Cells were analysed by flow cytometry and/or differential cytology using a Diff-Quik staining kit (Medion Diagnostics AG, Duedingen, Switzerland).

Plasma

Plasma samples were obtained by cardiac puncture with a heparinised needle and syringe. Heparinised blood was centrifuged at 4000rpm for 5min at 4ºC. Plasma was removed and aliquotted for storage at -80ºC for analysis by ELISA.

Lung wet:dry weight ratio

Following exsanguination of mice, lungs were excised and briefly rinsed in saline then blotted on tissue paper. Lungs were weighed and placed in an oven at 60-65ºC to dry overnight. Lungs were re-weighed and wet:dry weight ratios calculated.

2.3 *In vitro* pulmonary endothelial cell culture

The in vivo experiments with TNF receptor knock-out animals investigating the roles of individual TNF receptor subtypes in TNF-mediated activation of pulmonary endothelial cells showed interesting results as will be described in Chapter 3. However, genetic modifications in knock-out animals may cause chronic compensation as well as non-predictable changes in the expression of other, not directly targeted genes which may influence a specific biological response. Also, lack of TNFR signalling on cells other than PECs (e.g. circulating leukocytes) may also indirectly impact on TNF-mediated activation of PECs. Therefore to verify our *in vivo* findings, we used an in vitro pharmacological approach in collaboration with Dr Justina O Dokpesi utilising wild-type primary mouse pulmonary endothelial cell (PEC) culture.

Isolation and culture of primary mouse pulmonary endothelial cells

Wild-type mice were sacrificed, and 1ml of 1mg/ml Dispase (Gibco, Invitrogen Ltd, Paisley, UK) was instilled through tracheostomy into the lungs and left for 5 min, after which lungs were excised and washed in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) containing 0.5% FCS. Samples were finely minced with a pair of scissors, digested in 1mg/ml Dispase at 37°C for 45 min, and then

mechanically dissociated by triturating and filtered through a 40-µm nylon mesh (BD, Oxford, UK) to form a single cell suspension. PECs were isolated from the suspension by a magnetic labelling method, using the midiMACS separation unit (Miltenyi Biotec Ltd., Bisley, Surrey, UK) according to the manufacturer's instructions. In brief, depletion of $CD45^+$ (pan-leukocyte marker, clone: 30-F1, Biolegend, Uithoorn, The Nederlands) cells was followed by 2 repeated steps of enrichment of $CD31^+$ (PECAM-1, endothelial cell marker, clone: MEC 13.3, BD Pharmingen, Oxford, UK) cells. By this method I could obtain 6 million pulmonary endothelial cells on average per mouse (determined by flow cytometry using microsphere counting beads (Caltag Medsystems, Towcester, UK)) with a purity of 95% (Figure 2.3).

Cells were then cultured at 37°C in 5% CO_2 in DMEM supplemented with 1% non-essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 12U/ml heparin (Leo Laboratories Ltd, Princes Risborough, UK), 25mM HEPES (VWR, Lutterworth, UK), 10% heat inactivated FCS, 2mM glutamine, 100µg/ml streptomycin, 100U/ml penicillin (all from Gibco), and 50µg/ml endothelial cell growth supplement (Sigma) in flasks coated with 1% type B gelatin (Sigma). After overnight culture, cells were washed to remove non-adherent cells and fresh medium was added. Once the cells reached confluence, they were re-plated using trypsin-EDTA (Sigma), and used for experiments between passage 3 and 8.

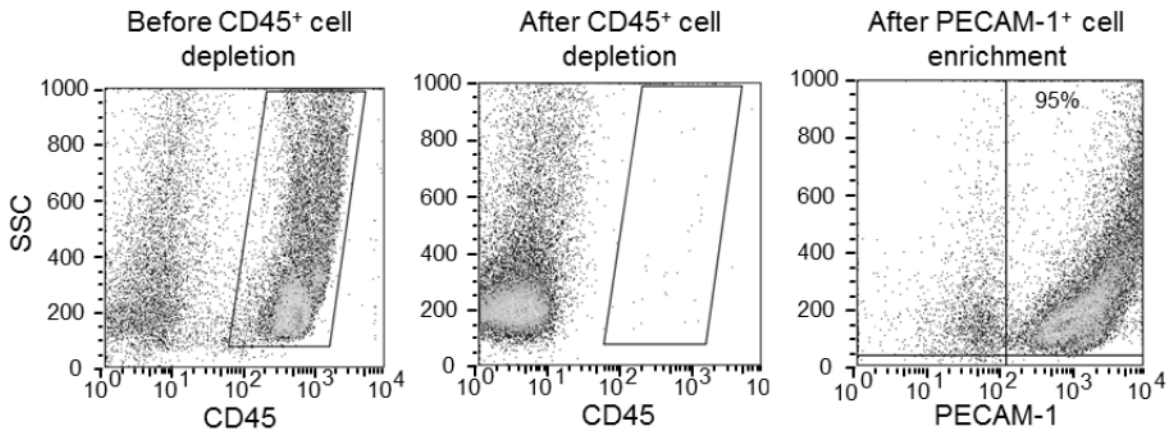


Figure 2.3 – Analysis of purification of mouse primary pulmonary endothelial cells by flow cytometry. Primary PECs were isolated from wild-type mice by enzymatic digestion and mechanical dissociation followed by depletion of $CD45^+$ cells and enrichment of PECAM-1^+ cells by a magnetic labelling method, using the midiMACS separation unit. This provided a 95% purity of PECs for culture. Dot plots represent composition of cell suspension *before* and *after* $CD45^+$ *cell depletion* and *after* PECAM-1^+ *cell enrichment*.

In vitro TNF-induced adhesion molecule expression by primary PECs

Cultured PECs were seeded at a density of 10^5 cells/well in a 24-well plate coated with 1% type B gelatin. After overnight culture, the medium was replaced with RPMI containing 10% FCS, and triplicate wells were allocated into one of four groups: no treatment (control); 10ng/ml recombinant mouse TNF for 4 h; and pre-incubation with anti-p55 (90µg/ml; 55R-170, BD Biosciences) or anti-p75 (20µg/ml; TR75-32, BD Biosciences) neutralizing antibody for 1 h before TNF treatment. The anti-TNF receptor antibodies are validated by the manufacturer as having no cross-reactivity between receptor subtypes. Cells were recovered/detached with 1% trypsin-EDTA at 37°C for 1 min and processed for flow cytometry.

2.4 Enzyme-linked immunosorbent assay (ELISA)

Colorimetric sandwich ELISA was used to quantify soluble mediators in BALF and plasma, in accordance with manufacturer's instructions. ELISA kits were purchased for murine interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC) and TNF (R&D Systems, Abingdon, UK), and analysed using a colorimetric plate reader (MRX II absorbance reader, Dynex Technologies, Magellan Biosciences, Worthing, UK).

2.5 Protein assay

Protein concentration in lavage fluid was determined by the Bradford method [829] using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hemel Hempstead, UK) with bovine serum albumin (Sigma-Aldrich, Gillingham, UK) as a standard. Samples were diluted with distilled water so that predicted protein concentrations would fall within the limits of the standard curve. Standard preparations of bovine serum albumin (BSA) were prepared by serial dilution (range 0.0156 mg/ml – 1 mg/ml, linear range 0.05 mg/ml – 0.5 mg/ml). Both samples and standards were plated in triplicate. Bradford reagent was added to each well and gently mixed. Plates were analysed using a colorimetric plate reader.

2.6 Lung histology

In some experiments, upon termination the lungs and heart were removed en bloc from the thoracic cavity, and lungs were instilled via the endotracheal tube with approximately 1 ml of melted (42ºC) 1 % low-melting point agarose in 4 % paraformaldehyde until moderate distension was gradually achieved (i.e. margins of

lobes became sharp, Figure 2.4). Lungs then were immersed in plastic tube containing 4 ºC cold 4 % paraformaldehyde to facilitate hardening of agarose gel and hence to maintain opened alveolar structure of lungs. After overnight incubation, samples were embedded in paraffin, and 5µm thick sections were cut using a microtome. Sections were mounted on glass slides and dried overnight before staining with haematoxylin and eosin (H&E) for histological evaluation [830].

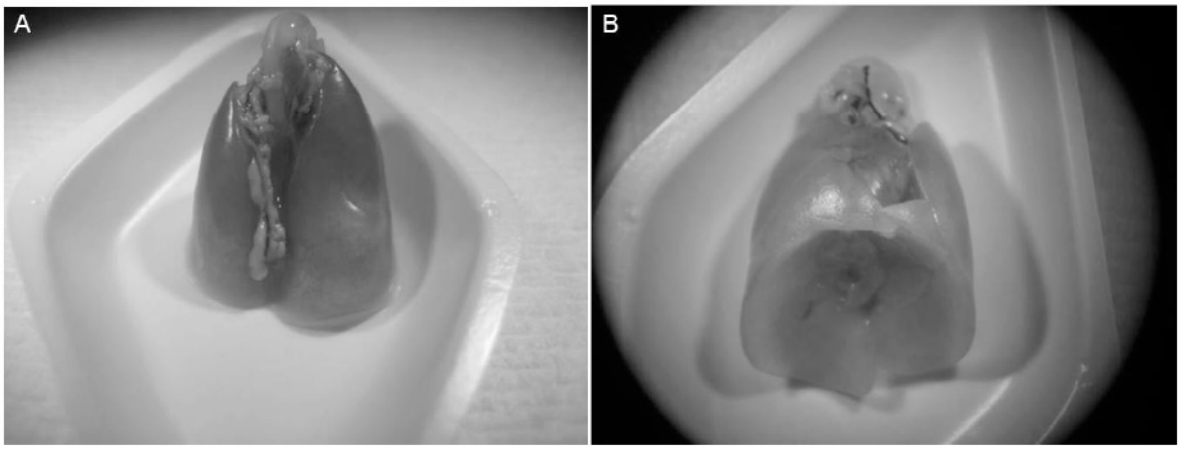


Figure 2.4 - Photographs of agarose instilled mouse lungs. For lung histology, lungs were excised from the thoracic cavity with heart en bloc and instilled with warm (42ºC) 1% low-melting point agarose in 4% paraformaldehyde via the endotracheal tube until gradual distension of lungs was achieved. Then lungs were immediately immersed into cold (4ºC) 4% paraformaldehyde to induce gelling of agarose in lung, thus fixing alveolar units in an open/inflated state (please note sharp edges of the lungs). Lungs were then kept in 4% paraformaldehyde at 4 ºC until processed for H&E staining.

2.7 Flow cytometry

Flow cytometry is a sensitive technique using immunofluorescence to measure expression of cell-associated antigens on a per cell basis. It is arguably the most quantitative way to assess this, because fluorescent signals are converted to numerical values. Cells are stained with antibodies specific for antigens of interest, conjugated to fluorescent dyes. A number of dyes can be used simultaneously, enabling co-staining for multiple antigens. Different cell types are identified based on their antigen expression and by their size and granularity. A disadvantage of flow cytometry is that samples have to consist of single cells, thus solid tissues have to be processed into single cell suspensions prior to scanning. Therefore, different tissue processing techniques may have to be employed depending on the antigen of interest.

We used flow cytometry to characterise TNFR expression profile and signalling on pulmonary endothelial cells under control and inflammatory conditions *both in vivo* and *in vitro*, and to identify and quantify inflammatory leukocytes in the lungs in different *in vivo* models of acute lung injury.

Preparation of lung single cell suspension

Lung single cell suspensions were prepared slightly differently depending on whether the number of leukocytes, the expression of adhesion molecules or TNF receptors was to be studied. For leukocyte counts and adhesion molecule expression, mechanical disruption and enzymatic digestion of excised lungs was carried out using an established technique, described and validated by our group previously [115]. Briefly, after harvest the lung was rinsed in saline and finely minced in a Petri dish by manual chopping using a surgical blade. The tissue was resuspended in

saline and incubated in 0.5mg/ml Collagenase (type IV, Sigma) at 37°C for 30 min. The resultant digest was then pushed through a 40-µm nylon mesh sieve (BD Falcon, Oxford, UK) with a syringe plunger and flushed through using FACS wash buffer (2% FCS, 1% sodium azide and 5mM EDTA), to produce the single cell suspension. Although it is possible that such a short warming period during the cell isolation procedure (i.e. collagenase digestion) could induce non-specific effects on PECs, we found in pilot studies that varying the incubation time between 15 to 45 minutes had no significant influence on adhesion molecule expression (Figure 2.5).

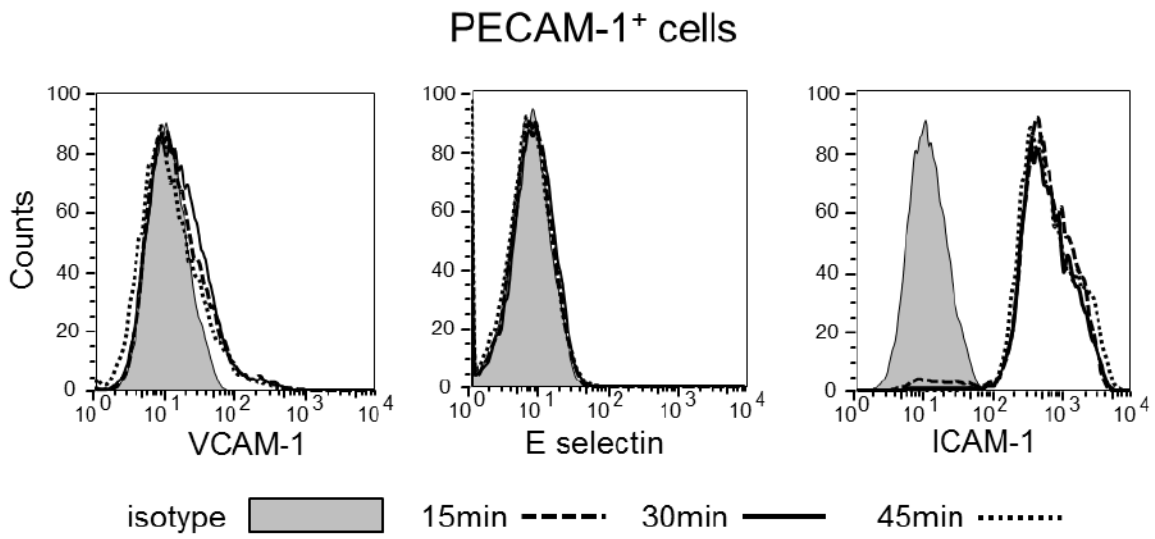


Figure 2.5 – Effect of collagenase digestion on adhesion molecule expression on PECs. Lung tissues were exposed to various length of collagenase digestion (15, 30 and 45min) at 37ºC then processed for flow cytometry to measure VCAM-1, E selectin and ICAM-1 expression on PECs (PECAM-1$^+$ cells, see below in 'Staining protocols'). Staining with respective antibody (*dashed, single* or *dotted line* as per digestion period) and isotype control (*grey fill*) are shown on histograms.

The tissue processing method was adapted slightly for assessment of TNF receptors. These molecules are highly sensitive to protease-induced shedding (much more so than adhesion molecules), and pilot experiments indicated that even a very short enzymatic digestion step had a major adverse effect on surface expression of the receptors (Figure 2.6). Therefore, to maximize cell yield whilst maintaining receptor expression, when mice were sacrificed, 1ml of ice-cold inhibitor cocktail was instilled through tracheostomy into the lungs. The cocktail consists of serine/cysteine protease inhibitor (1mM PMSF, Sigma-Aldrich Ltd., Poole, UK) and metalloprotease inhibitor (10μM BB94, British Biotech, Abingdon, UK) in PBS with 2% FCS, 1% sodium azide and 5mM EDTA. Lungs were then excised and chopped (without collagenase, in the inhibitor cocktail described above) using a gentleMACS tissue dissociator (Miltenyi Biotec Ltd., Bisley, UK), after which they were also filtered and flushed through a sieve. This 'non-collagenase' method resulted in better preservation of TNFR expression on PECs (Figure 2.6). In some technique validation experiments the liver was also processed the same way.

The lung single cell suspension was finally centrifuged at 1300rpm for 7 min, the supernatant discarded and the pellet resuspended in 900μl of flow cytometry washing buffer or inhibitor cocktail as appropriate. The samples were kept on ice throughout the procedure (except the short collagenase incubation period where utilized) until analyzed by flow cytometry.

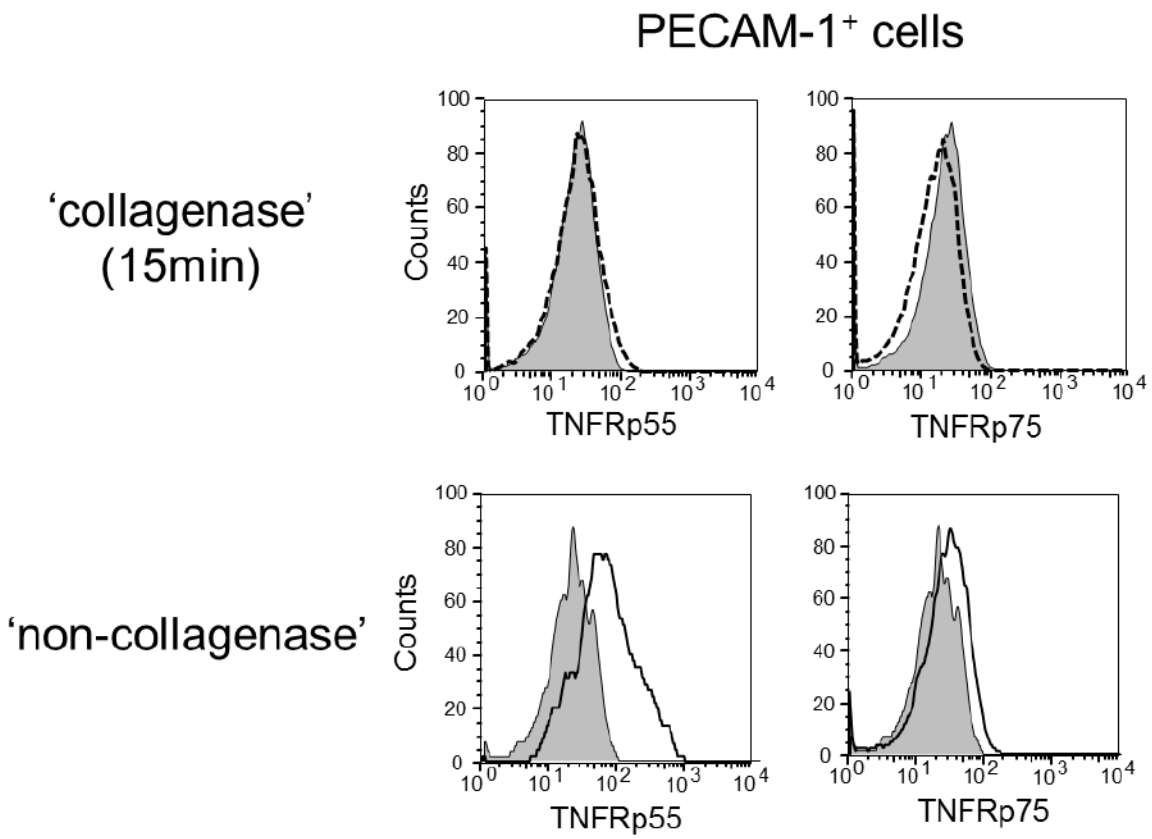


Figure 2.6 – Effect of different tissue processing methods on TNFR expression on PECs. Pilot studies indicated that even a short (15 minutes) exposure of lung tissue to collagenase results in shedding of TNFRs from PECs (PECAM-1$^+$ cells), whereas their expression is better preserved by the use of protease inhibitors and maintaining the working temperature close to 4^{o}C ('non-collagenase method'). Staining with respective antibody (*dashed* and *single line* for collagenase and non-collagenase methods, respectively) and isotype control (*grey fill*) are shown on histograms.

Staining protocols

Cells from BALF samples, *in vitro* culture and lung cell suspension were stained for flow cytometry as previously described [106, 114-116]. Cell-surface TNF receptor and adhesion molecule expression were quantified using fluorophore-conjugated antibodies for TNF receptor p55 (clone: 55R-286), p75 (clone: TR75-89), or their isotype controls (AbD Serotec, Kidlington, UK), and for adhesion molecule VCAM-1

(CD106, clone: 429), ICAM-1 (CD54, clone: 3E2) and E selectin (CD62E, clone: 10E9.6) or their isotype controls (antibodies all from BD Biosciences), respectively.

Different cell populations were identified based on size, granularity and expression of surface markers using the following antibodies: PECAM-1 (CD31, clone: MEC13.3), Endoglin (CD105, clone: MJ7/18), VE-cadherin (CD144, clone: BV13), ICAM-2 (CD102, clone: 3C4), Mucosialin (CD34, clone: RAM34) (BD Pharmingen, Oxford, UK), *Griffonia simplicifolia* lectin (Sigma), F4/80 (clone: CI:A3-1, AbD Serotec), CD11b (clone: M1/70), Gr-1 (clone: RB6-8C5), CD11c (clone: HL3) (BD Pharmingen), CD45 (clone: 30-F1, Biolegend, Uithoorn, The Nederlands).

Cells were stained in the dark at 4°C for 30min and then washed with flow cytometry wash buffer to remove unbound antibodies. In some experiments, cell numbers were determined using microsphere beads (Caltag Medsystems, Buckingham, UK) as previously described [114-116].

Samples were run on a FACSCalibur flow cytometer (BD) and analysed by FlowJo software (Tree Star, Ashland, OR, USA).

Identification of cell types

Pulmonary endothelial cells

Pulmonary endothelial cells were identified in lung cell suspensions using their characteristic expression of PECAM-1 (Figure 2.7), as previously described [115]. PECAM-1 positive cells were further characterised as endothelial cells by positive staining for Endoglin (CD105), VE-Cadherin (CD144) and ICAM-2 (CD102), confirming the endothelial origin of these cells. Additionally, cells were stained for mucosialin (CD34) and with lectin from *Griffonia simplicifolia*. Positive staining for both of these markers suggests that the vast majority of endothelial cells analysed

were microvascular in origin [831-833], although some contamination by macrovascular cells cannot be entirely excluded.

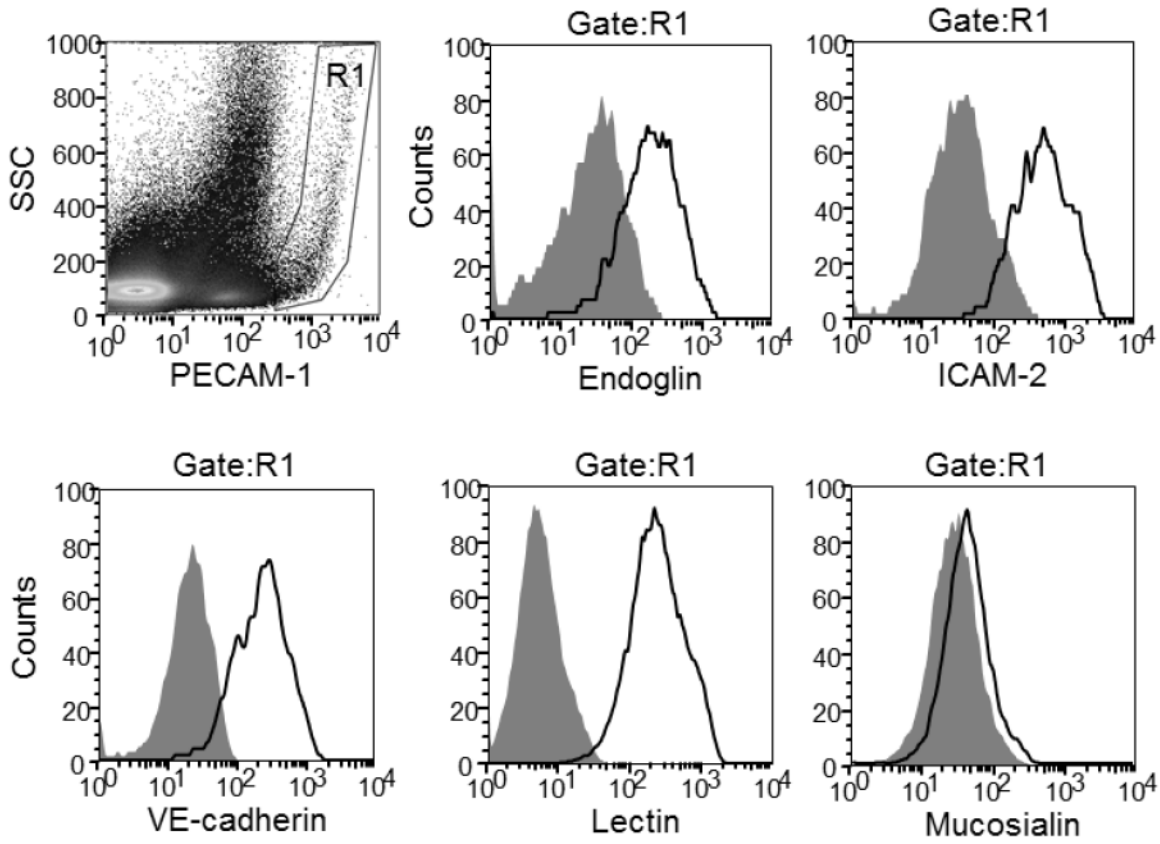


Figure 2.7 - Identification of pulmonary endothelial cells (PECs) in lung single cell suspension by flow cytometry. In characterization studies PECs were identified as PECAM-1+ events (R1) and their endothelial phenotype was confirmed by positive staining for endothelial cell markers, i.e. Endoglin (CD105), VE-Cadherin (CD144), ICAM-2 (CD102), *Griffonia simplicifolia* Lectin and Mucosialin (CD34). Staining with the respective antibody (*black line*) and isotype control (unstained in the case of lectin) (*grey fill*) is shown on histograms.

During the in vitro studies where cultured PECs were utilised, before each set of experiments, cells were also stained for a variety of these endothelial cell markers, to confirm both that they still retained their phenotype across passages, and that

measurement of adhesion molecule expression did not include the presence of any contaminating non-endothelial cells (Figure 2.8)

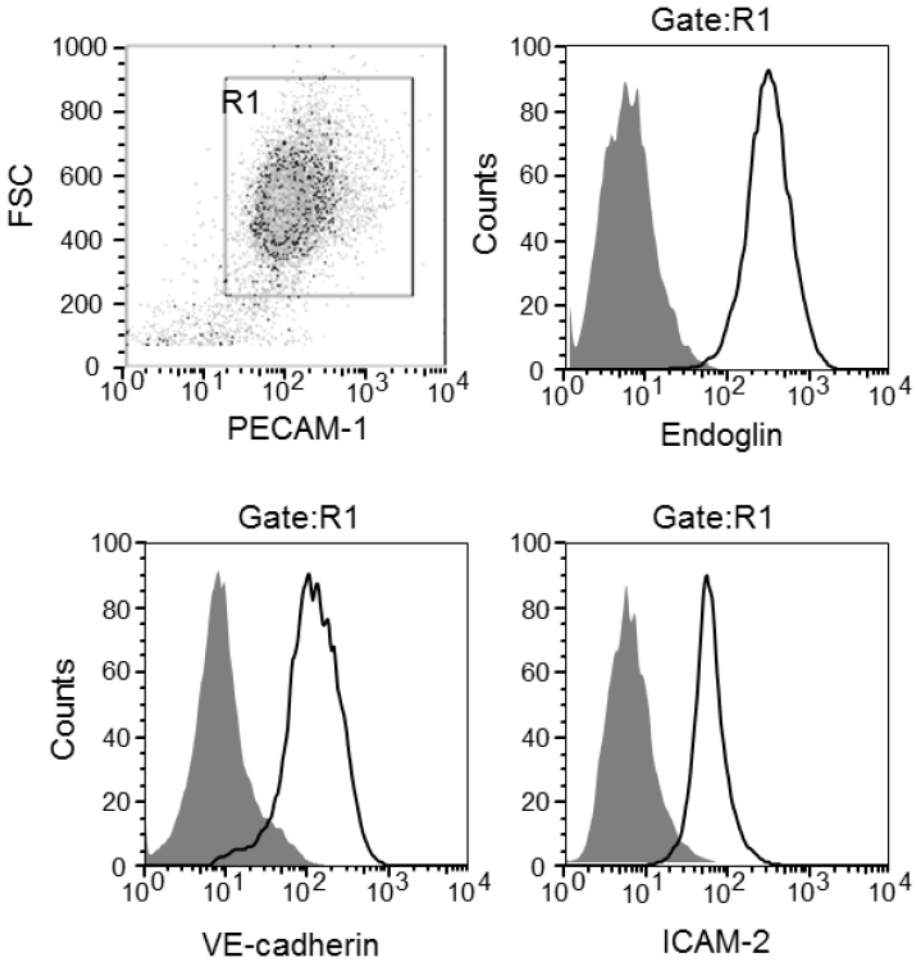


Figure 2.8 – Characterization of mouse primary pulmonary endothelial cells by flow cytometry. Before experiments (as well as at time of purification), PECAM-1$^+$ cells were characterized by staining for Endoglin, VE-Cadherin and ICAM-2 to ensure their endothelial phenotype was maintained from passage to passage. Staining with the respective antibody (*black line*) and isotype control (*grey fill*) is shown on histograms in passage #6 cells.

Lung leukocytes

Our group has previously demonstrated that neutrophils and inflammatory subset of monocytes (i.e. Gr-1^{high} monocytes) marginate from the bone-marrow to the pulmonary microvasculature in response to high-stretch ventilation and inflammatory stimuli such as LPS [106, 109, 114-116]. Leukocytes were identified in lung cell suspensions and BALF as previously described [114]. Lung-marginated and BALF neutrophils were identified by their high expression of CD11b and Gr-1 and absence of F4/80 expression (Figure 2.9/A-B). Lung-marginated monocytes were identified by their expression of CD11b and F4/80 (Figure 2.9/A). Monocyte Gr-1^{high} and ^{low} subpopulations were identified based on the relative expression of this antigen. Monocyte subpopulations were not present in BALF. Alveolar macrophages in BALF were identified as forward/side-scatter^{high}, F4/80^{+}, CD11c^{+} events (Figure 2.9/C).

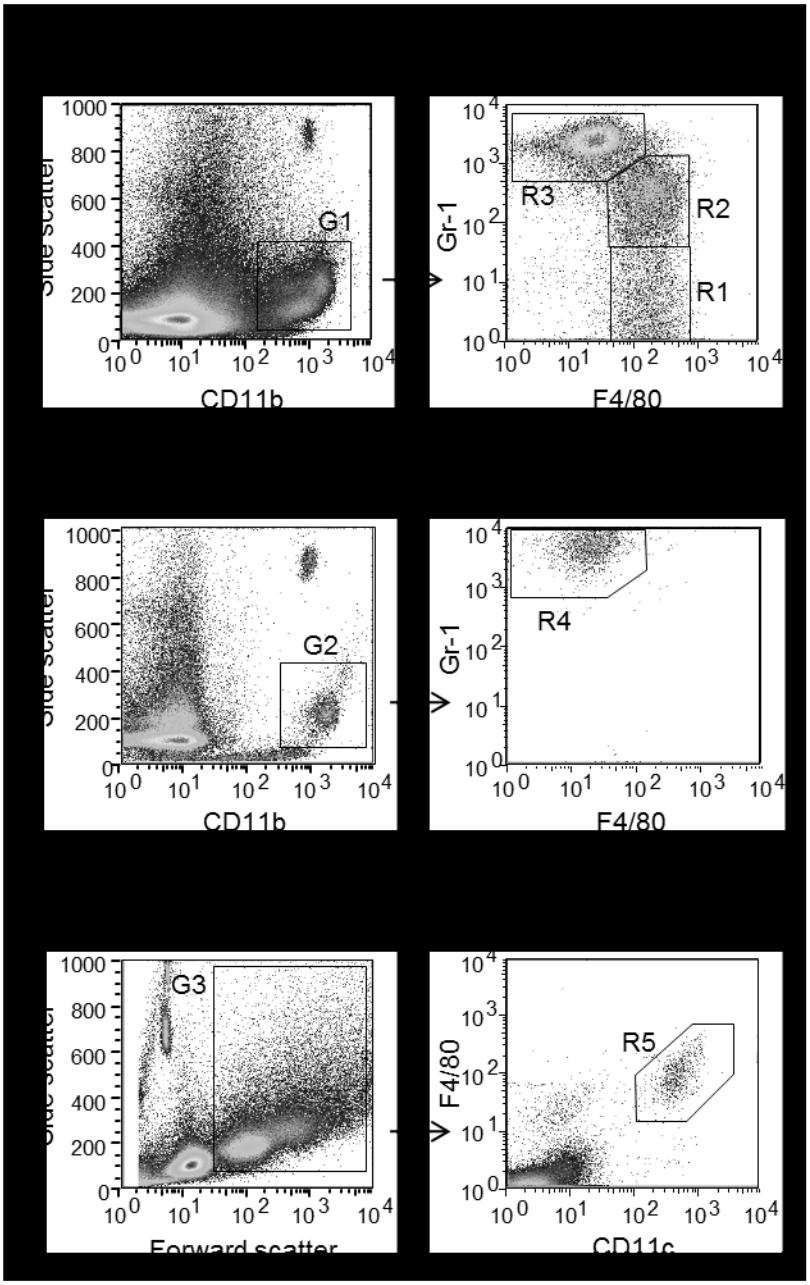


Figure 2.9 - Flow cytometric analysis of leukocyte subsets in the lungs and lavage fluid – representative sample from i.t. LPS + VILI model. (A) In lung single-cell suspensions monocytes were identified as $CD11b^{+}$, $F4/80^{+}$ events, and their subsets were defined as either $Gr\text{-}1^{low}$ (R1) or $Gr\text{-}1^{high}$ (R2), differentiated from $CD11b^{+}$, $F4/80^{-}$, $Gr\text{-}1^{very\ high}$ neutrophils (R3). (B) Neutrophils in lavage fluid were similarly identified as $CD11b^{+}$, $F4/80^{-}$, $Gr\text{-}1^{very\ high}$ events (R4), and their number varied model specifically (i.e. higher in i.t. LPS+VILI model than in 'pure' VILI), whereas monocytes were not present in BALF in any of the models investigated. (C) Alveolar macrophages in lavage fluid were recognised as forward/side-$scatter^{high}$, $F4/80^{+}$, $CD11c^{+}$ events (R5).

2.8 Data analysis

Data are expressed as means±SD. Normality was tested across the whole data set using Shapiro-Wilk test. Where data were not normally distributed, data transformation (followed by parametric tests, i.e. *t*-tests or ANOVA with Bonferroni tests where appropriate) or non-parametric analysis (i.e. Mann-Whitney U-test or Kruskall-Wallis with Dunn's post-test analysis) was carried out. A P value <0.05 was considered significant. All statistical analyses were performed using Prism software (version 5.0, GraphPad, La Jolla, USA) or SPSS (for normality assessment, IBM SPSS statistics 19.0, IBM Corporation, USA).

3. Characterisation of TNF receptor subtype expression and signalling on pulmonary endothelial cells

Both clinical and experimental evidence supports the pivotal role of TNF in the pathogenesis of ALI/ARDS. Cellular effects of TNF are mediated via two cell surface receptors, the p55 and p75. Using TNF receptor knock-out mice our group has demonstrated that signalling through the two receptors can have directly opposing roles during the progression of ALI/ARDS of different aetiologies [639, 814]. Specifically, signalling through the p55 receptor promoted pulmonary oedema, whereas p75 signalling opposed this, indicating that specific targeting of p55 signalling as opposed to total TNF blockade could be more beneficial in attenuating ALI.

However, due to the nature of using knock-out models, it remains unclear where these important signalling mechanisms take place, the endothelial or epithelial side of the alveolar-capillary membrane, thus where p55 signalling should be targeted. Therefore, over the past few years our group has been trying to further characterise the role of individual TNF receptors in the lung by assessing their expression levels on different cells and identifying receptor-specific cellular mechanisms using *in vivo* and *ex vivo* mouse models. As part of this on-going investigation, Dr Anthony D Dorr developed a flow cytometric technique during his PhD project that allows us to quantitatively assess the expression profile of TNF receptors on different cells in the mouse lung. This work, however, mainly focussed on the pulmonary epithelium and therefore the kinetics and roles of individual TNF receptors in the pulmonary microvasculature during ALI remains unknown.

Using this flow cytometric technique and developing it further we characterised the expression profile of TNF receptors on the surface of freshly harvested pulmonary endothelial cells (PECs) from mice, and found expression of both receptors with dominance of p55.

Then we treated wild-type and TNF receptor knockout mice with intravenous TNF and determined surface expression of adhesion molecules (E-selectin, VCAM-1, ICAM-1) on PECs by flow cytometry to characterise the specific role of each receptor. We found that TNF-induced upregulation of all adhesion molecules was substantially attenuated by absence of p55, whereas lack of p75 had a similar but smaller effect that varied between adhesion molecules. By pharmacological inhibition of individual TNF receptor signalling in wild-type primary PEC culture, we then confirmed that the in vivo findings were a result of direct TNF receptor inhibition on pulmonary endothelium and not on other cells (e.g. circulating leukocytes).

The role of TNF receptors has been shown to correlate with their surface expression in other models of TNF-mediated organ injury such as kidney and heart failure. Moreover, surface expression of TNF receptors may change dynamically in response to inflammatory stimuli. Therefore, we tested whether this also applies to the pulmonary endothelium in a scenario of generalised inflammation such as sepsis, a common cause of ALI/ARDS. We found that PEC surface expression of p55 dramatically decreased in the early stages of endotoxaemia following intravenous LPS, while no change in p75 expression was detected.

These data demonstrate a crucial in vivo role of p55 and an auxiliary role of p75 in TNF-mediated adhesion molecule up-regulation on PECs, however their overall roles may vary at different stages of pulmonary microvascular inflammation following changes in their relative expression.

3.1 Background

As discussed in Chapter 1, activation of the pulmonary endothelium is one of the key

features of the pathogenesis of ALI/ARDS, and involves the production of inflammatory cytokines/chemokines and upregulation of adhesion molecules on PECs. As a result, inflammatory leukocytes will marginate to the pulmonary microvasculature, become activated through cell-to-cell interactions with PECs and migrate into the lung interstitium and alveolar space, where they release their ROS and proteases to fight invading pathogens and to trigger further amplification of the inflammatory response [675]. There is mounting evidence that the pro-inflammatory cytokine TNF plays a key regulatory role in this process [109, 675, 686, 826].

Cellular effects of TNF are mediated via two cell surface receptors, TNFR p55 and p75 [649]. While early studies in the 1990s suggested that p55 is the major effector of TNF-mediated responses and p75 plays merely an auxiliary role by collecting and passing ligand to p55 (hence the name 'ligand-passing'), more recent evidence indicated that p75 can also mediate cellular responses partly or completely independently from p55 [643, 649, 737, 777]. For example, activation of p75 by receptor-specific muteins leads to activation of JNK (but not ERK or p38) MAPK in human rhabdomyosarcoma and cervical epithelial cell lines, and is sufficient to elicit cell death [737]. Also, receptor-specific activation of p75 in renal tubular epithelial cells results in activation of Etk/Bmx, leading to cell proliferation as opposed to apoptosis elicited by p55 via activation of apoptosis signal-regulating kinase 1 (ASK1) [777]. Additionally, it has been demonstrated that the two receptors can even act in a directly opposing manner in some acute inflammatory conditions including retinal ischaemia, sepsis, VILI and acid aspiration-induced lung injury [639, 729, 814, 817]. What is common in these models is that p55 seems to play a deleterious role, while p75 signalling is protective. Consequently, tissue specific expression of each individual TNF receptor may be a major determinant of an organ's inflammatory

response to TNF stimulation.

Indeed, Al-Lamki *et al.* demonstrated that TNFRs are differentially expressed on different cell-types of the myocardium and these expression patterns are changed in heart allograft rejection [834]. They found that in normal heart p55 but not p75 is predominantly expressed on cardiomyocytes, whereas cardiac allograft rejection was associated with a strong induction of p75 and diminished expression of p55 on these cells. The authors also demonstrated in wild-type mouse heart organ culture that TNF up-regulated p75 expression and activated both ASK1 and Etk/Bmx, causing both apoptosis and cell cycle entry. TNF treatment in heart tissue from p55 knock-out mice led to diminished apoptosis, activation of Etk and increased entry into cell cycle. In contrast, heart tissue from p75 knock-out mice showed increased ASK1 activation, apoptosis and lower level of cell cycle entry in response to TNF. The authors concluded that differential activation of ASK1 and Etk by regulated expression of TNFRs is an important determinant of the outcome of the heart allograft rejection process.

With regards to the roles of individual TNFRs in endothelial cells, the majority of previous studies have indicated that p55 receptor almost exclusively regulates TNF-induced inflammatory gene activation such as adhesion molecule expression [662, 724, 725, 750, 835], although such studies were primarily based on *in vitro* human umbilical vein endothelial cell (HUVEC) cultures. In direct contrast, Chandrasekharan *et al.* observed a critical role for p75 in regulating TNF-induced expression of adhesion molecules in aortic endothelial cells [749]. The authors found that when these cells were isolated from p75 knock-out mice, the TNF-induced up-regulation of E-selectin, VCAM-1 and ICAM-1 was completely abrogated compared to wild-type cells.

Such discrepancies underscore the crucial importance of endothelial cell heterogeneity across the different vascular beds [836]. Endothelial heterogeneity is present at structural, functional and gene expression level. For example, the endothelium of arteries and veins form a continuous uninterrupted layer of cells, held together by tight junctions, whereas the endothelial layer of capillaries might be fenestrated (e.g. in glomeruli, endocrine glands, choroid plexus or the intestinal mucosa) or discontinuous (e.g. in the liver sinusoids) according to the function of the underlying tissue [837].

Differential regulation of vascular permeability and haemostasis across vascular beds secondary to differential expression of junction proteins and coagulation factors, respectively, are great examples of functional heterogeneity. For instance, mice deficient for the tight junction protein claudin-5 have selective impairment in blood-brain barrier function [838], whereas systemic administration of anti-VE-cadherin antibodies to wild-type mice leads to increased vascular permeability preferentially in the lung and heart [44]. With regards to haemostasis regulation, while the anti-coagulant mediator endothelial protein C receptor (EPCR) is primarily expressed by large vessels [839], tissue factor pathway inhibitor (TFPI) is mainly produced in microvessels [840], whereas tissue-type plasminogen activator (t-PA) is predominant in pulmonary and cerebral arteries [841]. Moreover, in response to systemic inflammation, the expression of these factors changes in ways that differ across vascular beds, which may lead to differences in local thrombotic phenotype, such as that observed in sepsis [837]. These examples highlight the danger of extrapolating findings obtained in one vascular bed (e.g. HUVECs) to endothelial cells in other tissues.

The lung microvasculature in particular is known to be functionally and phenotypically distinct from most other vascular beds, for example in how leukocyte extravasation is regulated [86]. In the systemic circulation, binding of endothelial E- and P-selectin to their leukocyte ligands promotes rolling of leukocytes on the endothelium, whereas binding of VCAM-1 and ICAM-1 to leukocyte integrins mediates firm adhesion. Transmigration involves endothelial PECAM-1 (or CD31) and junctional adhesion molecule-1. Preferential expression of E-selectin, P-selectin, VCAM-1, and ICAM-1 in postcapillary venules means that leukocyte extravasation predominantly occurs at this site of the vascular tree [836, 842]. In direct contrast, in the pulmonary circulation leukocytes primarily extravasate in alveolar capillaries by a rolling- and E-selectin-independent but ICAM-1 dependent mechanism that involves trapping of poorly deformed activated leukocytes by the activated endothelium [86]. Haemostatic phenotype of the pulmonary microvasculature is also distinct from that of other tissues. For example, LPS- and TNF-induced pro-coagulant activity is higher in lung than in brain microvascular endothelial cells, reflected by differential expression of thrombomodulin, t-PA and PAI-1 [251]. In addition, pulmonary microvascular endothelial cells isolated from ARDS patients have a lower t-PA/PAI-1 ratio compared to controls, indicating a pro-coagulant phenotype [251].

While a small number of studies have attempted to investigate the specific role of pulmonary endothelial TNF receptors, they may be considered somewhat limited due to the methodologies used. Ermert *et al.* investigated the expression profile of TNFRs on rat and human pulmonary parenchymal cells using immunohistochemistry, and showed moderate to strong expression of p55 and a moderate levels of p75 receptor on endothelial cells [735]. However, they did not validate their staining technique in receptor knock-out animals; therefore the

possibility of non-specific staining for TNFRs cannot be ruled out with absolute certainty, which may have biased their results.

Neumann *et al.* investigated the role of p55 receptor in mediating TNF-induced up-regulation of adhesion molecules on endothelial cells in different organs, including the lungs, utilising p55 receptor knock-out mice and immunohistochemistry [695]. They demonstrated a crucial role for p55 in mediating TNF-induced E-selectin and VCAM-1 expression on pulmonary endothelial cells, but any conclusion regarding ICAM-1 was impossible to make using this technique, due to difficulties in detecting changes to its already high basal expression level. Moreover, the role of p75 receptor was not investigated.

The only study that quantitatively assessed the expression profile of individual TNFRs on pulmonary microvascular endothelial cells was that by Grau *et al.* [53]. The authors isolated and cultured pulmonary microvascular endothelial cells (PECs) from patients who died with ARDS and those who had lung cancer and underwent pulmonary lobectomy serving as controls, and used flow cytometry to measure surface TNFRs expression and adhesion molecules (E-selectin, VCAM-1 and ICAM-1) [53]. They found a constitutively up-regulated p75 and ICAM-1 on PECs from ARDS patients, and higher expression of IL-6 and IL-8 compared to controls, both under resting conditions and in response to TNF stimulus, but did not investigate the individual role of TNFRs [53]. More importantly, they assessed PECs after 2-3 weeks of culturing, which may result in changes in the phenotype of primary cells.

This is because endothelial cells actively sense and respond to the extracellular milieu, and differences in this will determine their phenotype and function [843]. Once they are removed from their native tissue, they lose these important extracellular cues and may undergo phenotypic changes [843]. Therefore

reconciliation of *in vitro* results with *in vivo* findings is essential to define the role of each TNF receptor during the activation of pulmonary endothelium. Thus, the relative expression of each TNF receptor within the pulmonary circulation and their precise roles in pulmonary microvascular inflammation *in vivo* still remain unclear.

3.2 Aims

In this study, we attempted to characterise the expression profile of TNF receptor subtypes on pulmonary endothelial cells and their specific roles in TNF-mediated cellular response. We used both *in vivo* and *in vitro* models utilising genetically modified animals as well as a pharmacological approach. There were two main aims:

1. Quantitatively assess the expression profile of TNF receptors on pulmonary endothelial cells both under control and inflammatory conditions.
2. Characterise the roles of individual TNF receptor subtypes in TNF-mediated activation of pulmonary endothelial cells.

3.3 Protocols

TNF receptor expression on PECs in vivo

Wild-type mice were sacrificed, and 1ml of ice-cold protease inhibitor cocktail was instilled through tracheostomy into the lungs to minimize TNF receptors shedding during lung tissue processing. Lungs were then excised to prepare a single cell suspension as described in Chapter 2, and TNF receptor expression on PECs was assessed by flow cytometry. In some animals the liver was processed and assessed by flow cytometry the same way for technique validation purposes.

In a separate series of experiments, mice were injected with 20µg LPS via the tail vein. After 2, 4, 8 or 24 h animals were sacrificed and the lungs processed for flow cytometry the same way.

Adhesion molecule expression on PECs in response to TNF in vivo

Wild-type or TNF-receptor knockout mice were injected with 500ng recombinant mouse TNF via tail vein. Two or four hours later animals were euthanised by isoflurane overdose, exsanguinated by cardiac puncture, the lungs were excised, and a single cell suspension prepared for flow cytometric analysis of adhesion molecules. Untreated animals served as controls.

Adhesion molecule expression on PECs in response to TNF in vitro

Primary pulmonary endothelial cells were isolated from wild-type mice and cultured under standard conditions. Cells were treated with TNF (10ng/ml for 4h) in the presence or absence of monoclonal neutralising antibodies for either the p55 or the p75 TNF receptor (chapter 2), and the expression of adhesion molecules VCAM-1, E selectin and ICAM-1 was quantitatively assessed by flow cytometry.

3.4 Declaration

The data contained within this chapter have been published in the *American Journal of Physiology – Lung Cellular and Molecular Biology* by Bertok *et al.* [1] and can be found in Appendix I. Where indicated, the figures in this chapter are taken from this manuscript. The investigation was a collaborative effort: Anthony D Dorr must be credited for developing the flow cytometry technique looking at surface TNF receptors on PECs, Justina O Dokpesi for the development of primary PEC culture,

Kieran O'Dea for his constant advice and guidance with flow cytometry, and Michael R Wilson and Masao Takata for designing and supervising the study.

3.5 Results

Basal expression of p55 and p75 receptors on PECs

Our first aim was to clarify the expression of individual TNF receptors on PECs *in vivo*. For this, we developed a quantitative flow cytometry technique which allows us to assess the expression of the two receptor subtypes in single cell suspensions freshly prepared from the excised lungs of mice (Chapter 2). PECs were identified based on positive staining for a wide range of endothelial markers, including *Griffonia simplicifolia* lectin and mucosialin (CD34) which are considered to be specific to endothelial cells of microvascular origin [831-833].

We found that under control conditions, both TNFRs were expressed on PECs, but expression of p55 was significantly higher compared to that of p75 (Figure 3.1/A). In order to rule out the possibility that the relative level of expression of the individual receptors was not due to differential susceptibility to shedding during tissue processing, we determined TNF receptor levels on endothelial cells harvested from the liver using the same methodologies. We found that these cells had similar p55 expression as pulmonary endothelial cells, but p75 expression was much higher, suggesting that the relative TNFR expression levels observed with PECs are unlikely to be an artefact of tissue processing (Figure 3.1/B).



Figure 3.1 - Flow cytometric analysis of TNF receptor expression on pulmonary and liver endothelial cells using the same tissue processing methodologies (i.e. ice-cold protease inhibitors and gentleMACS tissue dissociator). A. In control condition PECs showed greater expression of p55 (*white fill*) than p75 (*black fill*). Mean+SD, N=4 **B.** Liver endothelial cells expressed similar amount of the p55 (*white fill*) and p75 (*black fill*) receptors. Mean+SD, N=3, ***p<0.001 by paired t-test. Source – Bertok *et al.*[1]

We then assessed TNFR expression on PECs from genetically modified animals lacking TNF receptors, as a final validation of the flow cytometry technique (Figure 3.2). In p55KO mice we found no p55 signal, while p75KO animals showed effectively zero signal for p75 with a very small degree of non-specific staining with this antibody, indicating that the low level p75 expression on wild-type PECs was a genuine signal. Importantly, the histogram patterns of p55 or p75 staining in wild-type animals are consistent with normal distribution, suggesting that there were no subpopulations of TNFR positive and negative cells, but all PECs are expressing a moderate (p55) or low (p75) level of receptors.

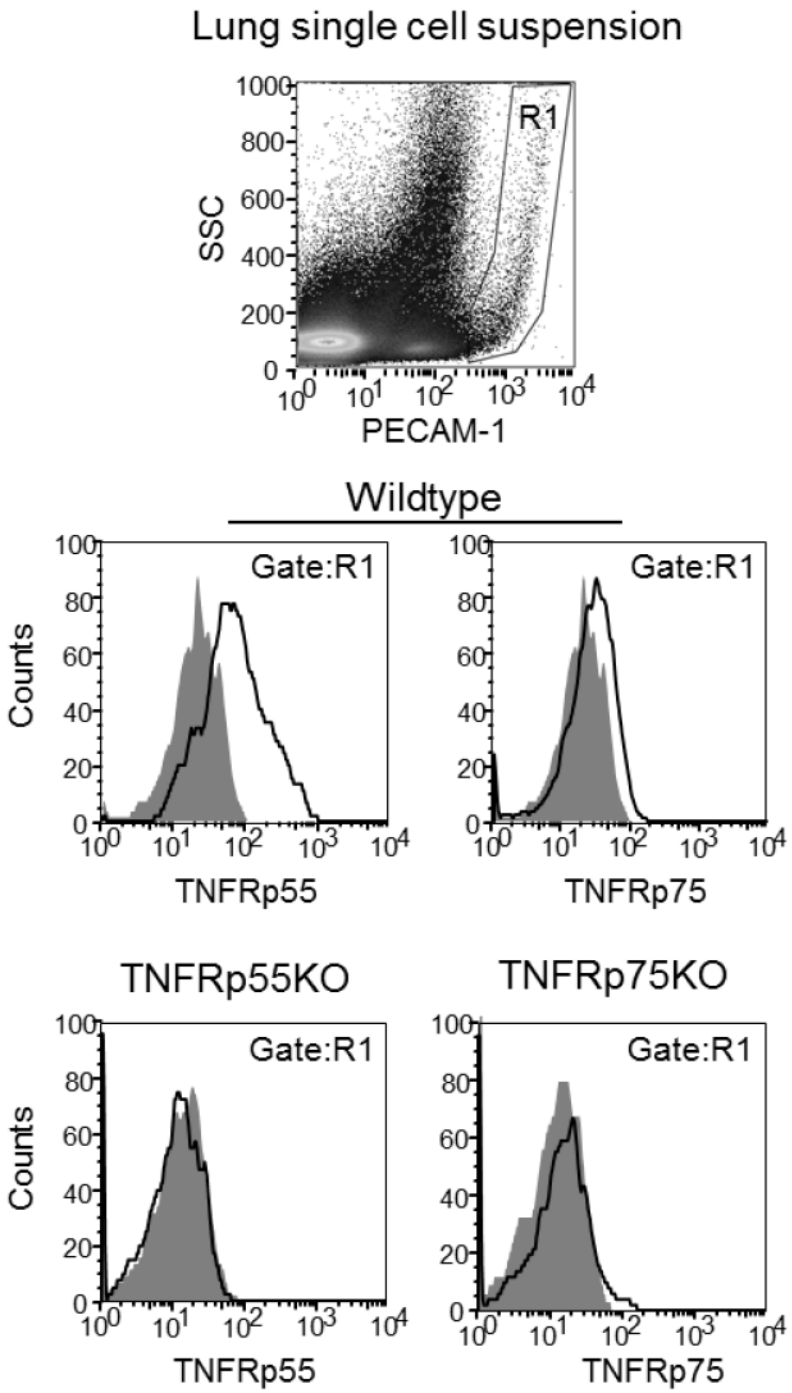


Figure 3.2 - Flow cytometric analysis of TNF receptor expression on pulmonary endothelial cells in wild-type and TNF receptor knock-out mice. Validation of flow cytometry technique using wild-type and p55 and p75 knockout animals (TNFRp55KO and TNFRp75KO, respectively) indicated small non-specific binding of the p75 antibody. Staining with the respective antibody (*black line*) and isotype control (*grey fill*) is shown on histograms. Source – Bertok *et al*. [1]

p55 and p75 TNF receptors both influence upregulation of adhesion molecules on PECs in vivo

Having characterised the surface TNFR expression profile of PECs, we then turned to investigate the individual roles of the receptors in pulmonary endothelial activation. For this, wild-type and TNF-receptor knockout mice were treated with i.v. TNF and the surface expression of leukocyte adhesion molecules VCAM-1, E-selectin and ICAM-1 was quantitatively assessed on PECs by flow cytometry as surrogate markers of cell activation. Although the cell harvesting process involved a short period of collagenase digestion, we confirmed that PECs had the same phenotypic characteristics – i.e. positive expression of ICAM-2, Endoglin and Mucosialin (Figure 3.3) when compared to the cells isolated during the TNFR expression studies.

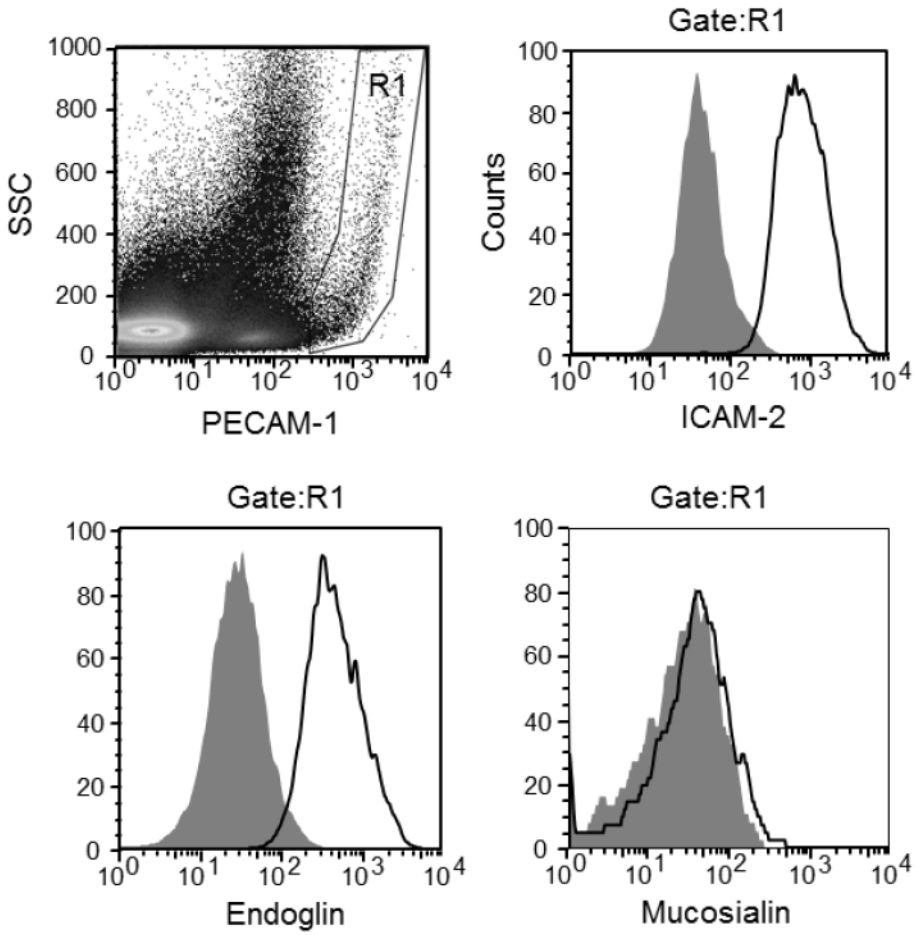


Figure 3.3 - Characterisation of pulmonary endothelial cells (PECs) in lung single cell suspension by flow cytometry using the collagenase method. PECs had the same phenotypic characteristics – i.e. positive expression of ICAM-2, Endoglin and Mucosialin as in the TNFR expression studies (see Chapter 2). Staining with the respective antibody (*black line*) and isotype control (*grey fill*) is shown on histograms.

In preliminary time course experiments using wild-type mice, we found that E-selectin had a peak expression on PECs at 2 h after TNF treatment, while VCAM-1 and ICAM-1 expression was greater at 4 h (Figure 3.4). Therefore, in subsequent experiments we compared E-selectin expression between strains at 2 h, and VCAM-1 and ICAM-1 expression at 4 h.

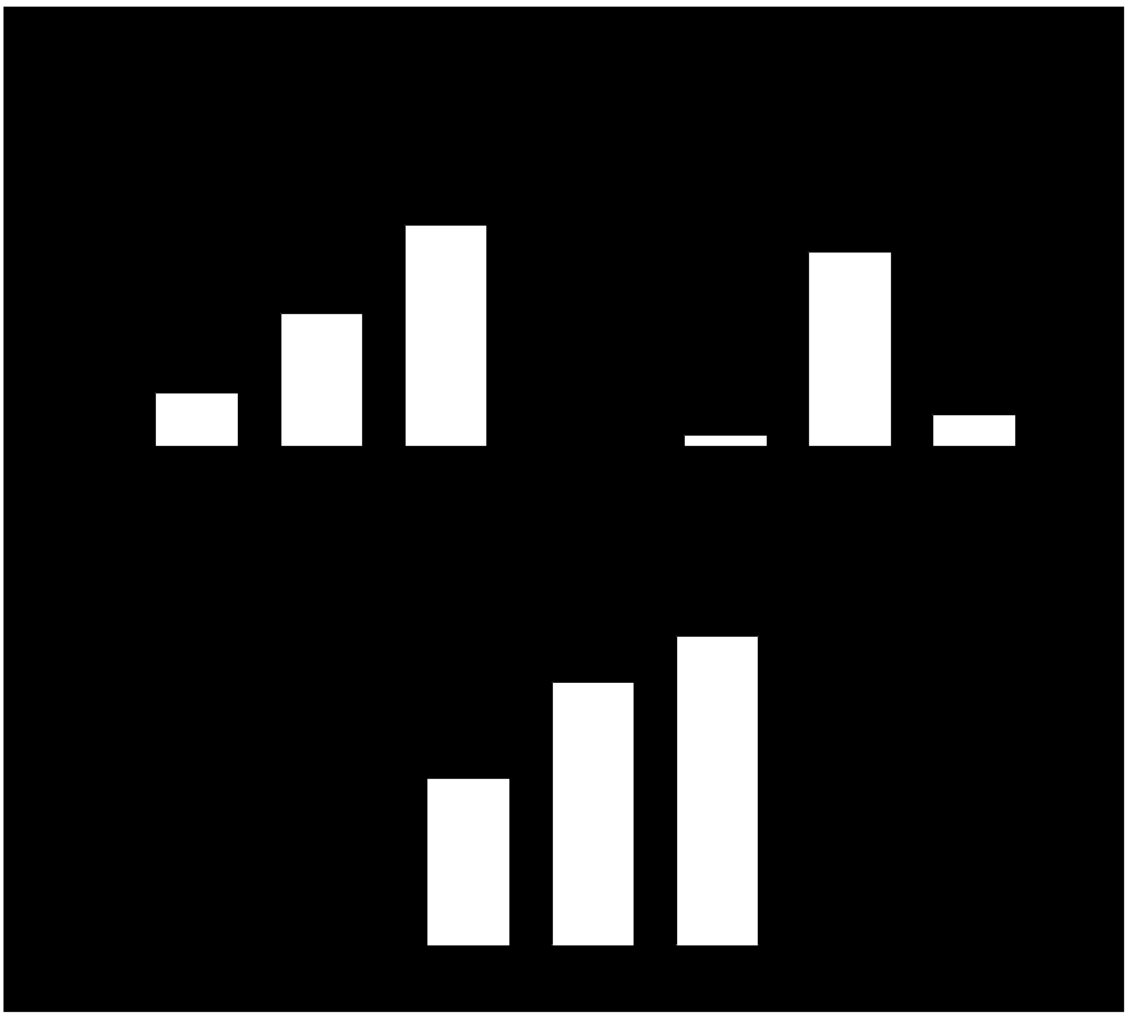


Figure 3.4 – Time course of adhesion molecule expression on PECs in wild-type mice following intravenous TNF. Pilot studies in WT mice indicated a peak expression for E selectin at 2 hours post-TNF i.v. (500ng), whereas it was 4 hours for VCAM- and ICAM-1. Therefore, in subsequent experiments these peak time points were used. Ideally time-matched controls should have been used for these experiments, however this would have significantly increased the number of animals required which was difficult to justify. Mean+SD of Mean Fluorescence Intensity (MFI), N=3-6/group.

PECs from all mouse strains had a moderate basal expression of VCAM-1, which was somewhat variable across the strains but in none of them was significantly different compared to wild-types (Figure 3.5/A). TNF treatment substantially up-regulated surface expression of VCAM-1 in wild-type mice, which was reduced in p75KO animals compared to wild-type mice, and decreased to an even greater extent in p55KO and DKO mice. In contrast to VCAM-1, E-selectin was not expressed under resting conditions (Figure 3.5/B), but it was induced in wild-type mice by i.v. TNF treatment. This TNF-induced response tended to be reduced (albeit not significantly) in p75KO mice compared to wild-type animals, whereas both strains of mice lacking the p55 receptor (i.e. p55KO and DKO) showed effectively no E-selectin expression. In contrast to VCAM-1 and E-selectin, ICAM-1 is expressed on PECs at a high basal level in all strains (Figure 3.5/C), and was further up-regulated upon TNF treatment in wild-type mice. While p75KO mice showed some tendency to decreased ICAM-1 expression, this was not significant. In contrast, animals lacking the p55 receptor (i.e. p55KO and DKO mice) had significantly lower ICAM-1 expression after TNF stimulation compared to wild-types.

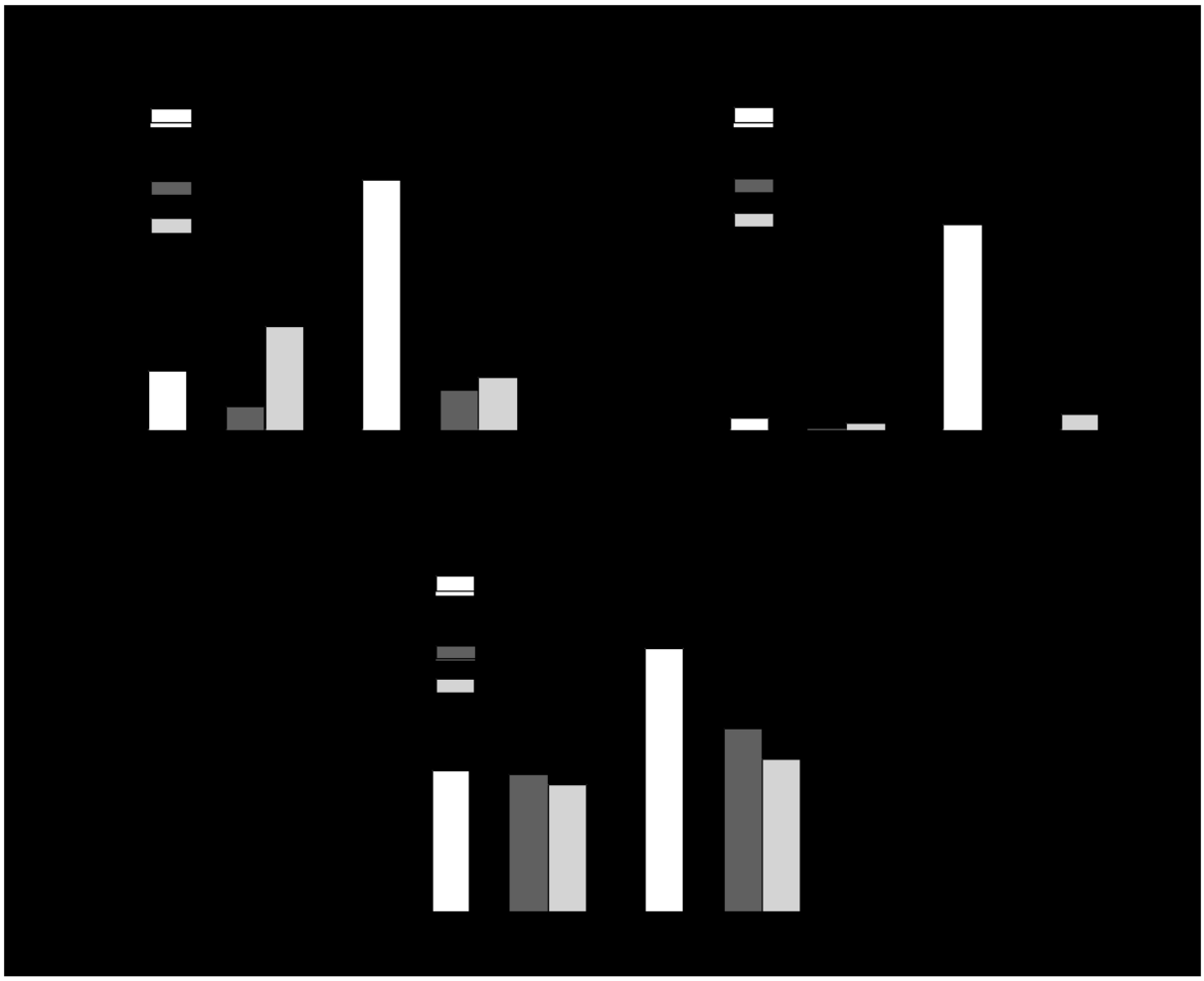


Figure 3.5 – Effects of chronic absence of TNF receptor subtype signalling on TNF-induced expression of adhesion molecules on pulmonary endothelial cells in vivo. Surface expression of VCAM-1 (**A**), E-selectin (**B**) and ICAM-1 (**C**) assessed by flow cytometry on pulmonary endothelial cells in different mouse strains either 2 (E selectin - **B**) or 4 h (VCAM-1, ICAM-1- **A** and **C**) after i.v. TNF injection (500ng). WT= wild-type, p75KO= TNFRp75 knockout, p55KO= TNFRp55 knockout, DKO= TNFRp55/p75 double knockout. Mean+SD of Mean Fluorescence Intensity (MFI), N=3-6/group, **p<0.01, ***p<0.001 vs TNF-treated WT by one-way ANOVA with Bonferroni post-tests (**A** and **C**) and by Kruskall-Wallis test with Dunn's post-hoc analysis (**B**) . Source – Bertok *et al.*[1]

Differences in adhesion molecule expression are due to TNF receptor signalling specifically on pulmonary endothelial cells

Because of the nature of using knock-out animals it was unclear, whether our *in vivo* findings reflected the importance of TNF receptors on endothelial cells *per se*, or the lack of TNFR signalling on other cells may have also influenced our observations. For example, leukocytes express both TNFRs [726], and Gr-1^{high} monocytes in particular are known to activate PECs in a TNF-dependent manner [115]. To dissect this out, we took an *in vitro* approach and used acute pharmacological TNFR blockade in primary PEC culture isolated from wild-type mice. Cells were treated with TNF in the presence or absence of antagonistic antibody for p55 or p75 receptors, and surface expression of adhesion molecules was quantified by flow cytometry as *in vivo*. The antagonistic TNFR antibodies used for this study were monoclonal antibodies (mAb) as opposed to domain antibodies (dAb) because anti-p75 dAb was not available at the time, and comparing the effect of anti-p55 dAb to that of anti-p75 mAb in our assay may have led to scientific biases. Although these monoclonal antibodies may not be as potent as their domain antibody counterparts, they were both shown to inhibit TNF-induced cytotoxicity *in vitro* to variable extent (>95% and up to 60% for anti-p55 and anti-p75 mAb, respectively) in a concentration dependent manner, as well as TNF-induced skin necrosis *in vivo*, whereas anti-p75 effectively inhibited TNF-mediated proliferation of T-cell-like CT6 cells [844]. Therefore, to achieve a good efficacy in blocking TNF activity in our model, we applied these antibodies in vast excess to TNF.

We found that p75 blockade, consistent with the *in vivo* findings, resulted in significant reduction of TNF-induced VCAM-1 and E-selectin expression by 28% and 23%, respectively ($p<0.05$), but did not influence ICAM-1 expression (Figure 3.5/A).

In contrast, p55 blockade significantly decreased the expression of all adhesion molecules ($p<0.01$) (Figure 3.5/B) in keeping with the *in vivo* results, although did not completely abolished the TNF-response.

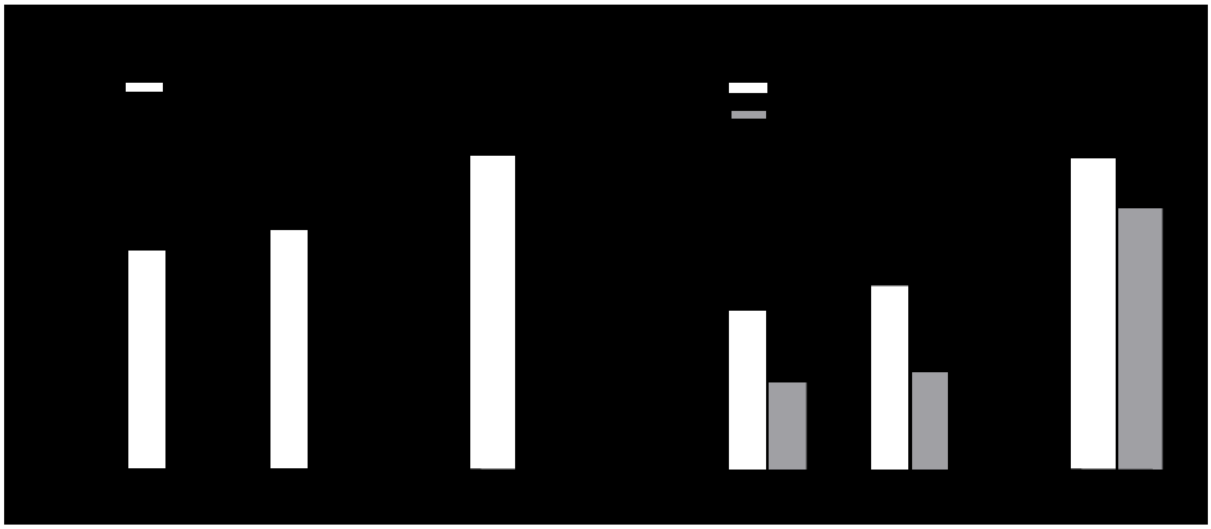


Figure 3.5 – Effects of acute TNF receptor inhibition on TNF-induced expression of adhesion molecules in primary pulmonary endothelial cell culture. Fold changes (compared to untreated cells in the same experiment) of adhesion molecule expressions on wild-type primary mouse lung endothelial cells following 4 h TNF treatment (10ng/ml) in the presence or absence of (**A**) p75 (20µg/ml) or (**B**) p55 (90µg/ml) receptor neutralising mAb. p55 and p75 blockade experiments were conducted separately. Mean+SD. N=3-4, * $p<0.05$, ** $p<0.01$, ***$p=0.001$ vs TNF by paired t-test. Source – Bertok *et al.* [1]

p55 receptor expression decreases, while p75 remains stable during acute endotoxaemia

Finally, we determined the influence of acute inflammation on the relative levels of TNFR expression. Historically, p75 was shown to be more readily up-regulated than p55 on monocytes and synovial fluid macrophages in response to LPS and IL-10 [53, 714], while both receptors also show a propensity to be shed from the surface of

neutrophils and monocytes in response to TNF and LPS [718, 721, 726], but such data for PECs have been lacking. This could be quite important, as dynamic changes in the relative level of TNFR expression could potentially influence the overall cellular response to TNF.

We found that p75 expression on PECs did not change substantially over 24 hours following i.v. LPS administration. In marked contrast, there was a dramatic decrease in p55 expression at 2 hours after LPS, which started to recover after 4 hours and reached nearly 80% of its baseline levels by 24 hours post-LPS (Figure 3.6).



Figure 3.6 – Changes in TNF receptor expression on pulmonary endothelial cells during endotoxemia *in vivo*. Mice were injected with i.v. LPS (20μg), and after 2, 4, 8, and 24 h the lungs were processed for flow cytometric analysis. Untreated animals served as controls. Ideally time-matched controls should have been used for these experiments, however this would have significantly increased the number of animals required which was difficult to justify. Surface expression of TNF receptor p55 and p75 was quantified on PECAM-1^+ events as described before. Mean±SD of MFI, N=4-5/each time point, ***$p<0.001$ vs control for p55 expression by one-way ANOVA with Bonferroni post-tests. Source – Bertok *et al.* [1]

3.5 Discussion

Although TNF plays an important role in the pathogenesis of sepsis/ALI and elevated levels in plasma and/or BALF are associated with poorer outcome [332, 678, 680, 795, 845], blockade of total TNF signalling in septic patients using anti-TNF monoclonal antibodies [616, 617, 620, 846] or TNFRp55-IgG fusion protein [623-625] proved to be clinically ineffective, suggesting that TNF signalling during sepsis/ALI could be more complex than originally considered.

Indeed, recent evidence indicates that the two TNFRs can play differential

and even opposing roles in a variety of acute inflammatory conditions, including sepsis [729, 817, 818]. For example, in a mouse model of sepsis induced by caecal ligation and puncture, Ebach *et al.* demonstrated that while p55 knock-out mice had prolonged survival compared to wild-type controls hence indicating a deleterious role for p55, p75 knock-out animals had increased mortality [817]. In contrast, the same group showed a protective role for p55 receptor and a deleterious role for p75 in a mouse model of T-cell mediated colitis [818], suggesting that the roles of individual TNFRs are model and/or disease specific.

Moreover, our group previously demonstrated that such opposing TNFR signalling also exists in ALI induced by high-stretch ventilation [639] and acid aspiration [814]. Using TNFR knock-out mice, we found in both models that signalling through p55 receptor promoted the development of pulmonary oedema and respiratory failure, whereas signalling through TNFR p75 opposed this, suggesting that selective targeting of p55 receptor signalling as opposed to total TNF blockade might be more effective in attenuating lung injury. Furthermore, these studies also indicate that the relative expression of p55 and p75 within the lung is critically important in determining the consequences of TNF signalling during the pathogenesis of ALI/ARDS. Whether such opposing TNFR signalling is involved in mediating pulmonary endothelium activation, an important step in the pathogenesis of ARDS, and the exact roles of TNFR subtypes in this process is unclear.

To address this, in this chapter we quantitatively assessed the expression profile of TNF receptors on the surface of freshly harvested mouse PECs, and explored their roles in mediating TNF-induced expression of adhesion molecules as surrogate markers of endothelial activation using flow cytometry. In control conditions, both receptors were expressed on PECs, with substantially higher

expression of p55 than p75. Both TNFRs played some role in mediating TNF-induced expression of adhesion molecules, but p55 receptor had a much greater impact than the p75 receptor *in vivo*. Finally, we demonstrated that in acute endotoxaemia p55 expression on PECs was rapidly down-regulated, but no change in p75 expression was observed, which may have important consequences for TNF signalling during pulmonary microvascular inflammation.

Expression of TNFR subtypes on PECs

Most of our current knowledge of TNF receptor expression on endothelial cells comes from studies using human umbilical vein endothelial cells (HUVEC). While TNF binding studies suggested marginally higher p55 expression on these cells [662, 847, 848], flow cytometric analysis showed similar expression of the two receptors or a slightly higher expression of p75 [724, 725]. TNFR expression profile on pulmonary endothelial cells is not clear. Previous studies suggested relatively similar expression of p55 compared to p75 on PECs of rats and humans, although these findings rely on data from prolonged in vitro cultures [53] which may significantly influence endothelial cell phenotype and function [843], and semi-quantitative immunohistochemistry technique [735]. Therefore quantitative in vivo analysis of TNF receptors on PECs has been lacking.

To address this, we established a flow cytometry method that enabled quantitative evaluation of TNFR expression on PECs in a single cell suspension. Positive cell staining for a panel of endothelial cell markers, including mucosialin and *Griffonia simplicifolia* lectin suggest that the PECs analysed were primarily microvascular in origin. This distinction is important, because phenotypic and functional heterogeneity has been described among pulmonary endothelial cells of

microvascular and macrovascular origin [685, 849, 850]. We found that TNFRs were labile and sensitive to conditions of tissue processing; however by using a mixture of protease inhibitors and keeping the working temperature close to 4°C, we managed to preserve their cell surface expression on PECs.

We found that both TNFRs were expressed on PECs, but expression of p55 was substantially higher than that of p75. Using receptor knockout, we demonstrated that the antibody-binding was specific to each receptor, with only a small non-specific signal for p75. We also measured the relative expression of TNF receptors on liver endothelial cells using the same tissue harvesting and processing technique, and found that while p55 expression was comparable to that found in PECs, p75 expression was greater and even exceeded the levels of p55. These data strongly support that the low signal of p75 on PECs is not due to processing artefacts, and more importantly, highlight the potential risks of extrapolating between endothelial cells from different sources.

Roles of TNFR subtype signalling in TNF-induced activation of PECs

A previous in vivo study by Neumann *et al.* using knockout mice suggested a crucial role of p55 in TNF-induced expression of E-selectin and VCAM-1 (measured by immunohistochemistry) and recruitment of leukocytes within the lung [695], however this study did not investigate the involvement of p75. To characterise the roles of the receptor subtypes, we injected TNF intravenously to wild-type and TNFR single and double knockout mice, and quantitatively evaluated E-selectin, VCAM-1 and ICAM-1 expression on PECs using flow cytometry. We found that p55 receptor signalling was necessary for the up-regulation of each adhesion molecule (in keeping with the study by Neumann) while p75 signalling contributed to VCAM-1 up-regulation, but any

effects on E-selectin and ICAM-1 expression were unclear. To rule out that our observations from genetically modified mice were not influenced by the lack of TNFR signalling on other cells, such as circulating leukocytes, we used *in vitro* receptor blockade experiments. We found that the consequences of individual TNF receptor signalling observed *in vivo* were very likely due to direct effects on PECs, and confirmed that p75 signalling influenced VCAM-1 and E-selectin expression. The influence of p75 signalling on ICAM-1 was however less clear, which may indicate some differential effect of p75 on the different adhesion molecules, or may reflect a relative lack of sensitivity due to already high basal expression of ICAM-1.

Our findings indicating a minor role for p75 in TNF-mediated adhesion molecule expression may be consistent with the ligand-passing theory of TNF signalling on PECs, whereby p75 functions primarily to modulate signalling through p55. Alternatively, it could be a reflection of the relative level of receptor expression (i.e. p55>p75) on PECs, and p75 may be more important in other organ beds, such as the liver, or conditions where the relative expression of the receptors are changed. Our finding that the p75 receptor does not contribute much to ICAM-1 up-regulation is in contrast with a previous study by Chandrasekharan *et al.* [749]. Isolating aortic endothelial cells from TNF receptor knockout animals the authors reported that p75 receptor signalling was the main determinant of TNF-induced ICAM-1 up-regulation, although they did not assess the relative levels of TNF receptor expression on these cells, and therefore it is unclear whether their findings may be due to a significantly higher expression of p75. This suggests that the role of p75 in signalling varies between vascular beds, which would argue against a generalised ligand-passing concept for this receptor.

Differential regulation of TNFR expression on PECs in inflammatory conditions

Expression of the TNF receptors is regulated by protein synthesis both at the transcriptional/translational level, by the action of sheddases such as TNF-alpha converting enzyme (TACE) [649, 758, 851] and by internalization mechanisms [727]. Both p55 and p75 may be shed or internalised from the surface of cells, and under certain circumstances both can be upregulated, p75 more dynamically than p55 [658, 712, 718, 721, 722, 727]. However, the vast bulk of this knowledge comes from hematopoietic cells and cancer cell lines, which may not be applicable to endothelial cells. To assess the changes in TNF receptor expression on PECs following an inflammatory stimulus, we treated mice with LPS and determined the expression of the two receptors over 24 h. For the first time in freshly harvested PECs, we showed that surface p75 expression was effectively unchanged while expression of the p55 receptor decreased greatly following LPS. Such a loss of surface p55 is likely to represent a regulatory mechanism to limit ongoing stimulation of the receptors following the initial ligand-receptor interaction. Indeed, TNF-induced TNF receptor shedding leading to deactivation of cells has been observed and investigated extensively in leukocytes [365, 721, 819], and impaired shedding of p55 leads to an exaggerated inflammatory state in humans and mice [850, 852]. Although mice lacking the p55 receptor show attenuation in pulmonary neutrophil accumulation following systemic LPS challenge [812], suggesting that TNF likely signals through the p55 receptor before shedding occurs, the subsequent downregulation of p55 receptors on endothelial cells may have significant long-term consequences (e.g. endothelial hyporesponsiveness to further TNF stimuli) beyond the effect on adhesion molecules we observed here.

Our findings regarding the changes in TNF receptor expression during inflammation are apparently inconsistent with those reported by Grau *et al.*

investigating the functional characteristics of PECs isolated from patients who died with ARDS [53]. They demonstrated greater p75 expression on PECs from the patients than controls, which appeared to positively correlate with the duration of the disease, while no difference in p55 was observed. However their study investigated TNF receptor expression on PECs in vitro after 2-3 weeks culture, which may not reflect the phenotype at the time of isolation. Both our study and that by Grau *et al*. do however indicate a change in the ratio of p55:p75 receptors on PECs during ALI/inflammation. The downstream consequences of such changes in the p55:p75 expression ratio remain unclear. As the current data indicate that p75 plays a similar role to p55 signalling in regards to adhesion molecule upregulation (albeit of lesser magnitude), it may be concluded that a relative shift in the contribution of signalling through p75 vs. p55 would have little impact. However, adhesion molecule upregulation is but one consequence of TNF receptor activation, and it is now clear that the two TNF receptors are able to signal independently. For example, TNF has been shown in neurons to mediate pro-apoptotic signalling through p55 but anti-apoptotic signalling through p75 [853]. Changes in the relative levels of TNFR expression may therefore have substantial consequences not apparent from the current observations.

In mouse models of VILI and acid aspiration-induced lung injury, our lab previously found that p75 signalling opposed, rather than complemented that of p55 during the development of high stretch-induced pulmonary oedema [639, 814]. One possible interpretation is that the 'protective' role of p75 signalling observed in our previous study is not related to endothelial cell adhesion molecule up-regulation (as we found p75 signalling plays a similar 'injurious' role to that of p55, albeit less potent), but a differential regulation of endothelial/epithelial cell permeability [689,

690, 694, 854] and fluid reabsorption [707-709].

In summary, the present study provides invaluable new data of individual TNF receptor expression and signalling on pulmonary endothelium in vivo, by quantitative assessment of TNF receptors and TNF-induced expression of adhesion molecules on freshly harvested PECs using flow cytometry. The results highlight the potential risks in extrapolating data obtained in cell lines/endothelial cells from other vascular beds or using semi-quantitative methods. Our findings add crucial pieces of information to our knowledge base of endothelial cell biology within the lung, which would lead to novel insights into TNF-mediated pathophysiology within the pulmonary microvasculature. Further work will need to be dedicated to elucidate the functional consequences of individual TNF receptor signalling during pulmonary inflammation.

4. Selective targeting of intraalveolar p55 TNFR signalling during ventilator-induced lung injury

Having characterised the expression profiles and roles of TNFRs on pulmonary endothelium, we then turned to investigate if specific targeting of TNFR subtype signalling could confer benefits in attenuating ALI of different aetiologies.

Studies in genetically modified mice show that the two TNF receptors play opposing roles during injurious high-stretch mechanical ventilation, with p55 promoting but p75 preventing pulmonary oedema [639], but it remains unknown by what mechanisms and in which pulmonary cells.

Evidence in the literature indicates that function of the TNF receptor subtypes correlates, at least in part, with their level of surface expression on cells [777, 834], which can change dynamically in certain conditions, especially during inflammation [658, 712, 718, 721, 722, 727]. Indeed, in Chapter 3 we have demonstrated that expression of p55 receptor on pulmonary endothelial cells rapidly decreases during systemic inflammation, whereas expression of p75 receptor remains unchanged. These findings suggest that targeting p55 receptor signalling during ALI from the intravascular space may not be beneficial. On the other hand there is evidence that surface expression of p55 in the alveolar space is well preserved during pulmonary inflammation [819]. Moreover, TNF is up-regulated within the alveolar space early in the course of ventilator-induced lung injury [337]. Therefore pharmacological blockade of intraalveolar p55 TNFR signalling could be a reasonable approach to attenuate VILI.

In this chapter, we investigated the effects of an intratracheally delivered, p55 TNFR targeting domain antibody in *in vivo* mouse models of VILI. We found that selective blockade of intra-alveolar p55 signalling reduced alveolar-epithelial barrier permeability and pulmonary inflammation, which suggests potential for new treatments of VILI and ALI.

4.1 Background

Mechanical ventilation is essential for the treatment of patients with acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), although this can often result in ventilator-induced lung injury (VILI) [329]. Although the initiating trigger is unique (i.e. mechanical stretch), the final pathophysiology of VILI is indistinguishable from that of ALI of other aetiologies, i.e. characterised by pulmonary oedema and inflammation [313, 330]. As the start of mechanical ventilation and thus VILI is predictable and physician-controlled, VILI may be particularly amenable to early pharmacologic intervention.

The pro-inflammatory cytokine tumour necrosis factor-((TNF) has been consistently implicated in the pathogenesis of ALI/VILI both clinically and in experimental models [322, 330, 334, 678]. Clinical trials of anti-TNF therapy in sepsis/ALI patients [616, 617, 624, 625] were unsuccessful, and in some cases may have led to increased morbidity. The reasons behind such a lack of benefit are unclear, but are likely to include issues relating to the site where TNF signalling was targeted (i.e. systemic vs. alveolar) and the complicated biology of TNF signalling.

TNF signals through two cell surface receptors, p55 and p75, and recent evidence suggests that signalling through these individual receptors may have differential, even opposing roles in a number of pathological conditions, including retinal ischaemia, heart failure and myocardial infarction [729, 834, 855]. In these models p55 receptor signalling was shown to be deleterious, whereas p75 receptor was protective. Using TNFR knockout mice our group demonstrated similar opposing roles for the two TNFRs during VILI and acid aspiration lung injury: p55 receptor knockout mice were protected while p75 receptor knockout animals were more prone to pulmonary oedema [639]. Moreover, we previously also demonstrated that

biologically active TNF is up-regulated within the alveolar space early during the development of VILI [337].

Dorr et al. showed that intrapulmonary inflammation induced by intratracheal administration of LPS (20ng and 20μg) in mice leads to increased levels of soluble p75 receptor in the alveolar space by 2 hours after challenge, while levels of soluble p55 do not change even after 6 hours, suggesting that surface expression of p55 in the alveolar compartment is well preserved [819]. In contrast, as we demonstrated in Chapter 3, following systemic administration of LPS (20μg) p55 receptor is rapidly shed from the surface of pulmonary endothelial cells by 2 hours post-LPS. Combining the findings of these two studies it is conceivable to think that targeting intraalveolar p55 signalling in ALI could be beneficial. We decided to test this hypothesis first in a VILI model.

However, conventional antibody technologies are not optimal for this purpose for a variety of reasons, but most importantly because monoclonal antibodies against TNFRs can induce receptor cross-linking, thereby activating rather than inhibiting TNFR signalling [820, 821]. For example, the anti-p55 mAb (clone 55R-170) used in our *in vitro* studies in Chapter 3 was shown to induce cell lysis in L929 cell line when cross-linked with anti-hamster IgG [844]. This may not be an issue for the *in vitro* studies where the presence of immunoglobulins can be controlled, but *in vivo* this would be impossible. In the lungs in particular, high levels of IgA is secreted into the mucous lining of the airways, which could possibly cross-link anti-TNFR monoclonal antibodies, and consequently lead to receptor activation. Indeed, when the anti-p55 mAb used in Chapter 3 was previously tested in a mouse VILI model in our lab, it caused more significant lung injury than its isotype control (interpersonal communication with Dr Wilson). It is important to note that as anti-TNFR mAbs

possess two antigen-binding fragments and can bind two TNFRs at once, their TNFR cross-linking ability does not necessarily require their own cross-linking by other immunoglobulins.

Therefore, to investigate the possibility of pharmacological inhibition of p55 signalling, we utilised a novel IgG fragment known as a domain antibody (GlaxoSmithKline, Stevenage, UK) that was selected to specifically bind and inhibit mouse p55 TNF receptor. As domain antibodies (dAbs) comprise only the variable domains of either the heavy or light chain of IgG [824, 825], they have monovalent ligand binding characteristics and lack the F_c portions ensuring high antigen specificity without the risk of receptor cross-linking. Their small size (~12kD) enables better tissue penetration, delivery at much higher concentrations per unit mass, and potentially less long-term adverse effects (e.g. immunosuppression) due to more rapid excretion. Nonetheless, they are suitable for local pulmonary delivery such as inhalation.

4.2 Aims

In this chapter we aimed to investigate the effects of an intratracheally delivered domain antibody that is targeted against mouse p55 TNF receptor on pulmonary oedema and inflammation during VILI using an *in vivo* mouse model. We had two main aims:

1. To develop a robust, reproducible *in vivo* mouse model of VILI that is suitable for intratracheal administration of antibodies in a fluid form.

2. To investigate the effects of blocking intraalveolar p55 TNF receptor signalling by domain antibodies on pulmonary oedema and inflammation in relation to VILI.

4.3 Model development and Protocols

The most challenging step of this project was to set up the high-stretch ventilation protocol. As we deliver our drug intratracheally in liquid form, it has an intrinsic effect on respiratory function as well as mechanics. It is likely that the fluid interferes with the surfactant layer, hence changing the elastic properties of the lung and introducing significant heterogeneity into the system. This is a very important point, because in these experiments, the degree of mechanical stress to produce VILI was standardised based on airway pressure, therefore any fluctuation of the baseline respiratory mechanics before introducing high-stretch ventilation may cause a substantial variability of the actually applied VILI stress. We also had to take into account that if the p55 receptor blocking antibody used has any protective effect on VILI in terms of respiratory physiology, it could be moderate to mild; therefore a model which causes a harsh injury may not be feasible to reveal these effects.

After investing a lot of time and effort, we managed to develop a robust model that can overcome the difficulties arising from the nature of intratracheal fluid intervention. Once the model was optimised great progress was made with some very exciting results.

This section will describe our main steps towards model development, whereas the main results achieved by the finalised protocol will be described in more detail in the subsequent 'Results' section.

Assessment of potential adverse effects of vehicle, p55 and dummy dAb

We first evaluated whether either of our agents *per se* would have any adverse effects on pulmonary physiology. For this, animals were given an intratracheal bolus (50µl) of vehicle, dummy or p55 specific dAb (1mg/kg) and ventilated for 3 hours with low stretch (8-10ml/kg, 100% O_2). None of the groups showed sign of pulmonary oedema formation as assessed by changes in PIP (Figure 4.1/A) and wet:dry lung weight ratio (Figure 4.1/B), nor caused inflammation as measured by percentage of neutrophils (PMN) in bronchoalveolar lavage fluid (Figure 4.1/C) after differential staining.

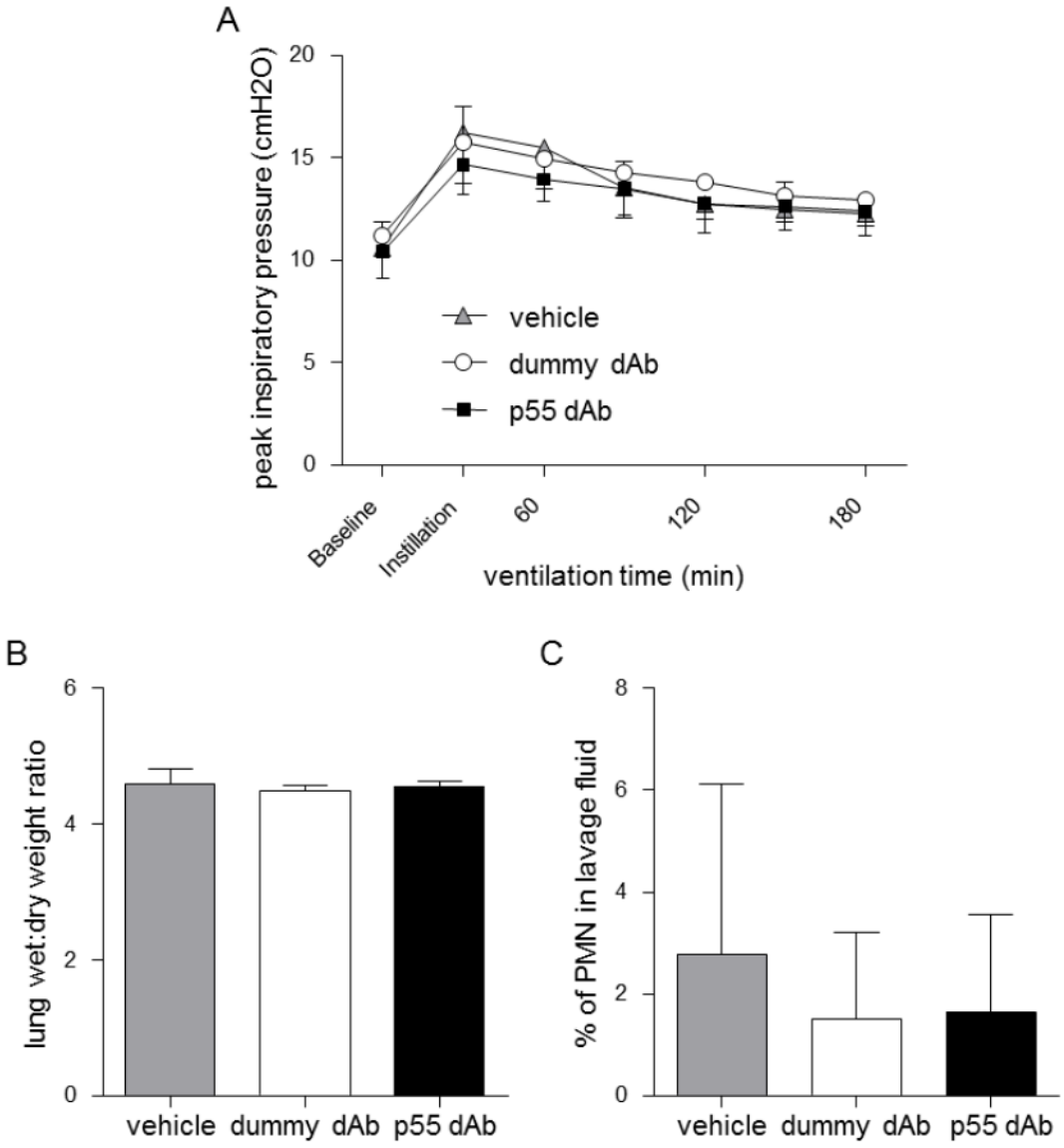


Figure 4.1 – Peak inspiratory pressure changes (A), lung wet:dry weight ratio (B) and percentage of lavage fluid neutrophils (C) during/after 3 hours low-stretch ventilation in vehicle, dummy dAb and p55 dAb treated mice. Mice were instilled intratracheally with 50µl of either vehicle or dummy dAb or p55 dAb and ventilated with low-stretch settings (V_T=8-9ml/kg, PEEP=3cmH_2O) for 3 hours. N.B. any values below 5 of lung wet:dry ratio does not indicate pulmonary oedema, also lavage PMN below 5% suggest no real inflammation. N=2-3/group.

Establishing high-stretch protocol using vehicle

In this model development stage the dAb and vehicle were given as intratracheal instillations 1 hour prior to the start of high-stretch ventilation. The rationale behind this was two-fold; firstly we considered that the dAb may need to be given prophylactically to have efficacy, and secondly (more importantly) that by carrying out the instillations in advance, the fluid would have time to dissipate and be absorbed, hopefully minimising its influence on respiratory mechanics thereby reducing the noise in our system. For these preliminary experiments, animals were given vehicle (100mM Na-citrate in 0.9% NaCl - to preserve dAb structure and function in solution) as a bolus (50µl) and ventilated with low stretch (V_T=8-9ml/kg, PEEP=5cmH_2O) for 60 min after which ventilation was set to produce high-stretch using different levels of peak-inspiratory pressure (PIP) and zero PEEP (ZEEP). This was achieved by increasing the gas flow in the ventilatory system until the desired PIP was reached, which was continuously measured during ventilation. Mice were then ventilated with this setting for up to 120min or until increase in PIP reached 30 per cent (indicating significant pulmonary oedema in our experience), whichever happened earlier. This 120min time frame was chosen to be consistent with our previous experiments showing protection from high-stretch-induced pulmonary oedema in TNFR p55 knockout mice [639]. We found that setting PIP below 32.5 cmH_2O at the start of high-stretch ventilation caused no significant lung injury, whereas any PIP above 33 cmH_2O resulted in rapid pulmonary oedema formation and mortality within 90min of high-stretch ventilation (Figure 4.2). Therefore in future experiments a target PIP of 32.5-33 cmH_2O (corresponds to V_T ~30ml/kg) was chosen to set high-stretch ventilation, as mice ventilated with these settings completed the 120min protocol and developed decent pulmonary oedema (Figure

4.2). Note that during these experiments pulmonary oedema formation was solely monitored by changes in respiratory mechanics (i.e. increase in PIP).

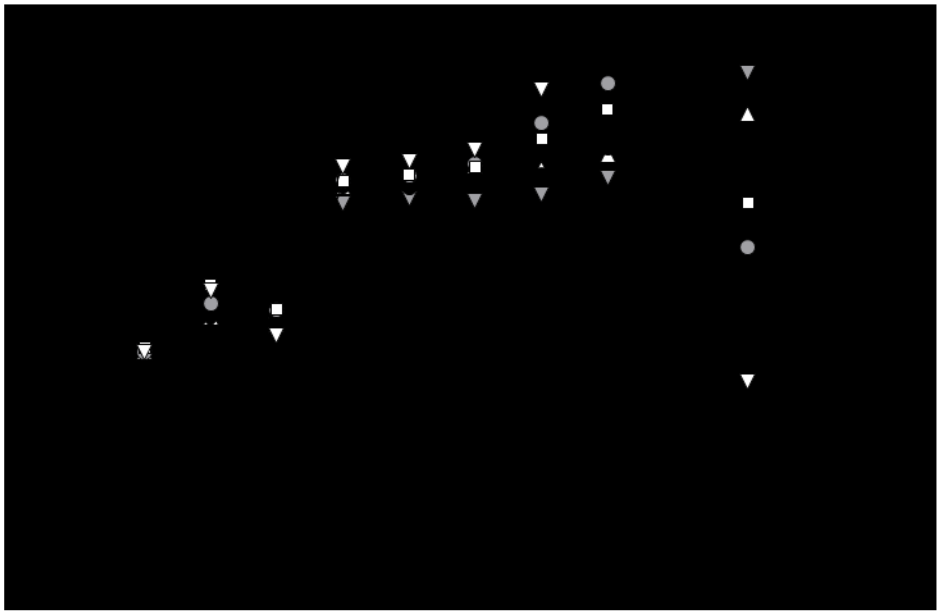


Figure 4.2 – Peak inspiratory pressure (PIP) changes in vehicle treated mice ventilated with high-stretch standardised by PIP. Mice were instilled with vehicle (50µl) intratracheally via the endotracheal tube and ventilated with low-stretch settings (V_T=8-9ml/kg, PEEP=3cmH_2O) for 60 minutes after which ventilation was set to produce high-stretch by increasing the gas flow to achieve predetermined PIP levels (between 30-35cmH_2O, with ZEEP). Setting PIP between 32-33cmH_2O produced substantial injuries over 120min without compromising the protocol length. 'Start'=start of high-stretch. N=1/each PIP level

Using these ventilatory settings we went on to test the p55 dAb using dummy dAb as control. Mice were instilled intratracheally with 50µl of either vehicle or dummy dAb or p55 dAb during low-stretch ventilation (V_T=8-9ml/kg, PEEP=5cmH_2O) and ventilated for 60min, after which settings were changed to produce high-stretch using a PIP of ~32.5cmH_2O (corresponding to a V_T of ~30ml/kg) and ZEEP. Again, experiments were stopped when PIP increased by 30% or at 120min, whichever

happened earlier. We found that experiments with p55 dAb-treated mice tended to last longer (meaning slower injury progression) as compared to dummy group, and seemed to have lower PIP (less oedema) at the 90min time point (Figure 4.3).

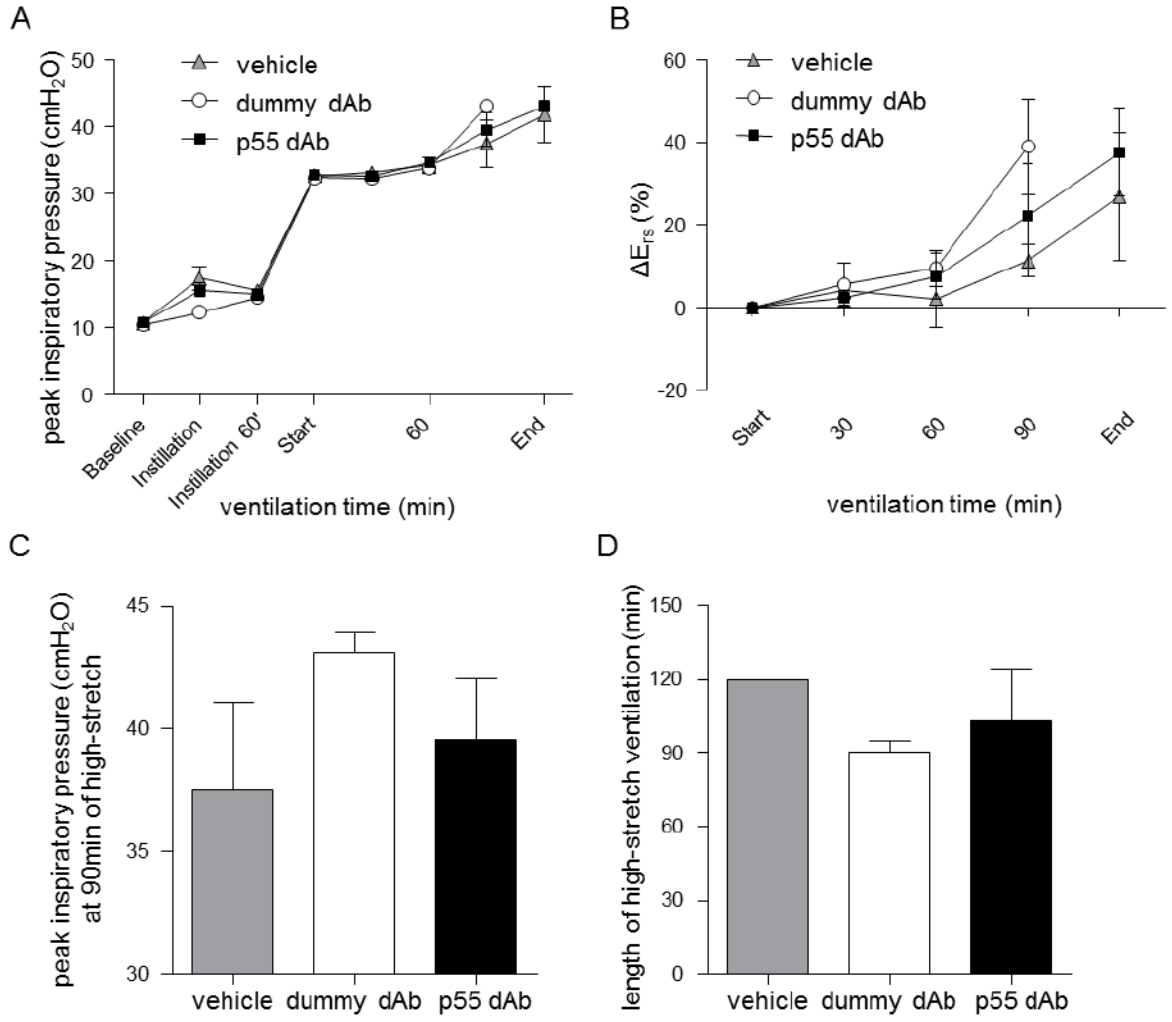


Figure 4.3 – (A) Peak inspiratory pressure (PIP) and (B) respiratory system elastance (E_{rs}) changes, (C) PIP values at 90 minutes and (D) length of high-stretch ventilation in vehicle, dummy and p55 dAb treated mice ventilated with high-stretch standardised by PIP. Mice were instilled intratracheally with vehicle, dummy dAb or p55 dAb (25µg in 50µl) and ventilated with low stretch (V_T=8-9ml/kg, PEEP=3cmH_2O) for 60 minutes. Then ventilation was changed to produce high-stretch by setting a PIP of 32.5cmH_2O with ZEEP. Changes in E_{rs} are expressed as percentage increase in relation to 'Start' values. N=3/group

However, the respiratory mechanics were highly variable, and hence differences between the groups were not clear. We also found that dummy dAb-treated mice were more injured than vehicle-treated mice. One possible explanation for this is that as domain antibodies are proteins, they may have presented oncotic forces thus caused slower fluid absorption/clearance from the airways compared to vehicle. The presence of fluid within the lung, somewhat moving along the major airways during the respiratory cycle results in variable airway resistance especially at high gas flow, which will ultimately produce more variability in peak pressure. Therefore we decided to fine tune the protocol using dummy dAb as control, and to use plateau pressure ($P_{plateau}$) instead of PIP to standardise stretch because $P_{plateau}$ is not influenced by airway resistance and is a surrogate for end-inspiratory alveolar pressure thus stress. Making these changes should consequently reduce some of the 'noise' in the model.

Fine tuning VILI using dummy as control and standardising with $P_{plateau}$

Mice were instilled with dummy dAb and ventilated for 60min with low-stretch (V_T= 9-10ml/kg, PEEP=5 cmH_2O) followed by 120min high-stretch standardised by $P_{plateau}$ (~25cmH_2O, corresponding to a V_T of ~30ml/kg) and ZEEP. We found that setting $P_{plateau}$ at 25-25.5cmH_2O produced substantial injury by the end of 120min high-stretch ventilation in dummy dAb-treated mice and only 2 out of 6 animals reached the 30% increase in PIP before 120min (Figure 4.4). However, variability in respiratory system elastance (E_{rs}) was still high.

Regrettably, when mice were given the p55 dAb using these settings, they showed no sign of protection compared to dummy, in fact these animals showed more rapid injury progression, meaning that none of the animals completed the

120min high-stretch protocol (Figure 4.4). Although these results may have reflected a true lack of efficacy of p55 dAb in attenuating VILI, the possibility that the model was too harsh to reveal any potential protective effect of the p55 dAb could not be ruled out. Nonetheless, the variability of respiratory physiology was still high and needed to be further reduced.

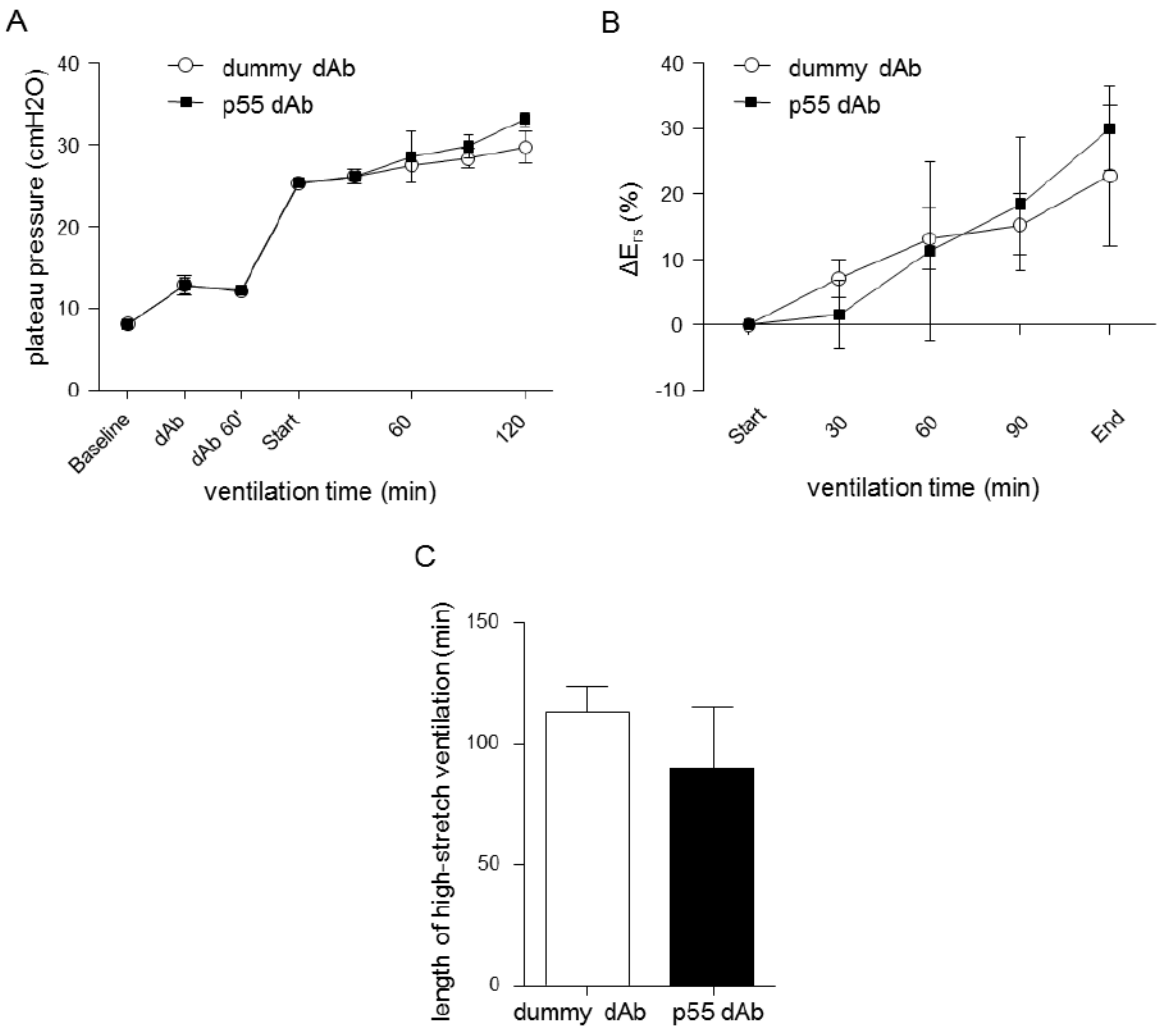


Figure 4.4 – (A) Plateau pressure ($P_{plateau}$) and (B) respiratory system elastance (E_{rs}) changes, and (C) length of high-stretch ventilation in dummy and p55 dAb treated mice ventilated with high-stretch standardised by $P_{plateau}$. Mice were instilled with dummy dAb and ventilated for 60min with low-stretch (V_T= 9-10ml/kg, PEEP=5 cmH_2O) followed by 120min high-stretch standardised by $P_{plateau}$ (~25cmH_2O, corresponding to a V_T of ~30ml/kg) and ZEEP. Changes in E_{rs} are expressed as percentage increase in relation to 'Start' values. 'Start' = start of high-stretch. N=5-6/group

Modification of the model – longer incubation period

Our first attempt to increase the reproducibility of the protocols involved increasing the period between instillation and high-stretch ventilation. In this set-up mice were instilled intratracheally with 50µl of either dummy dAb or p55 dAb during low-stretch ventilation (V_T=8-9ml/kg, PEEP=5cmH_2O) and ventilated for 120min (as opposed to

60min), after which settings were changed to produce high-stretch standardised by $P_{plateau}$ (~25cmH_2O, corresponding to a V_T of ~30ml/kg), and zero PEEP. Experiments were stopped when $P_{plateau}$ increased by 30% (indicating significant injury/oedema) or at 120min, whichever happened earlier. Only 1 out of the 4 dummy-treated mice completed the 120min high-stretch protocol, whereas p55 dAb-treated animals showed even more rapid injury progression indicating no sign of protection (Figure 4.5). This longer pre-incubation, however, somewhat reduced the variability in physiology.

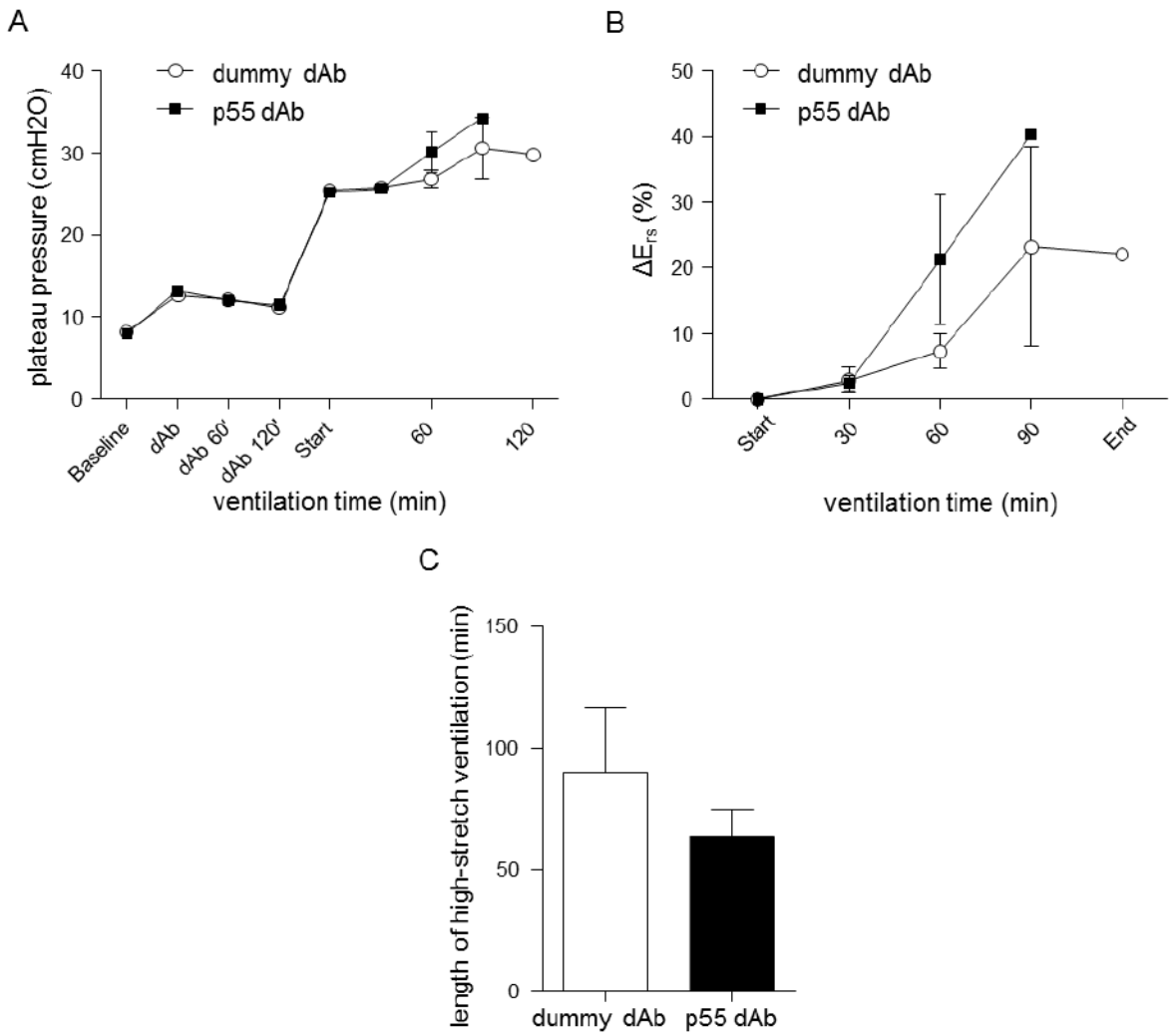


Figure 4.5 – (A) Plateau pressure ($P_{plateau}$) and (B) respiratory system elastance (E_{rs}) changes, and (C) length of high-stretch ventilation in dummy and p55 dAb treated mice ventilated with high-stretch standardised by $P_{plateau}$ after 120min incubation period. Mice were instilled intratracheally with 50µl of either dummy dAb or p55 dAb during low-stretch ventilation (V_T=8-9ml/kg, PEEP=5cmH_2O) and ventilated for 120min, after which settings were changed to produce high-stretch standardised by $P_{plateau}$ (~25cmH_2O, corresponding to a V_T of ~30ml/kg), and zero PEEP. Experiments were stopped when $P_{plateau}$ increased by 30% (indicating significant injury/oedema) or at 120min, whichever happened earlier. Changes in E_{rs} are expressed as percentage increase in relation to 'Start' values. N=3-4/group

Trials with different instillation methods - bolus vs microsprayer

Although longer incubation after dAb instillation helped, we felt that the protocol was still not ideal and had to be further refined. One area we tested was using a microsprayer (PennCentury) to nebulise the instillate. During this technique, mice were disconnected from the ventilator, placed in an upright position and the microsprayer probe was inserted into the endotracheal tube (ETT) until it just passed the inner orifice of the ETT (i.e. just above the tracheal bifurcation), then the instillate was rapidly injected. Then mice were reconnected to the ventilator and sustained inflations were applied to re-establish lung volume. While we found that this method substantially decreased variability compared to the bolus technique (Figure 4.6), its usage resulted in intratracheal haemorrhage (Figure 4.7). One possible explanation as to why the microspraying resulted in intraalveolar haemorrhage is that the ETT practically sealed around the microsprayer probe, preventing any leak of air during instillation, which may have led to excessively high airway pressures and consequently barotrauma. As this issue could not be solved, in subsequent experiments the bolus method was used for drug delivery.



Figure 4.6 – Percentage of standard deviation (SD) of different respiratory mechanics at 40 minutes post intratracheal instillation by the bolus method and the microsprayer technique. After finishing surgical preparation, mice were disconnected from the ventilator during low-stretch ventilation (V_T=8-9ml/kg, PEEP=3cmH_2O), placed in an upright position and the microsprayer (PennCentury) probe was inserted into the endotracheal tube (ETT) and the instillate was rapidly injected. Then mice were reconnected to the ventilator and sustained inflations were applied to re-establish lung volume, after which low-stretch ventilation continued for 3 hours. Values are normalised to that of the bolus method (average of N=4/group). C_{rs}=respiratory system compliance, R_{rs}=respiratory system resistance, PIP=peak inspiratory pressure, P_{aw}=airway pressure.

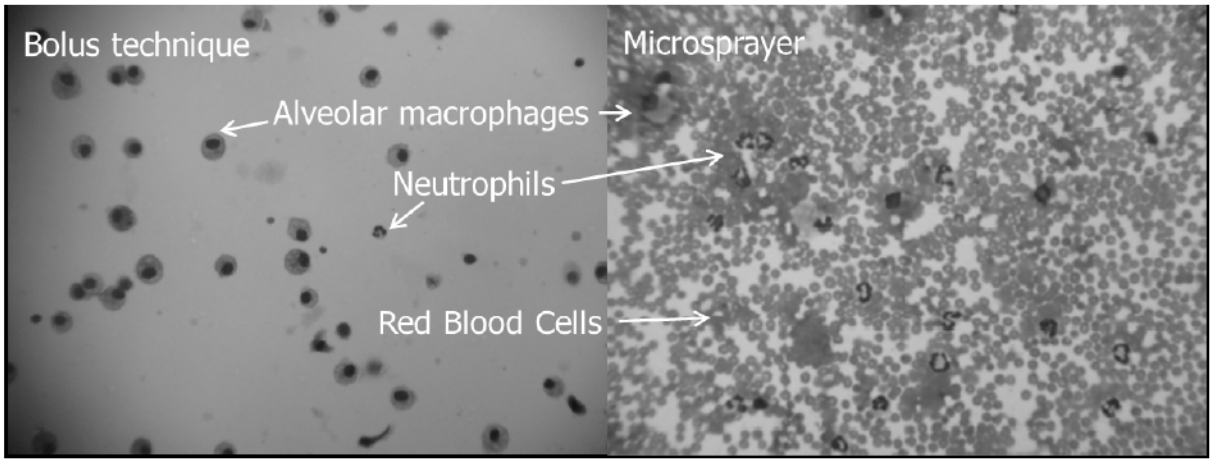


Figure 4.7 – Photomicrographs of cytospin slides showing lavage cells after Diff-Quick staining from mice instilled with dummy dAb using the bolus and microsprayer methods. Animals instilled with the microsprayer showed intrapulmonary haemorrhage and substantially increased neutrophil numbers compared to those in which the bolus method was utilised.

Conclusion

Our overall conclusions regarding model development at this stage were that the model was both too harsh (i.e. too rapid oedema development due to short protocol), and too variable to reveal any effects of the dAb on respiratory physiology and lung injury development. This variability in physiology, although somewhat reduced during model development, remained an issue that had to be appropriately addressed.

As mentioned previously, intratracheal fluid instillation has a great impact on the elastic properties of the lung, and because our standardisation of high-stretch is based on respiratory mechanics, such an impact must introduce substantial noise into the system. To avoid this, we decided to set high-stretch ventilation before any intrapulmonary fluid administration, as well as reducing the degree of stretch to make the model less harsh. By definition this means that the dAb would not have a pre-incubation period if prophylaxis was required, but we felt that it was more important at this stage to consider reducing model variability. Another major change to the previous model was to apply PEEP during high-stretch ventilation. This was necessary because of the increased propensity of the lungs to develop atelectasis and atelectrauma due to the effect of fluid instillation on lung mechanics. Moreover, in practice this increased susceptibility to airway collapse could have also squeezed some of the instillate out of the lungs straight away after instillation in an unpredictable manner, which would have likely introduced noise to the system. Please note, during model development when the antibodies were instilled during low-stretch ventilation, a PEEP of 5cmH_2O was applied, which was only changed to zero PEEP during high-stretch, by which time the instillate had been presumably absorbed, thus did not implicate such issues.

Finalised VILI protocol

Following surgical preparation for the *in vivo* mouse mechanical ventilation system (during which animals were ventilated with 8-9 ml/kg) and baseline measurements, ventilation was then set to produce VILI ('pure' VILI model), standardised by $P_{plateau}$ of 14 cmH_2O corresponding to V_T of ~22 ml/kg, with 3 cmH_2O PEEP, 90/min respiratory rate, and 96%oxygen+4% CO_2 (to prevent hypocapnia). Immediately following this, mice received an intratracheal bolus of either dummy dAb or p55 dAb (25µg), or either mouse anti-TNF monoclonal antibody (50µg) or its isotype control in a total volume of 50µl. In a separate series of experiments, a subclinical dose of LPS (20ng) was co-administered intratracheally with the dAb bolus to enhance the inflammatory component of the model. In this LPS+VILI model, slightly lower $P_{plateau}$ (13 cmH_2O) and V_T (~20 ml/kg) were used because LPS was expected to enhance the degree of VILI [116].

Ventilation using the same constant high V_T was continued in all groups for 240 minutes or until mean BP dropped below 45 mmHg [337]. A small number of animals displayed very rapid haemodynamic deterioration: in the 'pure VILI' model, 3 dummy dAb, 1 p55-specific dAb, 1 isotype control and 2 anti-TNF treated; in the 'LPS+VILI' model, 1 dummy dAb treated mouse. These prematurely terminated experiments were excluded from analyses. $P_{plateau}$, E_{rs} and R_{rs} were calculated every 20 minutes, followed each time by sustained inflation (35cmH_2O, 5 seconds) to avoid the development of atelectasis. Arterial blood gases (ABG) were assessed at predetermined points throughout the protocol (Figure 4.8).



Figure 4.8 - Scheme of ventilation protocol. Mice were anaesthetised, tracheostomised and ventilated with non-injurious ventilation for approximately 15-20min during instrumentation (*dotted line*). Injurious ventilation parameters using high tidal volumes (V_T) were then introduced, followed immediately by instillation of the antibody of interest. As a model of VILI with enhanced inflammation, a subclinical dose of LPS (20ng) was co-administered with the domain antibody bolus. After instillation of the reagents, 4 sustained inflations (35cmH_2O, 5 seconds) were applied to help the fluid distribute within the lungs. Respiratory mechanics were assessed every 20min, followed each time by sustained inflation. Arterial blood gases (ABG) were assessed at predetermined time points (*Start, 120min, End*). Source – Bertok *et al.* [2]

Upon termination of experiments, mice were euthanized by anaesthetic overdose and cardiac puncture and saline lavage performed, as described in Chapter 2. Samples were centrifuged and BALF supernatants aliquotted and stored at -80°C These were later quantified for total protein as a further indicator of pulmonary oedema/alveolar barrier dysfunction, and for IL-6, KC, MIP-2 and MCP-1 by colorimetric sandwich ELISA in the LPS+VILI model as indicator of intraalveolar inflammation. In the LPS+VILI model, lungs were excised and processed for flow cytometry along with cell pellets from BALF samples to assess the degree of leukocyte infiltration and activation state of alveolar macrophages. Alternatively, non-lavaged lungs were instilled with 1% low-melting point agarose in 4%

paraformaldehyde and processed for histological evaluation by H&E staining as described in Chapter 2.

4.4 Declaration

The data contained within this chapter have been published in *Thorax* by Bertok *et al.* [2] and can be found in Appendix II. Where indicated, the figures in this chapter are taken from this manuscript. The investigation was a collaborative effort: Peter Morley, Ruud de Wildt, Andrew Bayliffe, Michael Wilson and Masao Takata must be credited for the conception and design of the study as well as for the analysis and interpretation of results, whereas Alicia Waite for her assistance with some of the flow cytometry measurements.

4.5 Results

Anti-p55 domain antibody attenuates pulmonary oedema in the 'pure VILI' model

Mechanical ventilation with the high-stretch protocol resulted in a substantial deterioration of lung function in dummy dAb treated animals, consistent with the development of pulmonary oedema. This was represented by a rapidly increasing $P_{plateau}$, E_{rs} and R_{rs} towards the end of the 4h ventilation protocol (Figure 4.9/A-C). In striking contrast, no deteriorations were seen in respiratory mechanics in p55 dAb-treated mice.

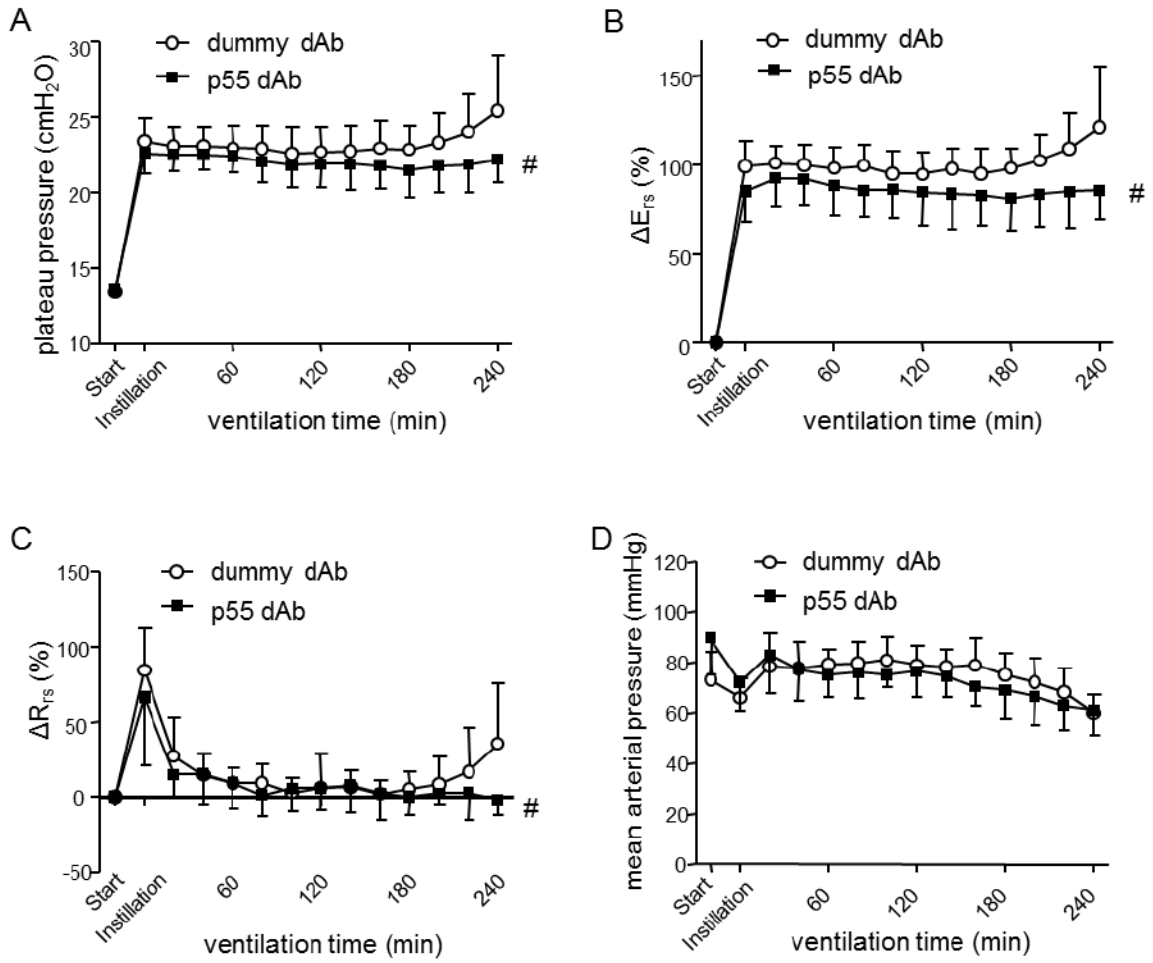


Figure 4.9 - (A) Plateau pressure ($P_{plateau}$), (B) respiratory system elastance (E_{rs}) and (C) resistance (R_{rs}), (D) mean arterial pressure were determined in ventilated animals treated intratracheally with either dummy or p55-specific domain antibody (dAb). After finishing surgical preparation during low-stretch ventilation (V_T=8-9ml/kg, PEEP=3cmH_2O), high-stretch was initiated standardised by $P_{plateau}$ (14cmH_2O, V_T~22ml/kg, PEEP=3cmH_2O). Immediately after setting high-stretch, mice were instilled intratracheally with either dummy or p55 dAb (25µg in 50µl) and ventilation continued with regular sustained inflations to prevent atelectasis for up to 240 minutes . Changes in E_{rs} and R_{rs} are expressed as percentage increase in relation to 'Start' (before instillation) values. $P_{plateau}$, E_{rs} and R_{rs} changes over time during ventilation were significantly different between treatment groups (# P<0.05 for interaction between 'treatment' and 'ventilation time' by 2-way ANOVA). N=8-11/ group. Source – Bertok *et al.* [2]

Arterial PO_2 and PCO_2 levels were similar across the treatment groups at the

start and 120 minutes after drug instillation (Figure 4.10/A-B). By the end of the protocol, oxygenation tended to deteriorate in the dummy dAb group (365±146mmHg), while PCO_2 increased. In contrast, PO_2 was better maintained in the p55 dAb group (440±41mmHg), and PCO_2 was unchanged from the 120 minute level. To assess alveolar epithelial permeability, we measured the protein content in BALF. High-stretch ventilation resulted in substantial protein levels in the dummy dAb group as compared to non-ventilated controls (2.82±1.73 vs 0.21±0.03mg/ml), and this was significantly attenuated by p55 dAb treatment (1.34±0.4mg/ml) (Figure 4.10/D). To confirm that high-stretch ventilation was associated with intraalveolar TNF production in dummy dAb treated mice, we measured BALF TNF at the end of the protocol, and found it to be up-regulated (147±144pg TNF, N=11) compared to untreated mice (undetectable).

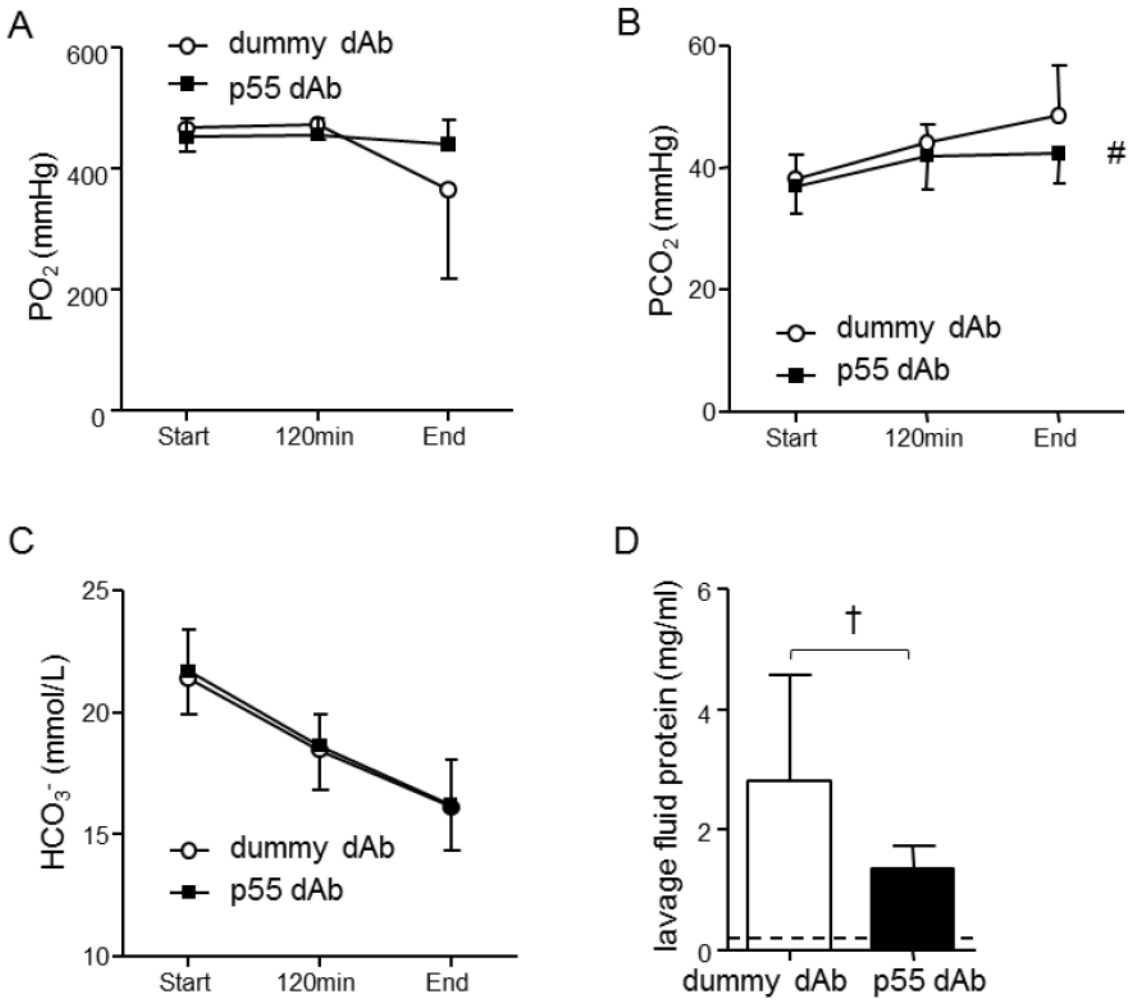


Figure 4.10 - (A) Arterial PO_2, (B) PCO_2, (C) bicarbonate (HCO_3^-) and (D) lavage fluid protein were determined in ventilated animals treated intratracheally with either dummy or p55-specific domain antibody (dAb). Arterial PO_2 showed a tendency to deteriorate in animals treated with the dummy dAb but not in mice given the p55-specific dAb. Changes in PCO_2 over time were significantly different between treatment groups (# $P<0.05$ for interaction by 2-way ANOVA), while changes in bicarbonate levels were similar across groups. Lavage fluid protein was significantly attenuated in the p55-specific dAb group († $P<0.05$ by unpaired *t* tests). Dotted line represents mean value in non-ventilated control animals. N=8-11/group. Source – Bertok *et al.*. [2]

Finally, we found on lung histology that VILI led to a moderate degree of injury in dummy dAb treated animals, characterised by neutrophil infiltration and protein-rich material in the alveolar space (Figure 4.11/A-B), that was substantially reduced

following p55 dAb treatment (Figure 4.11/C-D).

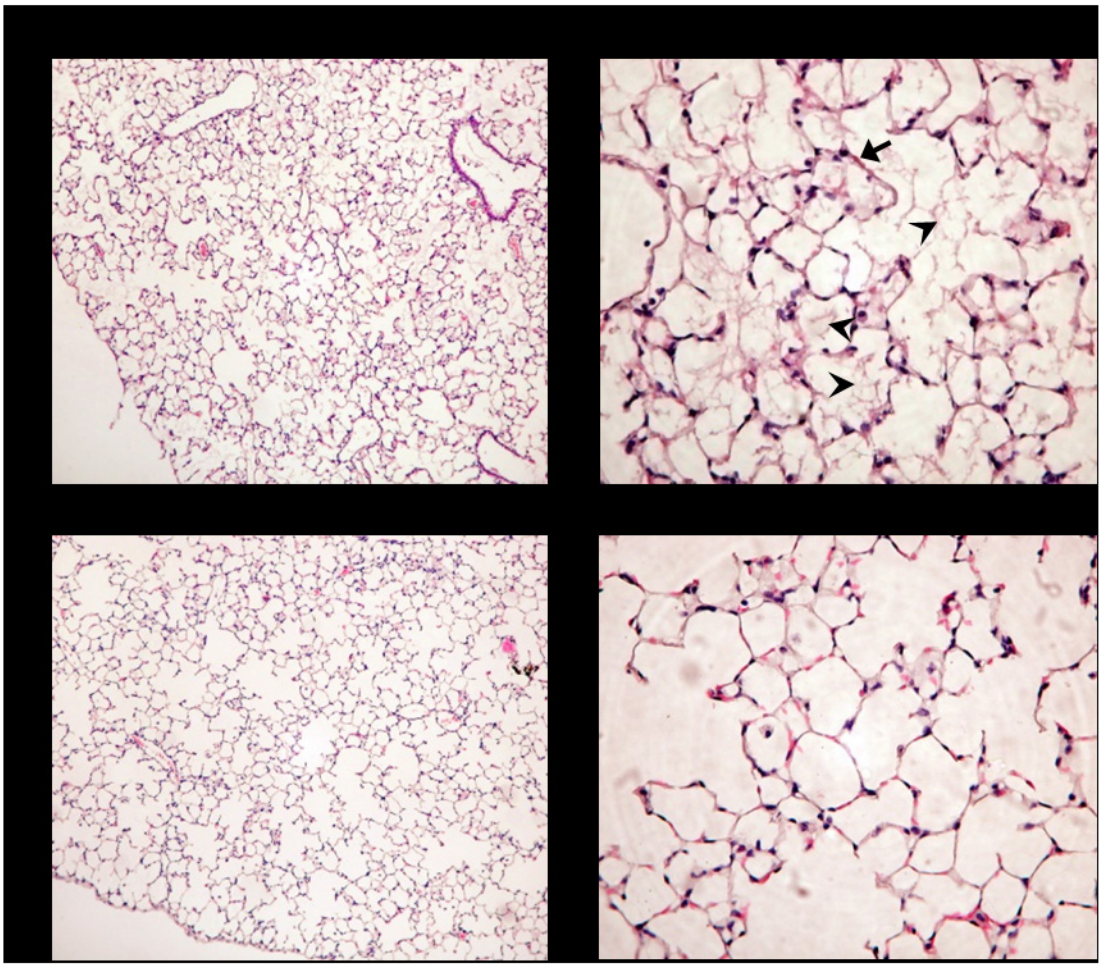


Figure 4.11 - Photomicrographs of lung tissue. Sample from dummy-dAb treated mouse (A, B) showed marked cellularity, alveolar wall thickening (arrow), hyaline membrane formation and intraalveolar debris (arrowhead), whereas minimal changes were seen in p55-dAb treated animal (C, D). Hematoxylin-eosin stain. Magnification x100 (A and C), x400 (B and D).

Blockade of total TNF-signalling by anti-TNF monoclonal antibody does not attenuate high-stretch induced pulmonary oedema

Having demonstrated that blockade of p55 TNF receptor signalling attenuated VILI, we investigated whether total TNF signalling blockade would be similarly effective. To address this, we treated mice with an anti-TNF monoclonal antibody (or isotype control) using the same instillation strategy and ventilation protocol as for the domain

antibody experiments. We previously demonstrated that this particular anti-TNF antibody, given the same route and dose, effectively reduced alveolar neutrophil infiltration associated with VILI [109].

We found that changes in respiratory mechanics in the isotype control treated group followed a very similar pattern as observed for the dummy dAb group (Figure 4.12/A-C). However, in marked contrast to the beneficial effect of p55 dAb, the anti-TNF mAb did not attenuate the changes seen in isotype-treated mice. Likewise, anti-TNF treatment had no beneficial effect on blood gases (Figure 4.13/A-C) or alveolar epithelial permeability (Figure 4.13/D).

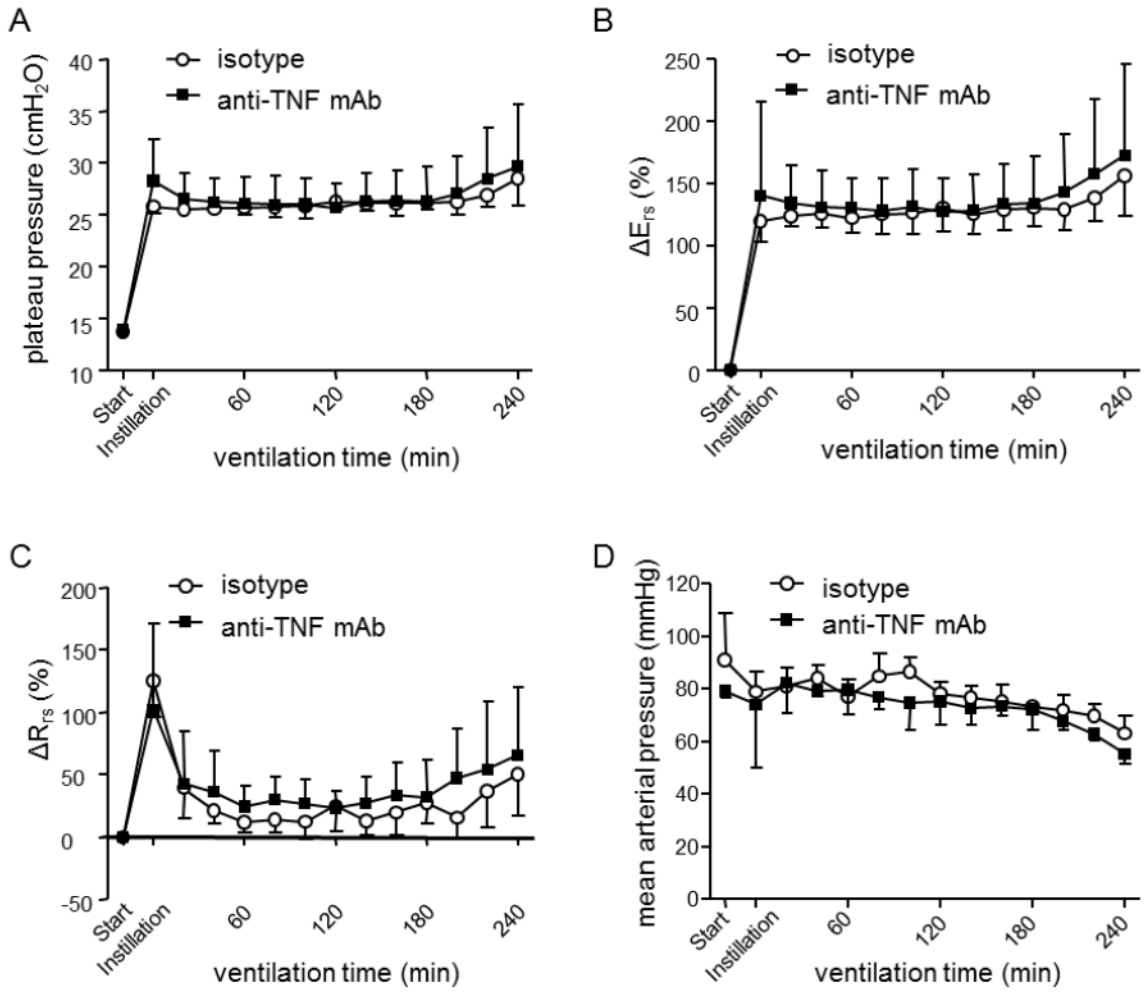


Figure 4.12 - (A) Plateau pressure ($P_{plateau}$), (B) respiratory system elastance (E_{rs}) and (C) resistance (R_{rs}), (D) mean arterial pressure in animals treated intratracheally with either isotype or mouse anti-TNF monoclonal antibody (mAb). After finishing surgical preparation during low-stretch ventilation (V_T=8-9ml/kg, PEEP=3cmH_2O), high-stretch was initiated standardised by $P_{plateau}$ (14cmH_2O, V_T~22ml/kg, PEEP=3cmH_2O). Immediately after setting high-stretch, mice were instilled intratracheally with either isotype or anti-TNF mAb (50µg in 50µl) and ventilation continued with regular sustained inflations to prevent atelectasis for up to 240 minutes. Changes in E_{rs} and R_{rs} are expressed as percentage increase in relation to 'Start' (before instillation) values. N=6/group. Source – Bertok et al. [2]

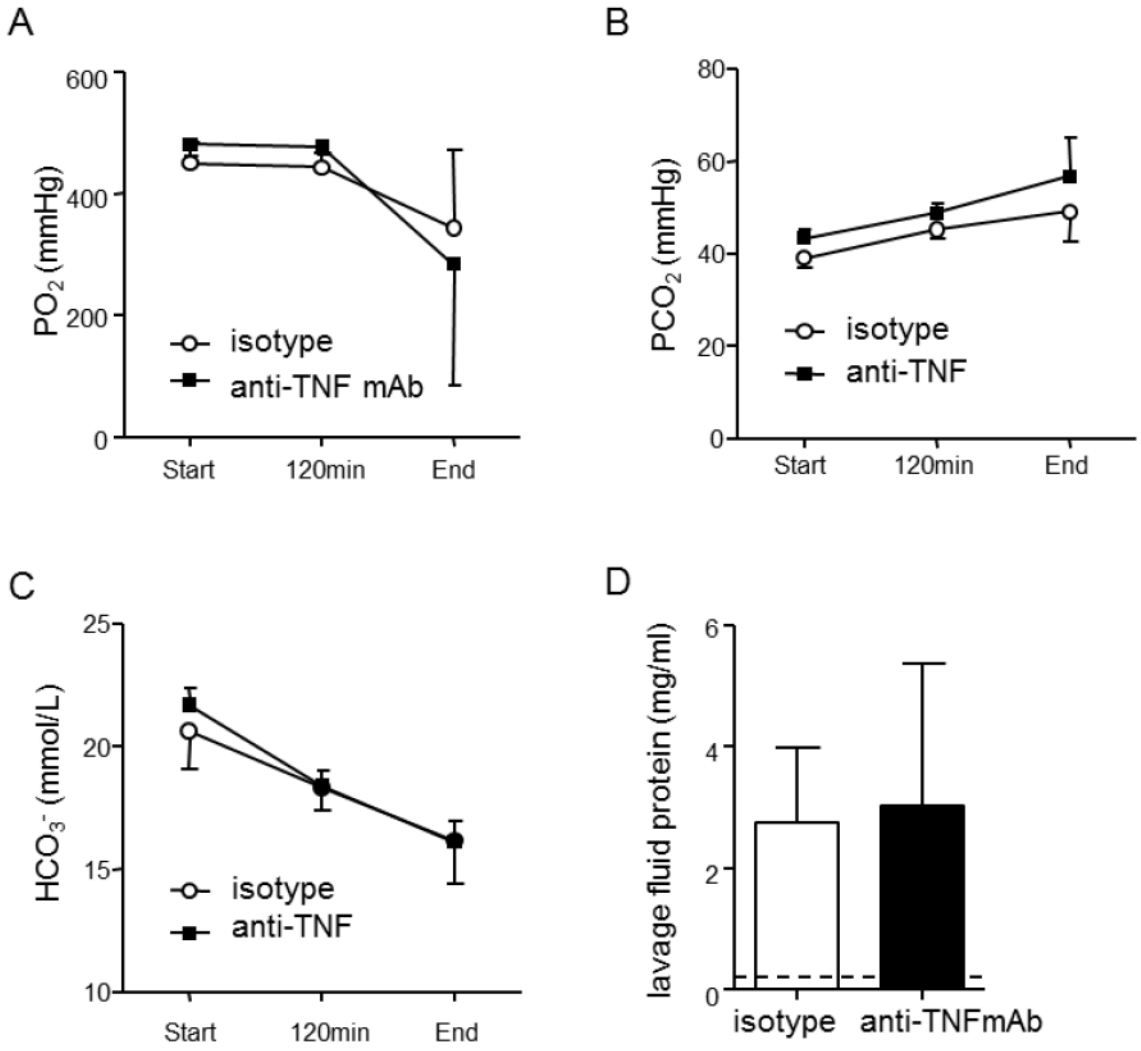


Figure 4.13 - (A) arterial PO_2, (B) PCO_2, (C) bicarbonate (HCO_3^-) and (D) lavage fluid protein in animals treated intratracheally with either isotype or mouse anti-TNF monoclonal antibody (mAb) and ventilated with high-stretch ventilation for 240min. Dotted line represents mean value in non-ventilated control animals. N=6 /group. Source – Bertok *et al*. [2]

Anti-p55 domain antibody reduces pulmonary inflammation secondary to LPS+VILI

Finally, we investigated if the p55-specific dAb would also have a beneficial effect on pulmonary inflammation associated with VILI. It was previously demonstrated by our group and others [856] that pure mechanical ventilation induces a relatively limited

degree of alveolar neutrophil infiltration (at least within the time frame of ventilation used currently), one of the hallmarks of clinical ALI. Therefore to enhance the degree of inflammation within the model we included a 'subclinical' dose of LPS (20ng) mixed with the dummy or p55 domain antibodies at the start of mechanical ventilation. Our preliminary experiments showed that this dose of LPS did not induce changes in respiratory mechanics with low tidal volume ventilation.

Similarly to the 'pure VILI' model, LPS+VILI induced substantial changes in respiratory mechanics which were significantly attenuated by p55 dAb (Figure 4.14/A-C).

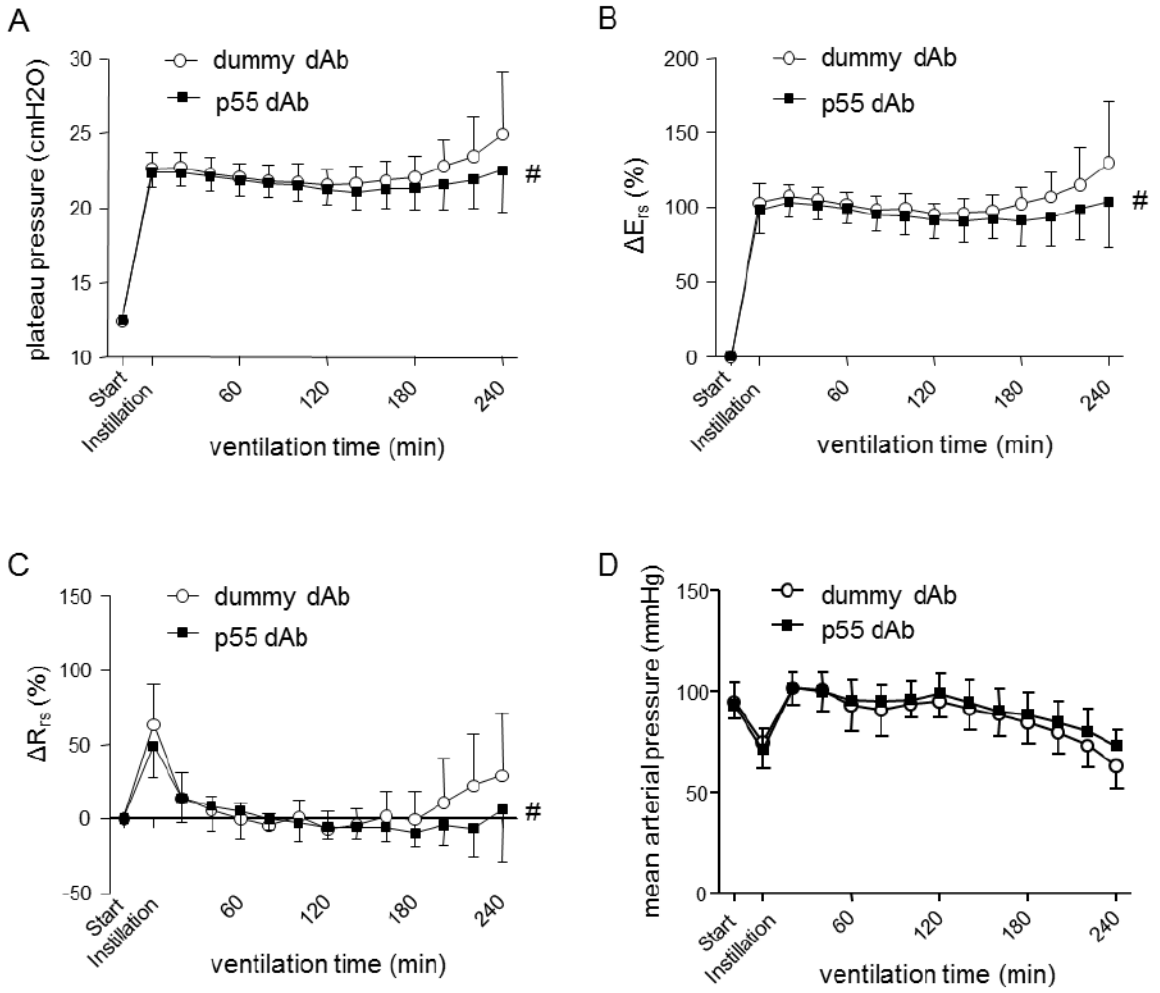


Figure 4.14 - (A) Plateau pressure ($P_{plateau}$), (B) respiratory system elastance (E_{rs}) and (C) resistance (R_{rs}), (D) mean arterial pressure in the LPS+VILI model. After finishing surgical preparation during low-stretch ventilation (V_T=8-9ml/kg, PEEP=3cmH_2O), high-stretch was initiated standardised by $P_{plateau}$ (13cmH_2O, V_T~20ml/kg, PEEP=3cmH_2O). Immediately after setting high-stretch, mice were instilled intratracheally with either dummy or p55 dAb (25µg) mixed with LPS (20ng) in a total volume of 50µl, and ventilation continued with regular sustained inflations to prevent atelectasis for up to 240 minutes.Changes in E_{rs} and R_{rs} are expressed as percentage increase in relation to 'Start' values. Changes in $P_{plateau}$, E_{rs} and R_{rs} over time were significantly different between treatment groups (# $P<0.05$ for interaction between 'treatment' and 'ventilation time' by 2-way ANOVA). N=8-11/group. Source – Bertok *et al.* [2]

Likewise, p55 dAb treatment prevented the deterioration of oxygenation (Figure 4.15/A), and tended to attenuate the rise in PCO_2 (Figure 4.15/B). There was

also a trend toward decreased protein levels in lavage fluid in p55 dAb-treated animals (2.94±1.62 vs 2.06±1.45mg/ml, for dummy and p55 dAb respectively), although this did not reach statistical significance (Figure 4.15/D).

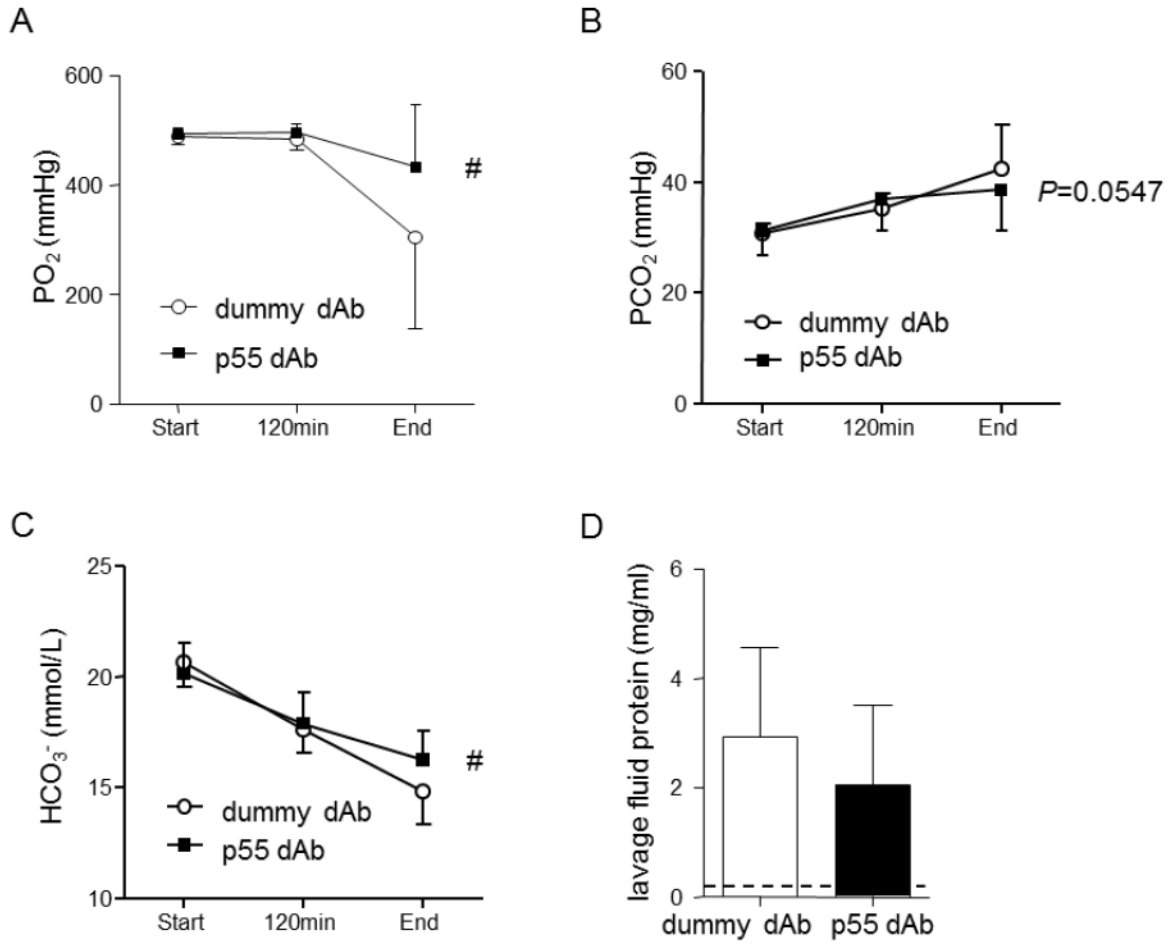


Figure 4.15 - (A) arterial PO_2, (B) PCO_2, (C) bicarbonate (HCO_3^-) and (D) lavage fluid protein in the LPS+VILI model. Arterial PO_2 and bicarbonate over time were significantly different between treatment groups (# P<0.05 for interaction between 'treatment' and 'ventilation time' by 2-way ANOVA). The p55-specific dAb tended to attenuate the rise in PCO_2, but this did not reach statistical significance (P=0.055 for interaction by 2-way ANOVA). Lavage fluid protein levels tended to be reduced in p55-specific dAb treated animals, but not significantly. Dotted line represents mean value in non-ventilated controls. N=8-11/group. Source – Bertok *et al.* [2]

Neutrophils and inflammatory subset Gr-1^{high} monocytes in lung single cell suspension and lavage fluid were identified based on characteristic antigen

expression using flow cytometry as discussed in Chapter 2, and their number or activation state quantitatively assessed. LPS+VILI significantly increased the number of lung-marginated neutrophils as compared to non-ventilated controls ($2.32\pm0.57x10^6$ vs $0.13\pm0.05x10^6$, respectively), which was significantly attenuated by p55 dAb treatment ($1.25\pm0.31x10^6$, $P<0.01$ vs dummy) (Figure 4.16/A). A similar trend was found with Gr-1^{high} monocytes, although this was not significant (Figure 4.16/A). Transmigration of neutrophils to the alveolar space was also greatly increased by LPS+VILI, and substantially attenuated by p55 dAb treatment (Figure 4.16/B), whereas Gr-1^{high} monocytes were not detected in BALF in any of the groups. Alveolar macrophages in BALF of dummy dAb treated mice showed up-regulated ICAM-1 expression compared to non-ventilated controls, indicating cell activation [107], which was also markedly reduced in the p55 dAb group (Figure 4.16/C).

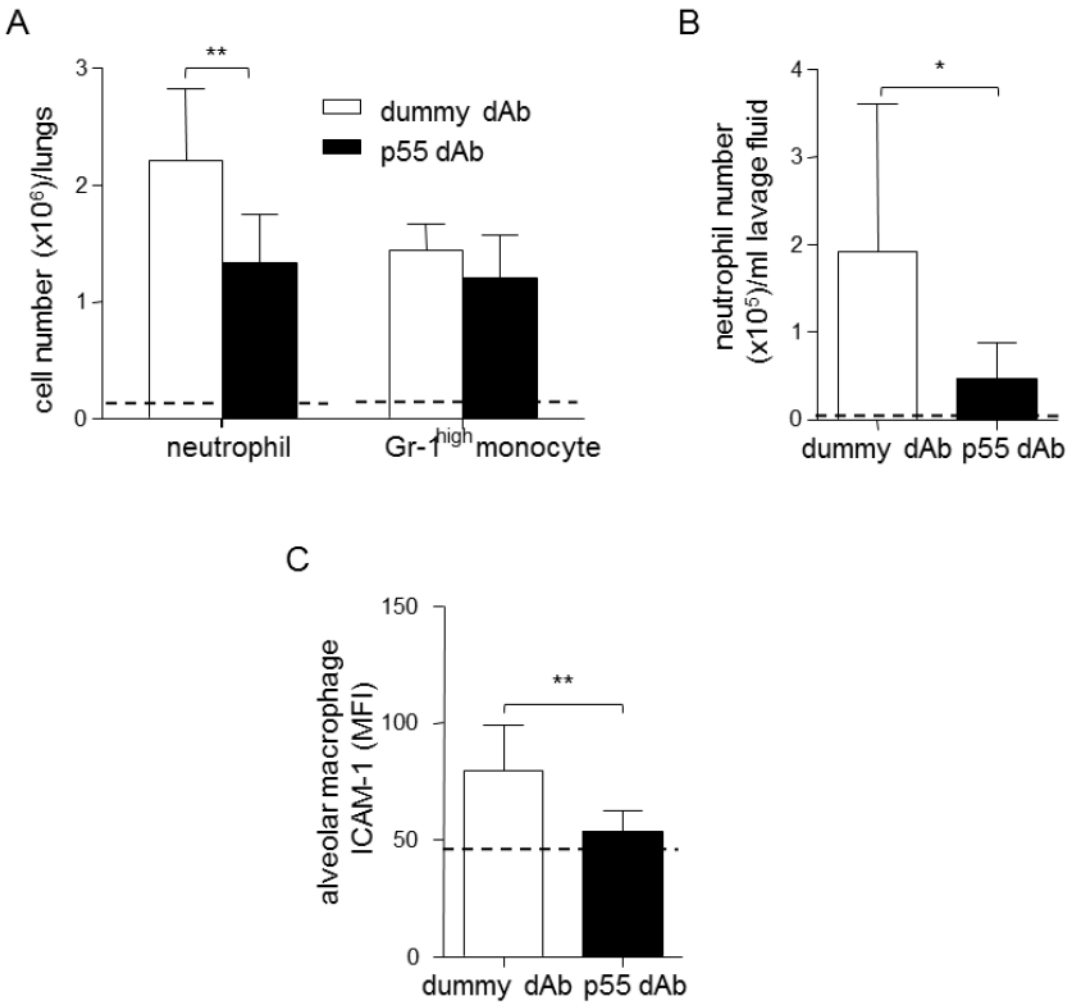


Figure 4.16 – Number of leukocyte subsets in the lungs and lavage fluid and ICAM-1 expression by alveolar macrophages of dummy-dAb and p55-dAb treated mice in LPS+VILI model assessed by flow cytometry. Treatment with p55-specific dAb attenuated the number of (A) lung-marginated and (B) intraalveolar neutrophils and (C) the activation state of alveolar macrophages by means of ICAM-1 expression (mean fluorescence intensity (MFI) after isotype subtraction). Dotted line represents mean value in non-ventilated control animals. *P<0.05, **P<0.01 by unpaired t-test (as lavage fluid neutrophil number data were non-parametric, these were transformed prior to t-test then transformed back for presentation). N=8-11/group. Source – Bertok *et al.* [2]

Finally, BALF levels of IL-6 and MCP-1 were also significantly decreased in p55 dAb treated mice, with similar trends for KC and MIP-2 (Figure 4.17).

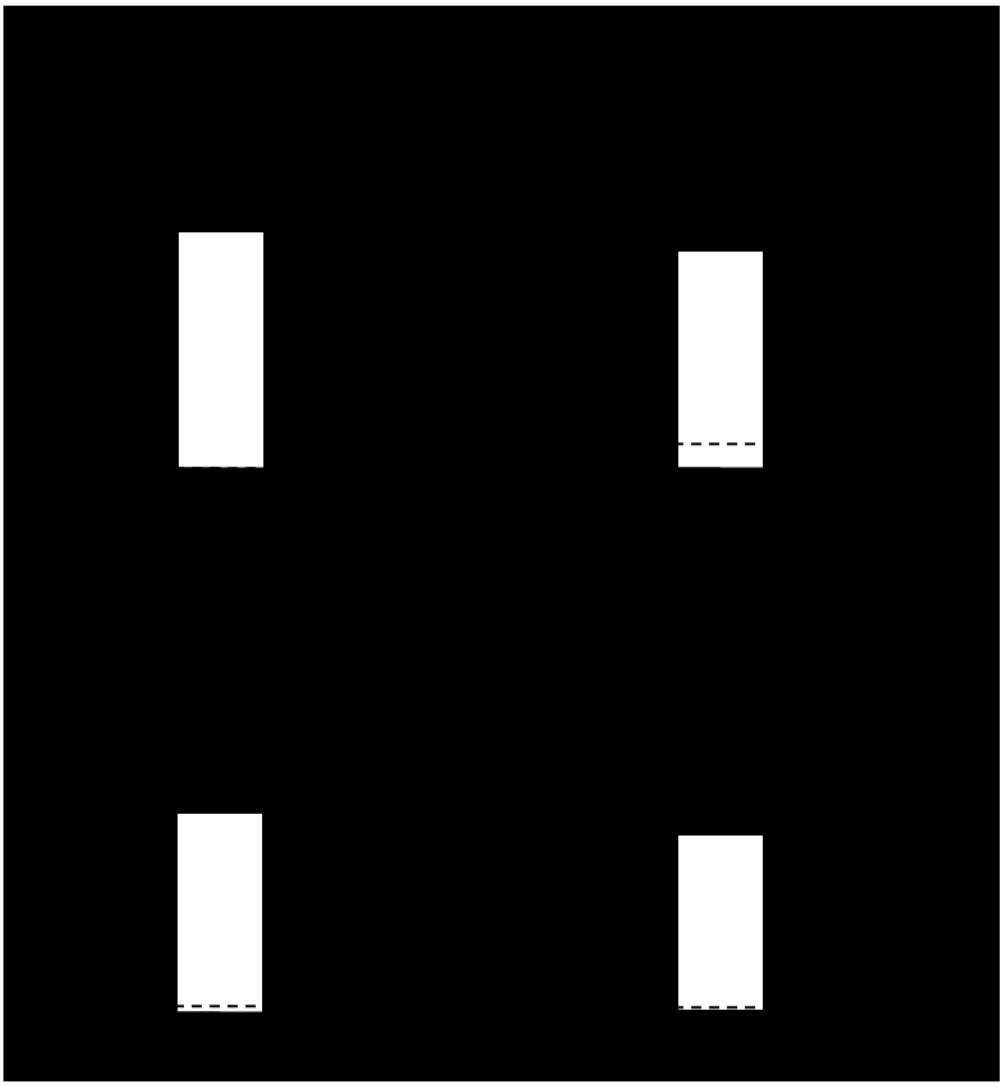


Figure 4.17 - Levels of lavage fluid (A) IL-6, (B) MCP-1, (C) KC and (D) MIP-2 in LPS+VILI model. Dotted line represents mean value in non-ventilated control animals (undetectable levels for IL-6). *P<0.05, **P<0.01 by unpaired *t*-test (A, B, C) and Mann-Whitney U-test (D). N=8-11/ group. Source – Bertok *et al.* [2]

Although the findings of the 'pure' VILI model would suggest that p55 dAb can attenuate high-stretch ventilation induced lung injury, the findings of this model (i.e. LPS+VILI) may represent an attenuation of the LPS effect primarily perpetuated by mechanical ventilation, rather than an effect on mechanical ventilation per se.

4.5 Discussion

Mechanical ventilation is an essential tool in the management of patients with ALI/ARDS, however this can exacerbate underlying lung injury and cause VILI. VILI can progress to systemic inflammation and multiple system organ failure (MSOF), which is a major cause of death amongst ARDS patients [291, 323]. Although low tidal volume ventilation in conjunction with other interventions reduced mortality in this patient population, it is not possible to eliminate completely the consequences of VILI in this manner. Manipulation of the inflammatory response to ventilation is considered to be the strategy most likely to be successful for the therapeutic treatment of VILI. In this chapter we investigated the use of a novel therapeutic technology, domain antibodies, for the first time in the area of acute lung injury, and showed that intratracheal administration of dAb targeting the p55 TNF receptor attenuated both pulmonary dysfunction and inflammation associated with VILI.

Model development

In order to test our hypothesis that intraalveolar targeting of p55 signalling could attenuate VILI, we first had to establish a robust model that allowed us to deliver the antibodies in fluid form into the alveolar space without significantly influencing respiratory mechanics. This was quite important for at least two main reasons. Firstly, standardisation of high-stretch was based on airway pressures wich are greatly influenced by respiratory mechanics, therefore high variability in these parameters could potentially introduce a lot of noise in the system and compromise standardisation. Secondly, our assessment of lung injury development relied, in part, on measuring respiratory mechanics, such as plateau pressure, resistance and elastance, and high variability in these parameters could potentially prevent us from

detecting a subtle effect of the antibody. Indeed, initial experiment in which high-stretch ventilation was initiated following some incubation period with intratracheally administered antibodies, failed to show any benefits of p55 dAb. We felt that this might be because intratracheal fluid instillation interfered with the surfactant layer of the lung hence changing its elastic properties. Also, fluid movements in the airways during respiration could result in variable airway resistance, particularly during high gas flow, which substantially influences peak inspiratory pressures.

To overcome these issues presented by fluid instillation to the airways, we 1) decided to standardise high-stretch by plateau pressure which is not influenced by airway resistance, 2) increased the incubation period before high-stretch ventilation and 3) tried microspraying to aid better fluid dissipation and absorption. Although, all these modifications somewhat helped to better standardise high-stretch ventilation and reduced the variability in respiratory mechanics, we still could not detect any positive effect of p55 dAb. We came to the conclusion that it was crucial to set high-stretch prior to antibody delivery to eliminate any influence of fluid instillation on model standardisation. We also decided to prolong the ventilation protocol, as too rapid injury development may have also masked any beneficial effects of p55 dAb. Having made these changes to the model design, we managed to produce a robust and reproducible VILI model, which allowed us to show some very encouraging and potentially clinically significant results.

The tidal volumes used in the finalised protocol (V_T 20-22ml/kg), although higher than used clinically in humans, would have induced only a moderately high degree of lung stretch in untreated healthy mice as mouse lungs are much more compliant than those of humans [857]. This was elegantly demonstrated by Wilson *et al.* who ventilated healthy mice with different level of V_T (10, 20, 30 or 40ml/kg), and

measured respiratory system compliance and arterial blood gases to evaluate physiological variables of lung injury, whereas lung wet:dry weight ratio, BALF protein and cytokines were measured to assess pulmonary oedema and inflammation [858]. They found that each ventilator setting reduced respiratory system compliance, but in mice ventilated with V_T of 10, 20 and 30 ml/kg, this was largely reversible by recruitment manoeuvre at the end of protocol, and only mice ventilated with 40ml/kg developed pulmonary oedema and inflammation [858]. The authors concluded that tidal volumes up to 20 mL/kg are unlikely to induce substantial lung overstretch in models using healthy mice, therefore signs of lung injury/inflammation using such models are likely to result from other factors, particularly alveolar derecruitment and atelectasis. However, in the current model due to the impact of the intratracheal fluid instillation, such settings were sufficient to induce injury within 4 hours ventilation. Since atelectasis was minimised by the use of PEEP and regular sustained inflations, the major component of the observed physiological changes can be ascribed to the development of stretch-induced pulmonary oedema.

While the timeframe of the current experiment is much shorter than ALI patients would be ventilated, it is comparable with the majority of published rodent studies of VILI and ALI.

Specific targeting of intraalveolar p55 signalling by dAb attenuates VILI

We found that high-stretch induced changes in respiratory mechanics and blood oxygenation, as well as production of pulmonary oedema were substantially improved when p55-specific blocking dAb was given intratracheally at the start of the 4 h high-stretch ventilation in the 'pure VILI' model. In complete contrast, total

blockade of intrapulmonary TNF signalling by a mouse anti-TNF mAb failed to exert protection.

Having demonstrated that intratracheally delivered p55 dAb attenuated the respiratory physiology in VILI, we questioned whether it could improve its inflammatory aspect. It is clear that ventilated patients are quite rarely without underlying inflammation the main causes of which are pneumonia and sepsis [22], and mechanical ventilation can exacerbate the inflammatory response [291, 859, 860] which may eventually lead to multiple organ failure (MOF), the main cause of high mortality amongst these patients.

Therefore to investigate the effects of p55-blocking dAb we chose a 2-hit model of VILI, which may better represent the clinical scenario. This comprised of an i.t. delivered LPS (subclinical dose) followed by 4h high-stretch ventilation (LPS+VILI). We found that in addition to the improvements in respiratory physiology, p55-dAb significantly reduced the number of marginated and transmigrated neutrophils, and decreased the levels of pro-inflammatory cytokine IL-6 and monocyte/neutrophil chemoattractant MCP-1 in lavage fluid, and showed strong trends towards decreases in the chemokines MIP-2 and KC.

While the precise sources of these inflammatory mediators in VILI remains uncertain, alveolar macrophages are likely contributors, and indeed we observed that alveolar macrophage activation (surface ICAM-1 expression [107]) was significantly attenuated by dAb treatment. Presumably as a consequence of this reduced intra-alveolar inflammation, margination and migration of neutrophils within the lung was also attenuated. The pivotal role of neutrophils and chemokines in the pathogenesis of VILI is well documented [105-109]. Recent evidence also indicates that margination of neutrophils to remote organs (including the lungs, heart and

kidney) during sepsis and thus their involvement in MOF is potentially triggered by induced expression of the MCP-1 receptor CCR-2 on neutrophils, which is mediated by Toll-like receptor signalling [861]. Therefore, reduction of intraalveolar MCP-1 by intratracheal p55-dAb treatment likely contributed to attenuated lung neutrophil numbers and injury in our study. However further investigations are needed to elucidate what significance of this reduced pulmonary inflammation may have in relation to other organ injury during VILI.

From the current data, it is not possible to precisely determine why the p55-specific dAb was clearly more effective in attenuating VILI than the anti-TNF mAb. One possibility is that this difference purely reflects greater biodistribution of the dAb compared to mAb due to differences in their biophysical properties. For example, in contrast to dAbs, mAbs possess an F_c fragment and are likely subject to elimination from the lung by different F_c-F_cR mediated mechanisms. Spiekermann *et al*. investigated the mechanisms by which IgG crosses epithelial surfaces in the mouse lung [822]. They identified an FcRn-mediated active cellular transport by bronchial epithelial cells, and using an erythropoietin-Fc fusion protein demonstrated that 12-15% of the intra-nasally delivered dose was absorbed to the systemic circulation. Although they ascribed this mechanism solely to bronchial epithelial cells based on their strong expression of FcRn, it cannot be excluded that alveolar epithelium contributed to the transport despite they are low FcRn expresser cells.

Another possibility by which intrapulmonary delivered monoclonal antibodies may be eliminated is their tendency to bind to surfactant proteins and being phagocytosed by alveolar macrophages, a mechanism which is also related to Fc-FcR interaction as shown by Lin *et al*. [823]. The authors demonstrated that surfactant protein-A (SP-A) binds to the F_c portion of IgG and increases the uptake of

IgG-coated particles by alveolar macrophages. However, this interaction of IgG with SP-A did not affect the antigen binding affinity in their study. Therefore, if any of these aforementioned mechanisms are involved, it is tempting to speculate that the poor results of anti-TNF treatment in our experiments were more likely due to a quicker elimination of the antibodies by bronchial epithelial cells or alveolar macrophages before the high-stretch induced production of TNF occured, rather than to impaired binding of TNF.

This lack of efficacy of anti-TNF treatment to attenuate the physiological manifestations of VILI are somewhat at odds with a previous study by Imai *et al.* showing a positive effect of anti-TNF in a rabbit model of saline lavage followed by injurious ventilation [334]. One possible explanation for this is that in their 2-hit model pulmonary inflammation (e.g. marginated neutrophils) played a more significant role in lung injury progression than in our 1-hit model, and this was significantly attenuated by anti-TNF mAb. Our results do, however, reflect the disappointing results from clinical trials of sepsis/ARDS patients [616, 617, 624, 625, 862].

In addition to the biophysical properties of the respective antibodies, we believe that target antigen specificity is vitally important and propose that the inefficacy of anti-TNF mAb in this model is potentially due to differential TNF receptor signalling. Our group previously showed using knockout mice that signalling through p55 promoted the development of high stretch-induced pulmonary oedema, while signalling through p75 protected against oedema formation, and that these opposing effects seemed to be independent of TNF-mediated leukocyte recruitment [639]. Importantly, mice lacking both TNF receptors showed no protection from oedema, a finding consistent with that of Yoshikawa *et al.* [863]. These data from TNFR knockout animals suggest that non-selective anti-TNF treatment would not be

effective to attenuate pulmonary oedema during VILI. Interestingly, in an earlier study, we showed that the monoclonal anti-TNF antibody used currently attenuated alveolar neutrophil accumulation in VILI [109]. Similarly, Wolthuis *et al.*, found that inhibition of total TNF signalling by a p75 receptor-Fc fusion protein (Etanercept), failed to attenuate high stretch-induced pulmonary oedema but reduced neutrophil influx and BALF cytokine levels [640]. Taken together, these data suggest that while therapy designed to block TNF *per se* may be effective in reducing pulmonary inflammation associated with VILI, it has little impact on pulmonary oedema, presumably as any beneficial effects of signalling through the p75 receptor on pulmonary fluid balance are inhibited. For example, it was demonstrated *in vitro* that activation of p75-associated Etk/Bmx tyrosine kinase [776] in epithelial cells results in cytoskeletal re-arrangement thus increased transepithelial resistance, protecting from hypoxia–induced permeability [778]. TNF receptor p75 may also protect from TNF-induced apoptosis in endothelial/epithelial cells, thus help to maintain alveolo-capillary barrier integrity and function [729].

Our study may look somewhat limited by not comparing the effects of p55 receptor inhibition by a dAb to that by a mAb. This was for two main reasons: First, as mentioned before, the use of monoclonal antibodies against the p55 receptor can result in receptor activation instead of inhibition due to receptor cross-linking as indicated in different cellular assays [820, 821, 844]. These findings are consistent with our previous preliminary studies where intratracheally given mouse anti-p55 mAb worsened VILI. Second, monoclonal antibodies are not suitable for pulmonary delivery for various reasons as described above, whereas previous data strongly suggest that TNF signalling should be inhibited in the alveolar space in order to improve VILI. We also aimed to further explore the role of differential TNF receptor

signalling in VILI. However, as neither anti-TNF nor anti-p75 dAb were available at the time of our study, we used a monoclonal anti-TNF antibody for this, which may not be ideal, but was previously proved to efficiently reduce VILI-induced inflammation by our group [109]. Despite these limitations, our study provides novel, valuable insight to the role of TNF signalling in VILI pathophysiology with potentially strong clinical significance.

In conclusion, we have shown for the first time that specific inhibition of intraalveolar TNFRp55 signalling by a domain antibody can ameliorate pulmonary oedema and inflammation during VILI in mice. Targeting intraalveolar p55 receptor signalling by domain antibodies may open new therapeutic approaches for ventilated patients with ALI.

5. Selective targeting of p55 TNFR signalling during ventilator-induced lung injury by systemically delivered domain antibodies

Having demonstrated that selective inhibition of intraalveolar p55 TNFR signalling by domain antibodies directly delivered to the lungs can significantly attenuate VILI, we wanted to investigate if targeting the same receptors via an indirect route of delivery, namely systemic as opposed to topical, would be similarly effective.

To address this, we treated mice with a continuous intravenous infusion of dAb whilst being exposed to high-stretch mechanical ventilation, and assessed development of lung injury by monitoring changes in respiratory physiology and measuring levels of inflammatory chemokines/cytokines in the lungs and in the plasma.

We found that, although similar amounts of p55 dAb could be delivered to the alveolar space by intravenous infusion as with the intratracheal delivery in Chapter 4 experiments, neither pulmonary oedema nor inflammation was attenuated. The reasons behind this lack of effect are not yet clear, and require further investigations before this route of delivery could be safely rejected.

5.1 Background

Using TNFR knock-out mice, our group has previously demonstrated that the two receptors of TNF play a directly opposing role in the pathogenesis of VILI, i.e. mice lacking the p55 receptor were protected, whereas p75 knock-outs were more prone to develop pulmonary oedema. Interestingly, lack of total TNFR mediated signalling in TNFR double knock-out animals led to similar amount of injury to that in wild-type mice, which may explain why clinical trials with anti-TNF antibodies failed to improve the outcome in ALI. These findings indicated that specific targeting of p55 receptor as opposed to total TNF blockade is potentially necessary to attenuate VILI. However, it was not clear from this study where exactly in the lung p55 signalling should be targeted, on the pulmonary epithelial or endothelial cells or both. Therefore further studies in the lab were dedicated to investigate and characterise the role of individual TNFRs in the lungs.

As discussed in Chapter 3, we found that p55 is the dominant receptor on pulmonary endothelial cells and the major regulator of TNF-induced adhesion molecule expression, which could be a reflection of its relative expression level to that of p75. However, this role may subside during inflammatory conditions such as sepsis, when its expression is dramatically decreased compared to p75, which remains unaffected. Similarly, using *in vivo* and *ex vivo* mouse models of VILI our group has previously demonstrated shedding of both TNFRs from the surface of pulmonary endothelial cells and lung marginated monocytes 2 hours after high-stretch mechanical ventilation (A Dorr – PhD thesis).

On the contrary, situation seems to be the opposite in the alveolar compartment: the same author found surface expression of both TNFRs on pulmonary epithelial cells and alveolar macrophages with a dominance of p75 (only

subtle in the case of pulmonary epithelial cells). He also demonstrated that direct inflammatory insult to the lungs, i.e. i.t. LPS instillation did not alter the expression level of the two TNF receptors on pulmonary epithelial cells [819]. Intratracheal LPS stimulation also resulted to selective up-regulation of soluble p75 receptors in the lavage fluid, lending further indirect support that cell surface expression of p55 receptor is well maintained throughout the alveolar compartment. All these findings let us to conclude that selectively targeting of p55 signalling in the alveolar space would be the most reasonable option to attenuate VILI pharmacologically. Indeed, when p55 receptor signalling was inhibited by intratracheally instilled domain antibodies both pulmonary oedema and inflammation were attenuated in a mouse model of VILI, as demonstrated in the previous chapter.

Intrapulmonary delivery of drugs in the clinical practice, especially in ventilated patients can be technically challenging thus the intravenous route would be more appealing at least from a practical point of view. Although the biophysical properties of domain antibodies allow penetration through the alveolar-capillary membrane and consequent accumulation in the alveolar space, it is not clear whether giving the p55 targeting dAb systemically would have similar benefits in VILI compared to direct pulmonary delivery. On the other hand, data in Chapter 3 demonstrated that p55 signalling may have an important role in pulmonary microvasculature activation, which is an important feature in the pathogenesis of VILI; therefore it is reasonable to think that giving the p55 dAb intravenously may even have additional benefits to the intratracheal route.

5.2 Aims

In this chapter I investigated the effects of systemically delivered p55-specific domain antibody on the development of VILI using the *in vivo* mouse model established in chapter 4. There were 2 main aims:

1. To identify an intravenous domain antibody dose that would ensure accumulation of substantial intraalveolar levels.

2. To compare the effects of i.v. vs. i.t. delivered p55-specific domain antibody on pulmonary oedema and inflammation during VILI.

5.3 Protocols

Pharmacokinetic studies to identify the adequate intravenous dAb dose

In this study I aimed to deliver the p55 receptor targeting domain antibody to the alveolar space indirectly by using the intravenous route. Because of its small size and biochemical properties, domain antibodies can cross the alveolar-capillary membrane from each direction. However, the half-life of p55 dAb in the plasma is extremely short ($T_{1/2}$ ~ 20min, personal communication with GSK) due to rapid renal excretion; therefore, in order to achieve significant amount of dAb in the alveolar space, it has to be given in a continuous intravenous infusion. Domain antibodies can be modified structurally, e.g. by PEGylation, to increase their plasma $T_{1/2}$ without affecting their antigen specificity or neutralising activity, however this has an impact on their tissue penetration properties given the increased molecular size. As in this particular study the aim was to get the dAb across the alveolar-capillary membrane, such a modified molecule could not be used.

In order to identify an intravenous dose that would ensure intraalveolar accumulation of the dAb in a comparable amount to that delivered in the i.t. dAb+VILI model, pharmacokinetic studies were carried out.

For this, mice were ventilated with high-stretch settings that would result in significant amount of injury within 240min, i.e. $P_{plateau}$=30cmH_2O equating to V_T=30.9±1.4ml/kg, PEEP=3cmH_2O [858]. Immediately after setting high-stretch parameters, a continuous infusion of p55-specific dAb was started via the jugular vein at a rate of 50µg/h (2µg/g/h at an infusion pump rate of 0.2ml/h). After 30, 60 or 120min experiments were terminated, mice euthanized by anaesthetic overdose and cardiac puncture and lung lavage performed. BALF and blood samples were centrifuged and the supernatant and plasma, respectively, stored at -80ºC until analysis for level of p55 dAb by ELISA (GSK, Stevenage, UK).

We found that p55 dAb given at this dose resulted in gradual accumulation of the antibody in the alveolar space reaching an average concentration of nearly 15ng/ml (1nM) by 120min, approximately 20% of the circulating level (Figure 5.1). However, this was felt to be too low to sufficiently inhibit p55 TNFR signalling, therefore, based on this pharmacokinetic study and calculations, a 5 times higher dose, i.e. 250µg/h was used in the subsequent experiments. Analysis of samples (performed by GSK) from these confirmed that a reasonable amount of alveolar p55 dAb (~500ng/ml) was achieved by the end of the 240min protocol with this intravenous dose, but it is unclear how does this compare to the intraalveolar levels of p55 dAb in the intratracheal model (Chapter 4), as we did not measure it.

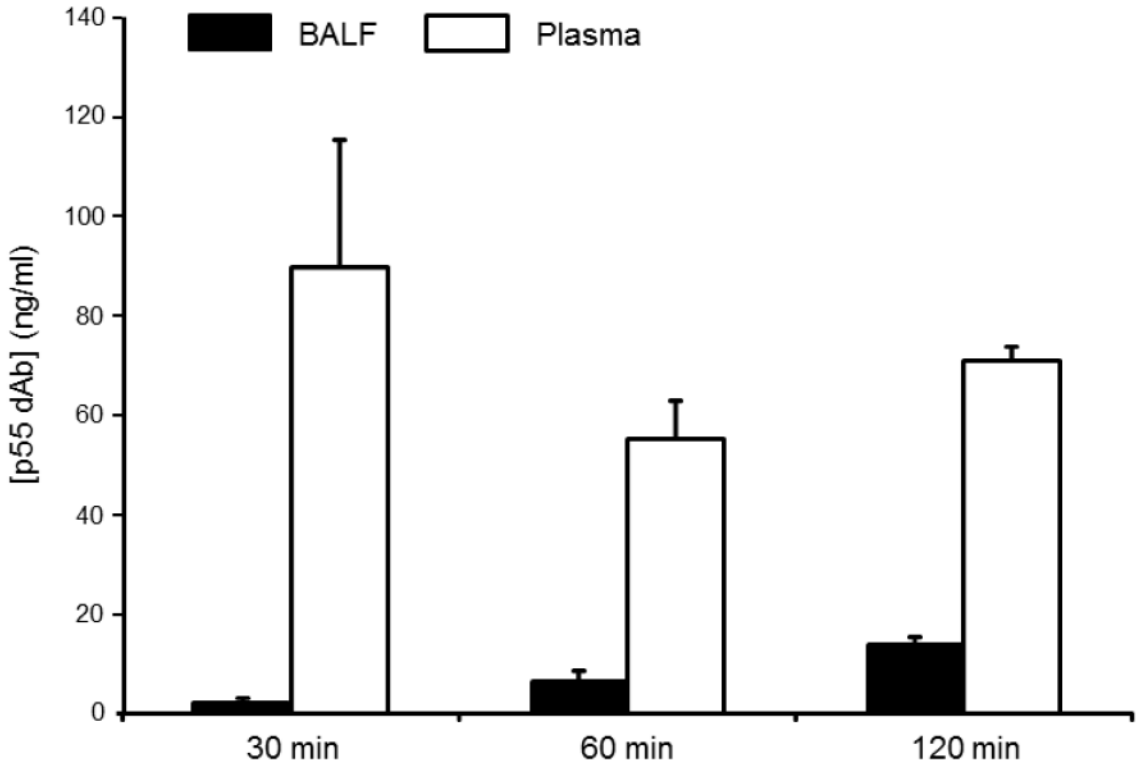


Figure 5.1 – Lavage fluid (BALF) and plasma levels of p55 dAb in mice with i.v. infusion of the antibody during high-stretch ventilation. Mice were ventilated with injurious settings ($P_{plateau}$=30cmH_2O, V_T~31ml/kg) whilst receiving a continuous infusion of p55 dAb (50µg/h) via the jugular vein, then killed after 30, 60 or 120min, BALF and plasma samples obtained and analysed for p55 dAb levels by ELISA. Intravenous infusion of p55 dAb resulted in a gradual accumulation of the antibody in the alveolar space (BALF), reaching approximately 20% of the plasma level by 120min of infusion. Mean+SD, N=3/each time point.

Ventilation protocol for i.v. dAb + VILI model

To be able to compare the injury progression and respiratory physiology changes in this model with that in the intratracheal model as directly as possible, it was necessary in this set of experiments to administer an intratracheal bolus of dummy dAb the same way as described in Chapter 2 and 4 after setting high-stretch ventilation, whereas either dummy or p55 dAb was given intravenously in a

continuous infusion (250µg/h, 10µg/kg/h) via the jugular vein using an infusion pump (0.2ml/h). Other protocol parameters were the same as described in Chapter 4.

In brief, following surgical preparation for the *in vivo* mouse mechanical ventilation system (during which animals were ventilated with 8-9 ml/kg) and baseline measurements, ventilation was then set to produce VILI standardized by inspiratory plateau pressure ($P_{plateau}$) of 14 cmH_2O equating to V_T of ~22 ml/kg, with 3 cmH_2O PEEP, 90/min respiratory rate, and oxygen+4% CO_2 (to prevent hypocapnia). Immediately following this, mice received an intratracheal bolus of dummy dAb (25µg, 50µl) and at the same time continuous intravenous infusion of either dummy dAb or p55 dAb was started through the jugular vein (250µg/h, 10µg/g/h, 0.2ml/h). To maintain the same fluid load as in the intratracheal model, saline infusion rate through the carotid line was set at 0.2ml/h.

Ventilation using the same constant high V_T was continued in all groups for 240 minutes or until mean BP dropped below 45 mmHg [337]. Anaesthesia was maintained by bolus administrations of intraperitoneal ketamine:xylazine every 20-25 minutes. Two animals displayed very rapid haemodynamic deterioration in the dummy group, whereas all p55 dAb treated mice completed the protocol. These prematurely terminated experiments were excluded from analyses. $P_{plateau}$, respiratory system elastance (E_{rs}) and resistance (R_{rs}) were measured every 20 minutes, followed each time by sustained inflation (35cmH_2O, 5 seconds) to avoid the development of atelectasis. ABG were assessed at predetermined points throughout the protocol (Figure 5.2).



Figure 5.2 - Scheme of ventilation protocol. Mice were anaesthetised, tracheostomised and ventilated with non-injurious ventilation for approximately 15-20min during instrumentation (*dotted line*). Injurious ventilation parameters using high tidal volumes (V_T) were then introduced, followed immediately by instillation of i.t dummy dAb and continuous i.v. infusion of either dummy or p55 dAb via the jugular vein. After instillation of i.t dummy dAb, 4 sustained inflations (35cmH_2O, 5 seconds) were applied to help the fluid distribute within the lungs. Respiratory mechanics were assessed every 20min, followed each time by sustained inflation. Arterial blood gases (ABG) were assessed at predetermined time points (*Start, 120min, End*).

Upon termination of experiments, mice were killed by anaesthetic overdose and cardiac puncture and saline lavage performed, as described in chapter 2. Samples were centrifuged and BALF supernatants and plasma samples aliquotted and stored at -80°C. Thawed samples were quantified for total protein as a further indicator of pulmonary oedema/alveolar barrier dysfunction, and for IL-6, KC, MIP-2 and MCP-1 by colorimetric sandwich ELISA as indicator of intraalveolar and systemic inflammation. Samples from non-ventilated animals served as controls.

5.4 Results

Intravenous anti-p55 domain antibody does not attenuate high-stretch induced changes in respiratory physiology and pulmonary oedema

All animals were ventilated with the same high stretch protocol (V_T 22.1±1.7 ml/kg vs. 21.9±2.6 ml/kg for dummy dAb and p55-specific dAb treated mice respectively; n=6-7/group) for up to 240min. High-stretch mechanical ventilation caused a substantial deterioration in lung function in dummy dAb treated animals (i.e. i.t.-dummy + i.v.-dummy) consistent with pulmonary oedema formation. This was represented by an exponentially increasing $P_{plateau}$, Ers and Rrs towards the end of the 4h ventilation protocol (Figure 5.3/A-C). The magnitude and nature of these ventilation-induced changes were very similar to that observed in the 'intratracheal' model (chapter 4). In contrast to our findings using direct intratracheal blockade, inhibition of p55 signalling by i.v. p55-dAb did not attenuate the high-stretch induced changes seen in dummy dAb-treated animals.

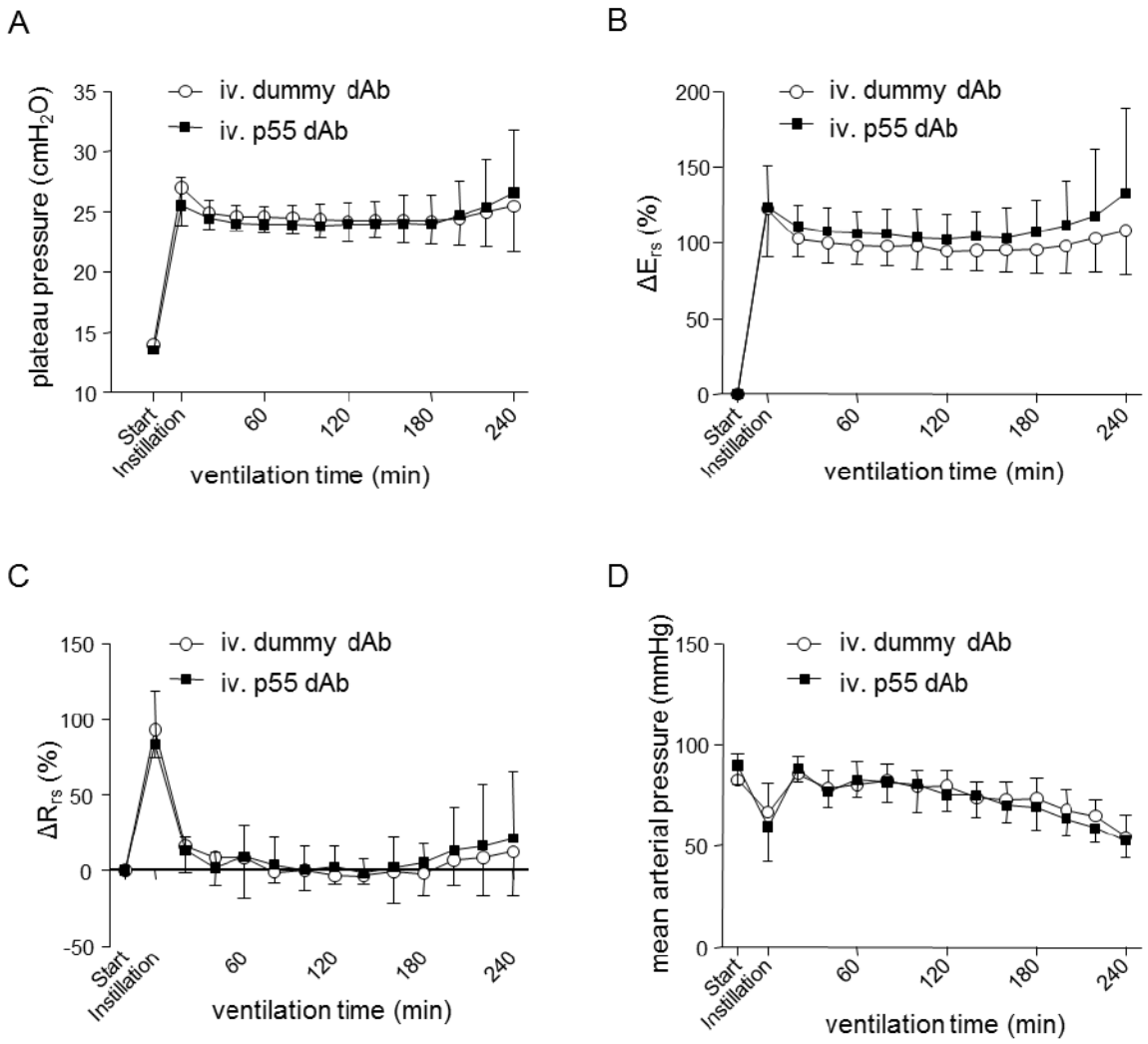


Figure 5.3 - (A) Plateau pressure ($P_{plateau}$), (B) respiratory system elastance (E_{rs}) and (C) resistance (R_{rs}), (D) mean arterial pressure in mice treated with i.v. dummy or p55 dAb and ventilated with high-stretch settings. After completing surgical preparation ventilation settings were changed to produce high-stretch ($P_{plateau}$=14cmH_2O, V_T~22ml/kg, PEEP=3cmH_2O) and mice received an intratracheal bolus of 25µg (50µl) dummy dAb and a continuous intravenous infusion of either dummy or p55 dAb (250µg/h) was started at the same time, and animals ventilated for up to 240min. Changes in E_{rs} and R_{rs} are expressed as percentage increase in relation to 'Start' values. Treatment groups showed no significant difference in these parameters. N=6-7/group.

Similarly, p55 dAb treatment had no beneficial effect on gas exchange (Figure 5.4/A-C) or alveolar epithelial permeability as indicated by lavage fluid protein (Figure 5.4/D).

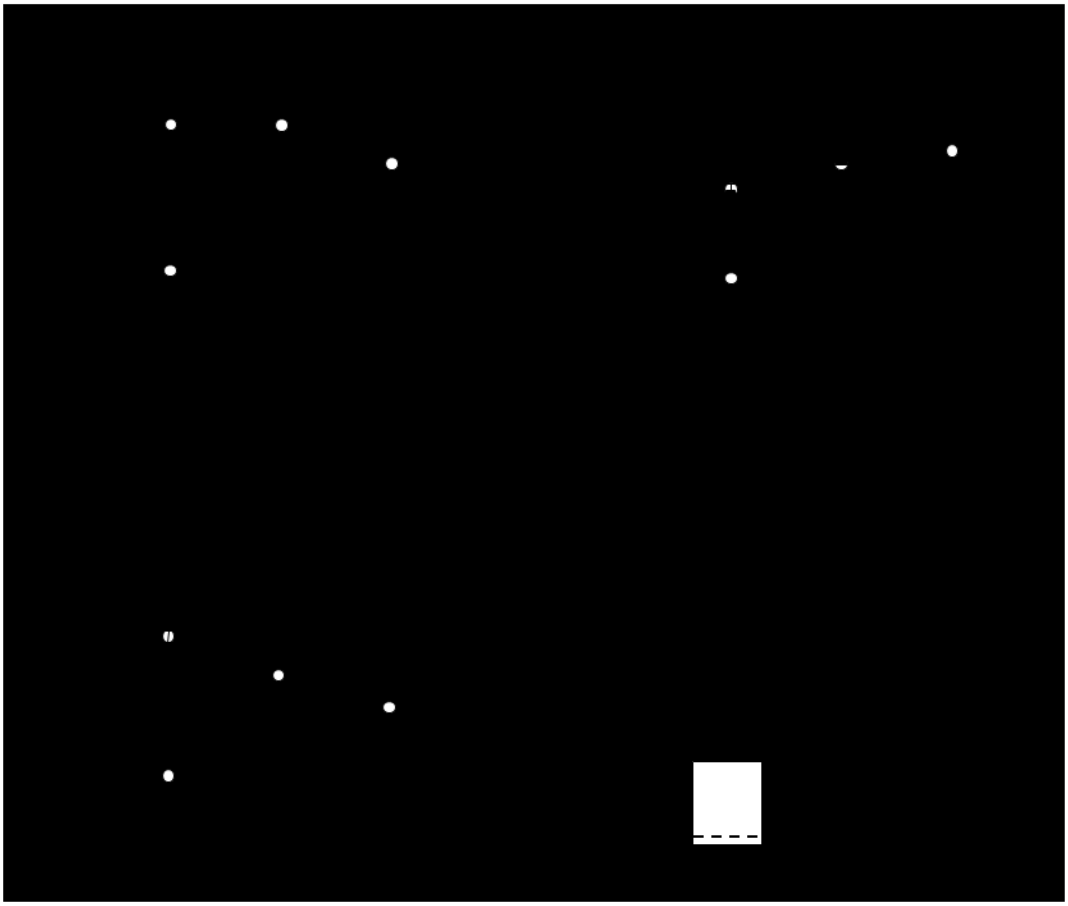


Figure 5.4 - (A) arterial PO_2, (B) PCO_2, (C) bicarbonate (HCO_3^-) and (D) lavage fluid protein in mice treated with i.v. dummy or p55 dAb and exposed to high-stretch ventilation. VILI-induced changes were not attenuated by intravenous p55 dAb infusion, in fact decrease in arterial bicarbonate over time was significantly worse in the p55 dAb group (# $P<0.05$ for interaction between 'treatment' and 'ventilation time' by 2 way ANOVA). Dotted line represents mean value in non-ventilated control animals. N=6-7/group.

Continuous i.v. infusion of p55-specific domain antibody does not attenuate levels of intraalveolar or systemic inflammatory cytokines/chemokines

Having found that intravenous infusion of p55 targeting dAb had no effect on respiratory physiology, I wanted to see whether it had any positive effects on the inflammation aspect of VILI. Similarly to the 'pure' VILI model described in chapter 4, this VILI model did not result in substantial leukocyte infiltration to the lung based on BALF neutrophil counts in preliminary studies, therefore flow cytometry was not

performed on lung samples. Instead, levels of the same inflammatory cytokines/chemokines were analysed as in the LPS+VILI model in chapter 4, i.e. IL-6, MCP-1, KC and MIP-2.

High-stretch ventilation resulted in significant increase in IL-6, KC and MIP-2 in the lavage fluid as compared to non-ventilated controls; however, there was no difference between treatment groups (Figure 5.5). Interestingly and unexpectedly, level of MCP-1 was lower in both treatment groups compared to non-ventilated control, however still without difference between dummy and p55 dAb treated animals.

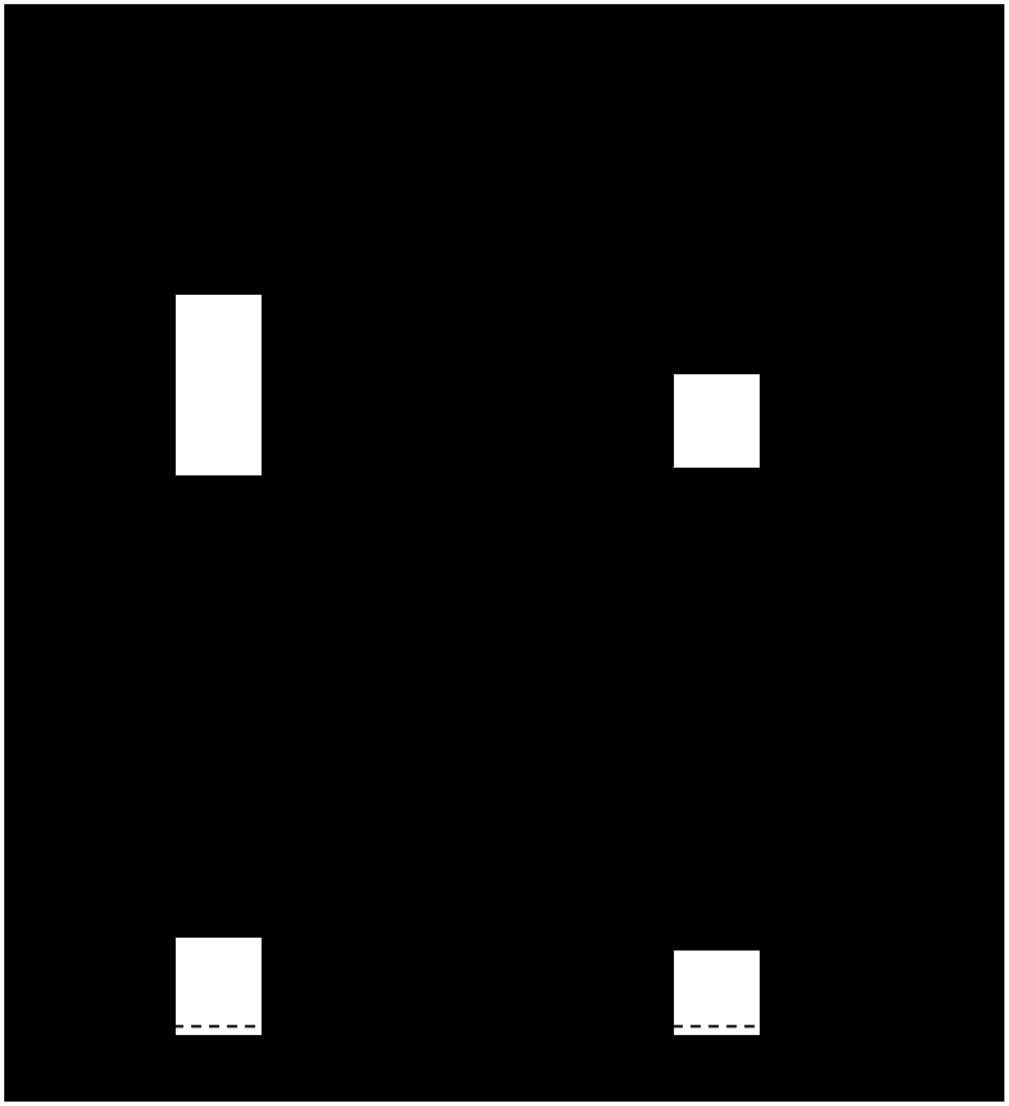


Figure 5.5 - Levels of lavage fluid (A) IL-6, (B) MCP-1, (C) KC and (D) MIP-2 after 240min high-stretch ventilation in mice treated with intravenous dummy or p55 dAb. No significant differences were detected between treatment groups. Dotted line represents mean value in non-ventilated control animals (undetectable levels for IL-6). Mean+SD, N=6-7/group.

Although i.v. p55 dAb infusion had no effect on the levels of inflammatory cytokines/chemokines in the alveolar space, it could have a positive impact on systemic inflammation. To address this, plasma levels of the same cytokines/chemokines were also measured. Levels of IL-6, MCP-1 and KC were significantly higher as compared to non-ventilated controls - which had undetectable levels - and IL-6 and KC were not different across the treatment groups (no MCP-1

data for p55 to compare) (Figure 5.6). Interestingly, MIP-2 levels were below the detection limit of the ELISA assay in both treatment groups (not shown).

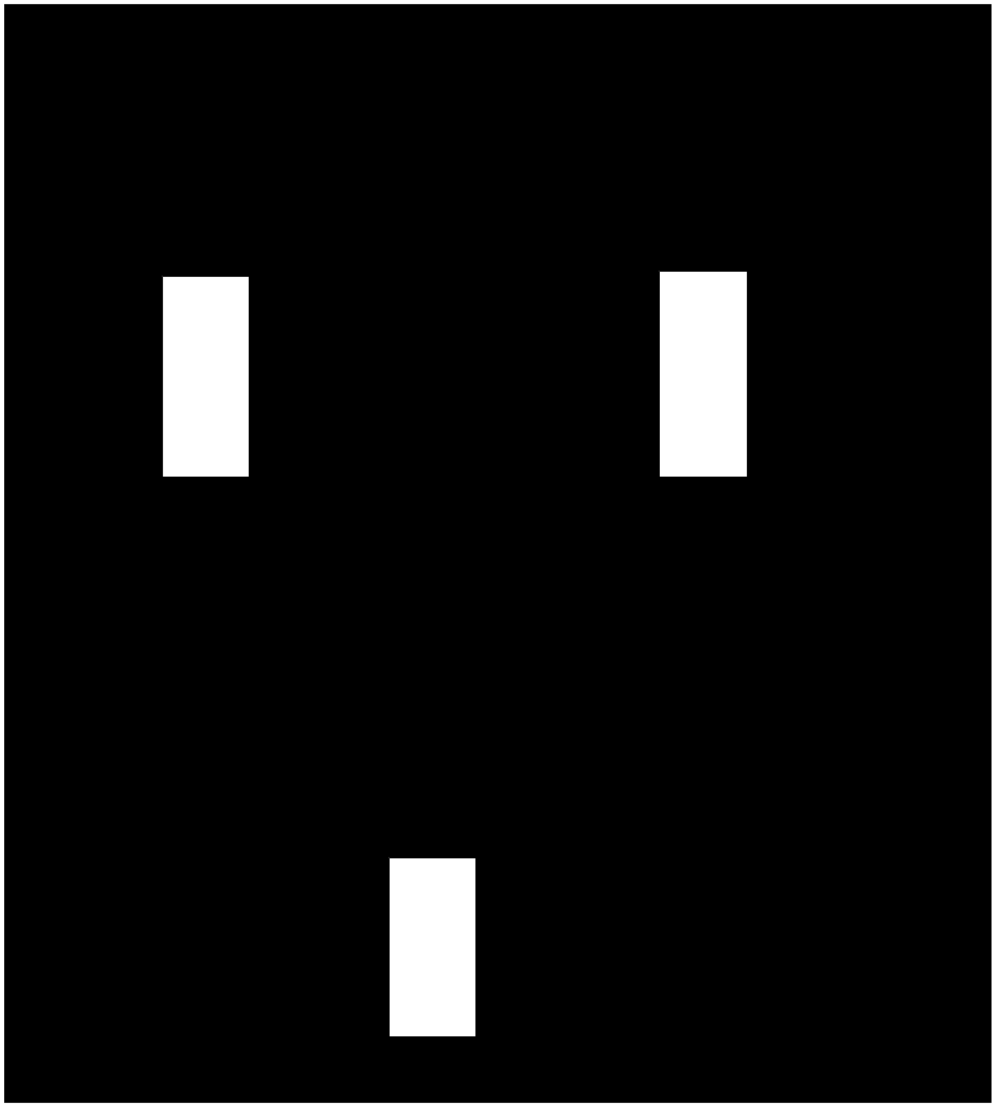


Figure 5.6 - Levels of plasma (A) IL-6, (B) MCP-1 and (C) KC at the end of 240min high-stretch ventilation in mice treated with intravenous dummy or p55 dAb. Note data is not available for plasma MCP-1 in p55 dAb treated group due to limited amount of sample (also used for p55 dAb detection). Treatment groups were not significantly different. Levels were undetectable in non-ventilated control animals. Mean+SD, N=6-7/group.

5.5 Discussion

ARDS patients have elevated TNF in their BALF [200], and levels are also increased when patients are ventilated with higher V_T as compared to those ventilated with

lung-protective, i.e. low V_T settings [330, 331]. Similarly, high V_T ventilation produces increases in BALF TNF levels in *in vivo* and *ex vivo* animal models of VILI [186, 322, 334, 335, 351, 681]. Using a 'pure' VILI model where mice are exposed to high V_T ventilation without any underlying lung injury, our group previously demonstrated that this up-regulation of biologically active intraalveolar TNF is transient and happens before lungs are flooded with oedema fluid [337]. Using genetically modified mice, the same authors later showed that the two receptors of TNF play directly opposing roles during injurious high-stretch mechanical ventilation, with p55 promoting but p75 preventing pulmonary oedema [639]. These findings suggest that targeting p55 receptor signalling specifically rather than total TNF blockade, particularly in the alveolar space, could be beneficial in VILI. Data from Chapter 4 confirmed this hypothesis; however it remains unclear whether targeting p55 receptors systemically per se or in addition to intraalveolar p55 blockade could have further advantages.

Therefore in this chapter we investigated the effects of systemically delivered p55 dAb on the progression of VILI with a primary focus on respiratory physiology. For the sake of comparability with the 'intratracheal' model (in terms of lung mechanics, tidal volumes, etc), it was necessary in this set of experiments to administer an intratracheal bolus of dummy dAb the same way as in the previous model, whereas either dummy or p55 dAb was given intravenously in a continuous infusion via the jugular vein using an infusion pump. The selected dose of intravenous dAb was based on preliminary pharmacokinetic studies and designed to achieve a reasonable concentration of p55 dAb (~500ng/ml) in the alveolar space. By this, at least theoretically, we could inhibit p55 signalling not only systemically (with a primary focus on pulmonary microvasculature) but also in the alveolar compartment. This is an important point as ventilator-induced lung injury is

considered to be a combined epithelial and endothelial injury at cellular level [283]. Other protocol parameters were the same as in the previous 'intratracheal' model in Chapter 4.

Effects of i.v. p55 dAb on respiratory physiology

The magnitude and nature of the ventilation-induced changes (respiratory mechanics, gas exchange, BAL protein) in control animals (i.e. i.v.-dummy dAb treated) were very similar to that observed in the 'intratracheal' model, however, in contrast to our findings using direct intratracheal blockade, inhibition of p55 signalling by i.v. p55-dAb did not attenuate these. To see if systemic administration of p55 blocking dAb had any effect on pulmonary and/or systemic inflammation, we assessed levels of IL-6, MCP-1, KC and MIP-2 in bronchoalveolar lavage fluid and plasma, but found no differences between treatment groups.

The apparently lacking benefits of intravenously given p55 dAb in VILI are not fully understood. From the aspect of targeting intraalveolar p55 signalling, the dAb should be able to enter the alveolar compartment. Although preliminary pharmacokinetic studies did indeed indicate accumulating concentrations of p55 dAb in the alveolar space over time during high-stretch ventilation, TNF may have signalled through the p55 receptor already before effective concentrations of p55 targeting dAb were achieved. Our group previously showed that TNF is produced early at the course of high-stretch ventilation before significant pulmonary oedema develops [337], indicating an intraalveolar source, but this study only looked at TNF levels 2 hours after the start of high V_T ventilation and at later time points. Therefore it remains unclear how soon upon mechanical stretch TNF is produced, particularly in the model we used currently.

Using a mouse isolated perfused lung (IPL) model, von Bethmann and co-workers showed that TNF is up-regulated almost 2-fold as early as 30 minutes after high-stretch mechanical ventilation at the transcription level, which then returns to control values by 150 minutes [336]. Although this study also examined changes in TNF protein in the perfusate, it lacked investigations of alveolar TNF levels. A later study by Tremblay *et al.* using a mouse isolated non-perfused system not only confirmed this transient up-regulation of TNF mRNA in the lungs, but also showed production of TNF protein in the alveolar space after 30 minutes of high-stretch ventilation with a gradual increase over time until the end of the 180 minutes ventilation protocol [682]. It is not clear why the protein expression did not follow the trend of mRNA, but it could be due to reduced removal of the cytokine by blood flow and lymphatic drainage [610]. More importantly from the perspective of our study, these findings by Tremblay and co-workers indicate that high-stretch ventilation can induce a very rapid release of TNF into the alveolar space.

Although our preliminary pharmacokinetic studies demonstrated detectable concentration of p55 dAb in the alveolar compartment as soon as 30 minutes after starting the i.v. infusion (and high-stretch ventilation in fact), this was probably inadequate to sufficiently inhibit p55 receptor signalling. There are two possible ways to overcome this issue. One is to increase the dose of i.v. p55 dAb and thus achieving higher and potentially more sufficient intraalveolar antibody concentrations. The second is to start the systemic administration of dAb some time before the start of high-stretch ventilation allowing the antibody to accumulate in the alveolar compartment before active TNF is produced. With VILI, this kind of prophylactic approach is possible even in the clinical setting. The most likely scenario is that both of these approaches are necessary to succeed. However,

further investigations will first need to clarify the kinetics of alveolar TNF production in this model in order to adequately design an experimental protocol to sufficiently inhibit alveolar p55 signalling by intravenously administered domain antibodies.

Both the work by Dorr (PhD thesis) and our characterisation study in Chapter 3 clearly indicated that p55 receptor is shed from the surface of pulmonary endothelial cells early in the course of VILI and endotoxaemia, respectively; hence its blockade may not confer further benefits in ALI. However, in both of the above studies the receptor expression was assessed only 2 hours after the initiation of insult, and it is not clear how rapidly p55 was down-regulated on the cell surface. Therefore, it is still possible that this receptor has a role in activating the pulmonary endothelium at a very early stage of VILI, thus contribute to injury progression.

For this, however, TNF should be present in the circulation, either in a soluble or membrane bound form, almost at the start of high-stretch ventilation. Von Bethmann *et al.* showed in the study mentioned above that TNF is increased in the perfusate of the mouse IPL system as early as 30 minutes from the start of high-stretch ventilation [336]. Although the source of TNF was not investigated in that study, as any increase in alveolo-capillary permeability is very unlikely at such early stage of VILI progression, an alveolar source is unlikely. Previous work from our group suggests that residing leukocytes in the pulmonary microvasculature, particularly monocytes could be the primary source (Wakabayashi – thesis).

In our study, plasma content of TNF was only assessed at the end of the four hours ventilation protocol which showed undetectable levels, but this does not mean that TNF could not be present in the circulation at earlier stages of high-stretch ventilation. However, no matter how quickly TNF is produced systemically in VILI, in the current study p55 blocking domain antibodies were present in the circulation in

high concentrations from the very beginning of high-stretch ventilation, and should be able to block p55 signalling on PECs at any stage of the injury development. Unfortunately, lung injury in this model was primarily assessed by development of pulmonary oedema (and consequent respiratory physiology changes), and although PECs have a very important role in maintaining alveolar-capillary integrity, this is but one of their many roles. For example, we did not investigate whether p55 dAb had any effect on activation of PECs specifically, like up-regulation of adhesion molecules (Chapter 3) and subsequent leukocyte margination or release of cell-specific activation markers such as von Willebrand factor [864-869], the latter of which has a predictive value for mortality and organ-failure in ARDS [63, 64, 869]. Overall, however, it seems that systemic TNF and thus p55 signalling has little impact on the acute physiological changes in this and our previous VILI models, possibly because epithelial damage is the main pathogenesis.

Effects of i.v. p55 dAb on respiratory physiology

We also measured the inflammatory cytokine IL-6 and inflammatory chemokines monocyte chemoattractant protein-1 (MCP-1), keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2) (the murine counterparts of human IL-8) both in bronchoalveolar lavage fluid and plasma.

These inflammatory mediators are increased in ARDS patients and their levels correlate with MOF and hence mortality [330, 333]. It was also demonstrated that high-stretch mechanical ventilation leads to increased pulmonary production of these substances, which then, along with other inflammatory materials such as pathogens and endotoxin, translocate to the systemic circulation [331, 335, 351, 356-358]. This is the fundamental element of the biotrauma hypothesis of VILI [291,

318]. Indeed, those patients who were ventilated with lung-protective strategy had lower levels of these cytokines/chemokines in their BALF and plasma and less likely developed organ-failure [330, 333]. Alveolar epithelial cells and macrophages are considered primary producers of these cytokines/chemokines [341, 342], nevertheless pulmonary endothelial cells also release them in response to stretch [343]. Their production is mediated at least in part by TNF signalling [660, 661, 663], and certainly their levels were reduced in the LPS+VILI model in mice given the p55 blocking dAb (Chapter 4).

In the current model BALF levels of these mediators were not reduced by p55 dAb treatment, suggesting that the antibody was not present in sufficient amounts in the alveolar compartment when their production and/or release by alveolar epithelial cells and macrophages was triggered. Another potential explanation is that in the LPS+VILI model the inflammation was more pronounced so that TNF signalling may had a more significant role in mediating cytokine production thus p55 receptor blockade could be more effective.

With regards to the plasma concentrations of these mediators, we found that they reflected the picture in BALF and p55 dAb was without effect. This suggests that PECs were either not major producers, or if were, then p55 blockade had no significant effect on their chemokine release. However, it is not clear why MIP-2 was not detectable in the systemic circulation. One possible explanation is that MIP-2, although produced in the alveolar compartment, did not cross the alveolar-capillary membrane. Quinton *et al.* demonstrated in i.t. LPS challenged rats that both MIP-2 and cytokine-induced neutrophil chemoattractant (CINC, rat homologue of KC) is produced and detectable in the alveolar compartment (BALF), but only CINC could be found in plasma [870]. They had the same findings when rats were instilled i.t.

with either recombinant or radiolabelled forms of these chemokines, and concluded that CINC is selectively transported from the lungs to the systemic circulation [870]. The same group later raised the possibility that MIP-2 may have entered the circulation but it was undetectable because it bound to cellular receptors both on parenchymal and circulating cells [871]. For example, these chemokines are known to have high affinity to cell surface receptors such as CXCR2 on vascular endothelial cells [872] and leukocytes [873], and to Duffy antigen receptor for chemokines (DARC) on red blood cells [874, 875] and endothelial cells [876].

DARC is considered to be working as a sink for CXC chemokines and possibly plays a role in sequestering circulating chemokine concentrations to cellular elements [874, 875]. Additionally, DARC on venular endothelium has been proposed to mediate transcytosis of CXC chemokines from the abluminal to luminal surface [263]. To test this possibility, Zamjhan *et al.* treated rats with i.t. LPS and looked at plasma as well as cellular MIP-2 and CINC levels in isolated erythrocytes and leukocytes [871]. While they found CINC both in plasma and cells, MIP-2 was only detectable in the cellular fraction of blood, indicating differential cellular binding and uptake dynamics between these chemokines. Indeed, it was demonstrated by Murakami *et al.* that CXCR2 receptors possess differential affinities for each chemokine, with MIP-2 exhibiting 70-fold greater binding affinity over CINC [877].

Although it is most likely that our current findings with undetectable plasma MIP-2 can be explained by these mechanisms, an isolated perfused lung preparation would be a useful tool to confirm this, as any influence of these 'sink receptors' on circulating cells can be excluded. And truly, our group's experience with a mouse IPL indicated that MIP-2 is detectable and increased in the perfusate in response to VILI

[878], suggesting that circulating cells are likely responsible for the undetectable plasma MIP-2 levels *in vivo*.

Due to the dilution effect of bronchoalveolar lavage and the 'sink' effect of red blood cells, it is not possible to tell from the whether the measured plasma cytokines/chemokines were originating, at least in part, from the alveolar compartment. However, while i.t. p55 dAb attenuated the level of these cytokines/chemokines, whereas i.v. p55 dAb did not, an alveolar source is likely.

In conclusion, although systemic targeting of p55 signalling by domain antibodies did not mitigate the currently investigated aspects of VILI, these data, in conjunction with findings of Chapter 4, support the hypothesis that specific inhibition of intraalveolar p55 signalling is essential to attenuate VILI. However, it is possible that targeting p55 receptor signalling systemically may have positive effects not clear from this study, and could offer additional benefits to sole intraalveolar p55 blockade. Moreover, systemic administration as a route of delivering p55 dAbs to the alveolar space should not be condemned based on this study, as it still may offer practical/technical advantages over pulmonary delivery. Further investigations are therefore a must to clarify the potential benefits of systemically delivered p55-blocking dAb.

6. Selective targeting of intraalveolar p55 TNFR signalling during acid-aspiration induced lung injury

Having successfully demonstrated in Chapter 4 that selective targeting of p55 TNF receptor signalling by a domain antibody can ameliorate VILI, in this chapter we investigated if it would be similarly beneficial in ALI of other aetiology.

A significant role of TNF signalling has been also implicated in the pathology of acid-induced ALI. Using TNF receptor knock-out mice our group has previously demonstrated that opposing TNF receptor signalling also occurs in acid-induced ALI in a similar manner as in VILI, where p55 signalling promoted, whereas p75 signalling protected against pulmonary oedema.

To address the potential usefulness of the domain antibody, I developed an in vivo mouse model of acid-induced ALI in collaboration with Dr Brijesh Patel and Dr Kenji Wakabayashi where mice are treated with i.t. p55-specific domain antibody in a prophylactic manner prior to i.t. acid instillation.

My model produced a substantial amount of pulmonary oedema, leukocyte infiltration and intraalveolar cytokines/chemokines 3h after i.t. acid instillation compared to saline control, which was significantly attenuated by pre-administration of i.t. p55-specific domain antibody. The findings suggest that targeting intraalveolar p55 TNFR signalling by domain antibodies may be a useful approach to prevent ALI of various aetiologies.

6.1 Background

Acid aspiration induced lung injury or aspiration pneumonitis was first described by Mendelson in 1946 who reported 66 women who developed ARDS resulting from aspiration of gastric contents into the lungs during labour [879]. Since then this condition continues to be recognised as a potentially lethal complication of the perioperative period. Its incidence was 1 in 2,131 patients receiving general anaesthesia according to a study in 1986 [880], which later reduced to approximately 1 in 3,216 after the introduction of prophylactic measures such as the 'nil by mouth' order prior to anaesthesia [881]. However, the true incidence of clinical gastric aspiration is likely to be even higher as suggested by the findings of Warner *et al.* [881]. They have studied 67 patients who have aspirated while undergoing general anaesthesia. Sixty-three per cent (42 patients) of these cases had no symptoms at all despite arterial desaturation and the radiological evidence of aspiration. On the other hand, aspiration pneumonitis can be so severe that 10 to 30% of the witnessed aspiration cases progress to ALI and ARDS with a mortality rate of up to 30% [881, 882]. Overall acid aspiration induced lung injury accounts for 11% of all ALI cases and up to 20% of all anaesthesia-related death [7, 881-883].

Aspiration of gastric contents causes a chemical burn to the pulmonary parenchymal cells, leading to an intense inflammatory reaction. An experimental study by Kennedy *et al.* using rats showed that acid aspiration induced lung injury has a characteristic biphasic pathogenesis [884]. The first phase, which peaks 1 to 2 hours after acid aspiration to the lungs, is a result of the direct, caustic effect of the low pH of the aspirate on the cells lining the alveolar–capillary interface leading to increased permeability and acute epithelial dyfunction [815]. The second phase, which peaks 4 to 6 hours post-aspiration, is characterised by neutrophil infiltration to

the lung parenchyma and alveoli, with a consequent destruction of alveolar-capillary walls, necrosis of parenchymal cells, fibrin deposition and haemorrhage.

The mechanisms of the lung injury after gastric aspiration involve a spectrum of inflammatory mediators including chemokines and cytokines, inflammatory cells, adhesion molecules, complement, cyclooxygenase and lipoxygenase products, destructive enzymes and reactive oxygen/nitrogen species released by inflammatory cells [80, 513, 685, 885-892]. Based on these experimental investigations numerous potential therapies were identified, such NOS inhibition [513], cytokine release inhibitor [890], P-selectin blockade [110] etc., however, to date no definite treatment exists, only supportive therapy. This and the fact that in many patients aspiration is, in theory, predictable (i.e. during or after anaesthesia) point to the potential application of a prophylactic approach, where the aspiration induced pathological process may be abrogated in a very early stage, thus preventing injury development. TNF seems to be a good candidate for such intervention, as it is one of the proximal, initiating pro-inflammatory cytokine in the pathogenesis of aspiration pneumonitis [685, 686, 893] and is likely to be involved in both phases.

Our group has previously developed an in vivo mouse model of acid aspiration induced lung injury, in which anaesthetised, ventilated mice have hydrochloric acid (HCl) administered directly into the trachea [814]. Pulmonary oedema develops as a consequence (within 2-3 hours), which we have shown to be mediated by TNF signalling. Specifically (and similarly to the situation with VILI), we demonstrated using TNF receptor knockout mice that blockade of p55 signalling protected from oedema formation, while blockade of p75 signalling may have exacerbated oedema. This suggests that pharmacological blockade of p55 TNFR

signalling might be beneficial in preventing and/or attenuating lung injury caused by acid aspiration.

As the pathogenesis of acid aspiration induced lung injury is (considered to be) primarily triggered by the initial pulmonary epithelial cell injury it is reasonable to target p55 TNFR signalling in the alveolar compartment of the lungs if one wants to prevent or ameliorate injury progression.

6.2 Aims

In this chapter we attempted to investigate the effects of blocking intraalveolar p55-signalling by a domain antibody on pulmonary oedema and inflammation during ALI induced by acid-aspiration. There were two main aims:

1. To develop a robust, reproducible in vivo mouse model of acid-induced ALI that is suitable for intratracheal administration of antibodies in a fluid phase.

2. To investigate the effects of blocking intraalveolar p55 TNF receptor signalling by domain antibodies on pulmonary oedema and inflammation during acid-induced ALI.

6.3 Protocols

Model development

As dAb could be potentially damaged when co-administered with HCl, we decided animals should receive dAb and HCl in separate boluses. However, intratracheal fluid instillation has certain impact on lung mechanics most likely secondary to

interference with the alveolar lining fluid, specifically with surfactant proteins. Because of this, lungs are more prone to suffer injury from a further insult (the basic principle of VILI models with saline lavage followed by mechanical ventilation). Therefore, to achieve similar kinetics of lung injury as in our previous knock-out model where only one intratracheal fluid instillation took place (i.e. HCl), in this model we had to titrate the dose of HCl given following the initial dAb instillation.

For this, after surgical preparation for the *in vivo* ventilation system (during which mice were ventilated with low-stretch settings, i.e. V_T=8-9ml/kg, PEEP=3cmH_2O, 100% O_2) and baseline readings, mice received an i.t. bolus of 25µg dummy dAb in a total volume of 50µl as described in chapter 2. Then low-stretch ventilation continued for 60min after which mice received an i.t. bolus of HCl varying in concentration (0.05-0.1M) and volume (50-70µl) or normal saline (50µl) as control. Mice were then ventilated for further 180min or until BP dropped below 45mmHg, whichever happened earlier. BP, airway pressure and gas flow was monitored throughout, and sustained inflations (35cmH_2O, 5sec) were given every 20min to prevent atelectasis. Development of lung injury was determined by changes in respiratory mechanics and deterioration in oxygenation.

Initial experiments with varying concentration of HCl (0.05-0.1M, or saline) but the same volume (50µl) suggested that the dose response is very tight as 0.05M and 0.075M HCl caused almost negligible amounts of injury based on $P_{plateau}$ increase and decrease in PO_2, while administration of 0.1M acid led to substantial injury, and some animals did not even complete the 180min protocol (Figure 6.1/A-B).

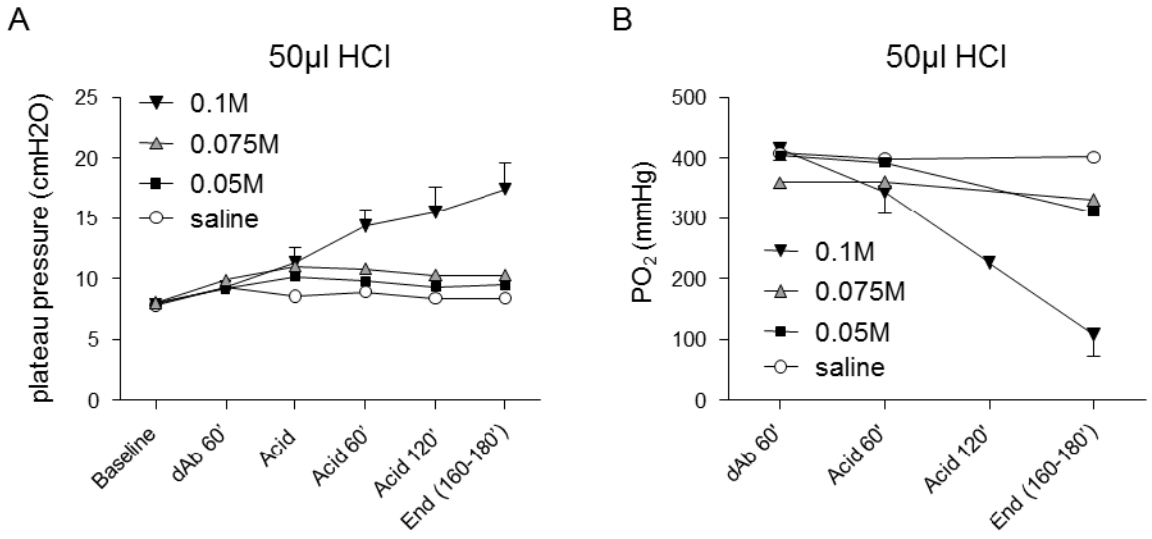


Figure 6.1 – Plateau pressure and PO_2 changes in mice treated with i.t. dummy dAb followed by 50µl i.t. hydrochloric acid (HCl) of different concentrations or normal saline. After surgical preparation (i.e. V_T=8-9ml/kg, PEEP=3cmH_2O, 100% O_2) and baseline readings, mice received an i.t. bolus of 25µg dummy dAb in a total volume of 50µl. Then low-stretch ventilation continued for 60min after which mice received an i.t. bolus of HCl varying in concentration (0.05-0.1M) or normal saline (50µl) as control. Mice were then ventilated for further 180min or until BP dropped below 45mmHg, whichever happened earlier. Mice treated with saline or HCl of 0.05 to 0.075 molar concentrations did not exhibit signs of lung injury as measured by (A) increase in plateau pressure and (B) decrease in PO_2, while animals receiving 0.1M HCl showed such substantial injury that some of these did not even complete the 180min protocol. N=1-3/group.

To reduce the harshness of the model, we therefore had to either use 0.1M acid but in a smaller volume or one of the lower concentrations in increased volume. We chose the latter, as lowering the volume of the i.t. bolus may result in uneven distribution of acid in the lungs and thus inconsistent pulmonary injury and increased noise across experiments. In the following experiments therefore mice received 0.075M HCl in volumes of 50 to 70µl the same way as described. We found that

administration of 65µl of 0.075M HCl resulted in substantial lung injury within 180min without affecting the lifespan of the model (Figure 6.2/A-B); therefore in further experiments this dose of acid was used.

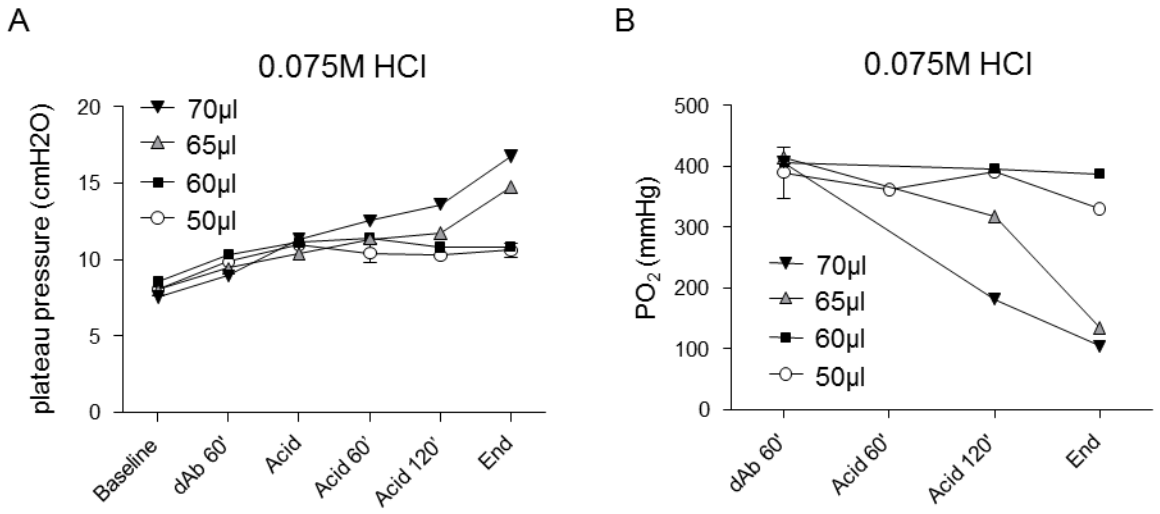


Figure 6.2 – Plateau pressure and PO_2 changes in mice treated with i.t. dummy dAb followed by 0.075M i.t. hydrochloric acid (HCl) of different volumes. Mice treated with 50 or 60µl of 0.075M HCl did not develop lung injury as measured by (A) increase in plateau pressure and (B) decrease in PO_2. Animals receiving 65µl acid showed gradual but significant deterioration in these parameters, while those treated with 70µl of acid developed more severe injury with more rapid dynamics. N=1-3/group.

Finalised protocol for acid aspiration induced lung injury

Following surgical preparation for the *in vivo* mouse mechanical ventilation system (during which animals were ventilated with V_T=8-9ml/kg) and baseline measurements, mice received an intratracheal bolus of either dummy (non-targeting) dAb or p55-specific blocking dAb (25µg) in a total volume of 50µl as previously described, then ventilation continued with the same settings (i.e. V_T=8-9ml/kg, PEEP=3cmH_2O, 100% O_2).

After 60min mice received a second i.t. bolus of 0.075M HCl in a total volume of 65µl, then ventilation continued for another 180 minutes or until mean BP dropped below 45 mmHg [337]. Anaesthesia was maintained by bolus administrations of intraperitoneal ketamine:xylazine every 20-25 minutes. One animal from the p55-secific dAb group displayed very rapid haemodynamic deterioration. This prematurely terminated experiment was excluded from the analyses. Plateau pressure ($P_{plateau}$), respiratory system elastance (E_{rs}) and resistance (R_{rs}) were measured every 20 minutes, followed each time by sustained inflation (35cmH_2O, 5 seconds) to avoid the development of atelectasis. ABG were assessed at predetermined points throughout the protocol (Figure 6.3).



Figure 6.3 - Scheme of ventilation protocol. Mice were anaesthetised, tracheostomised and instrumented for the ventilation system in approximately 15-20min (*dotted line*). Then mice were instilled intratracheally with either dummy or p55-specific dAb and ventilation continued for 60 minutes after which a bolus of hydrochloric acid or saline was instilled the same way. After instillation of the reagents, 4 sustained inflations (35cmH_2O, 5 seconds) were applied to help the fluid distribute within the lungs. Mice were ventilated with non-injurious settings throughout (V_T=8-9ml/kg, PEEP=3cmH_2O) and 100% oxygen. Respiratory mechanics were assessed every 20min, followed each time by sustained inflation. Arterial blood gases (ABG) were assessed at predetermined time points (dAb 60', *Acid 60', Acid 120', End*).

Upon termination of experiments, mice were killed by anaesthetic overdose and cardiac puncture and saline lavage performed, as described in chapter 2. Samples were centrifuged and BALF supernatants aliquotted and stored at -80°C, whereas cell pellets were used for quantification of neutrophils by manual counting after differential staining (chapter 2). Thawed samples were quantified for total protein as a further indicator of pulmonary oedema/alveolar barrier dysfunction, and for IL-6, KC, MIP-2 and MCP-1 by colorimetric sandwich ELISA as indicator of intraalveolar inflammation. Lungs were excised and processed for flow cytometry to assess the degree of leukocyte infiltration.

6.4 Results

While saline instillation had no significant impact on respiratory mechanics, acid instillation caused a substantial deterioration in lung function in dummy dAb treated animals, consistent with pulmonary oedema formation. This was represented by a rapidly increasing $P_{plateau}$ and E_{rs} that occurred towards the end of the 3h ventilation protocol (Figure 6.4/A-B). In marked contrast, these parameters showed only a non-significant tendency to increase in the p55 dAb group. Changes in R_{rs} and arterial pressure were not different across the treatment groups (Figure 6.4/C-D).

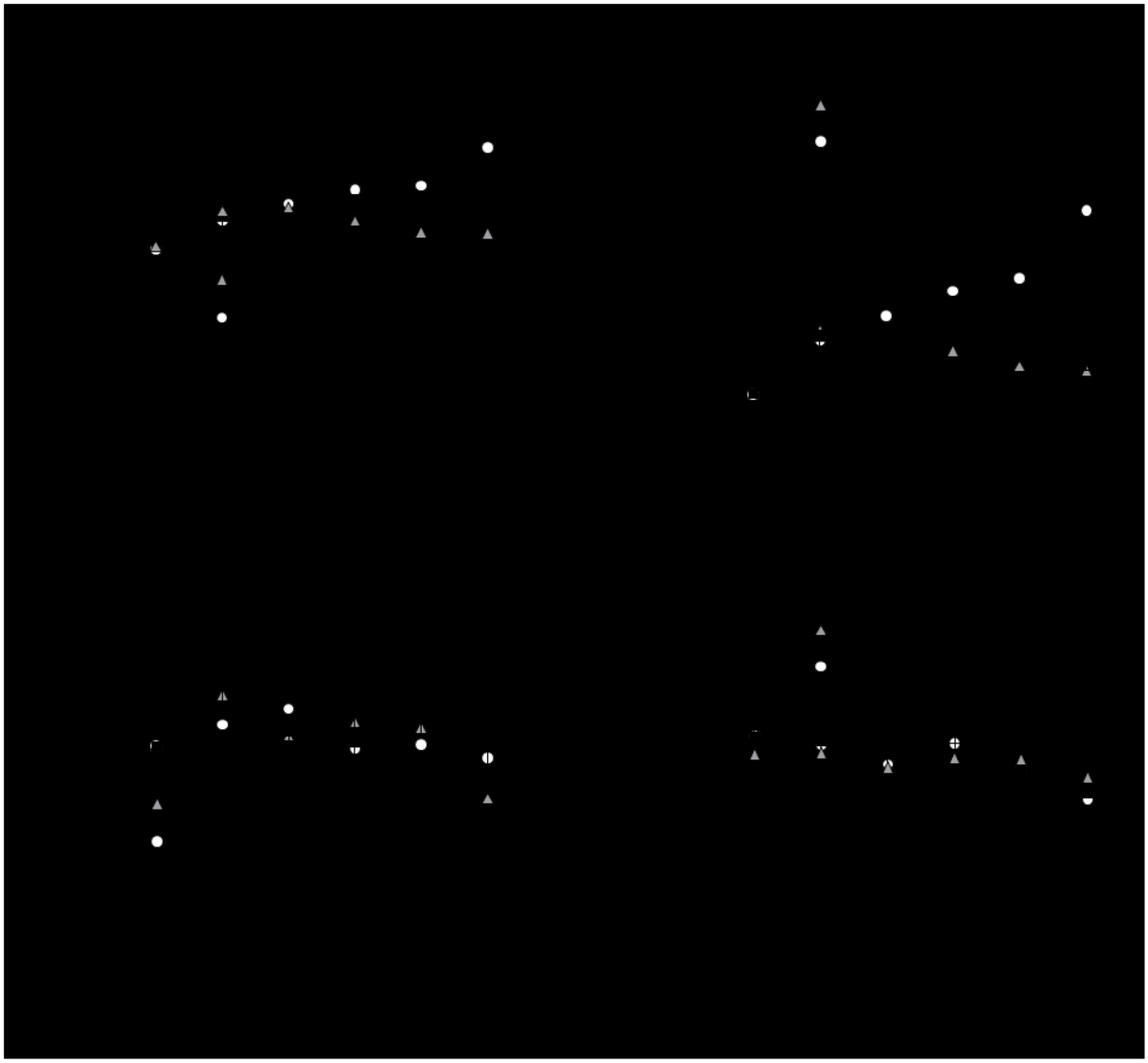


Figure 6.4 - (A) Plateau pressure ($P_{plateau}$), (B) respiratory system elastance (E_{rs}) and (C) resistance (R_{rs}), (D) mean arterial pressure were determined in ventilated animals treated intratracheally with either dummy or p55-specific domain antibody (dAb) followed by HCl acid instillation or saline. Mice had i.t. dummy or p55-specific dAb and ventilation continued for 60 minutes after which a bolus of hydrochloric acid or saline was instilled the same way. Mice were ventilated with non-injurious settings throughout (V_T=8-9ml/kg, PEEP=3cmH_2O) and 100% oxygen. Changes in E_{rs} and R_{rs} are expressed as percentage increase in relation to 'Start' (before instillation) values. Saline instillation had no deteriorating effect on lung mechanics, whereas intratracheal acid instillation resulted in significant increase in $P_{plateau}$ and E_{rs} in dummy dAb treated mice. Changes over time during ventilation were significantly different in p55 dAb treated group in these parameters (# $P<0.05$ for interaction between 'treatment' and 'ventilation time' by 2 way ANOVA). N=4-6/group.

Saline instillation did not affect gas exchange significantly throughout the ventilation protocol. Arterial PO_2 and PCO_2 levels were similar between the two acid treated groups at 60 minutes after the antibody and acid instillations, but the trends started to diverge 120 minutes after acid instillation and were overall significantly different (Figure 6.5/A-B). By the end of the protocol, oxygenation deteriorated in dummy dAb treated animals (183±110mmHg), while PCO_2 increased. In contrast, PO_2 was better maintained in the p55 dAb group (304±124mmHg), and PCO_2 was unchanged from the Acid 120' level. To assess alveolar epithelial permeability, we measured the protein content in lavage fluid. Acid instillation resulted in substantial protein levels in the dummy dAb group as compared to saline controls (4.53±2.27 vs 0.33±0.10mg/ml), and this was tended to be lower in mice given the p55 dAb (2.51±1.38mg/ml) without statistical significance (Figure 6.5/D).

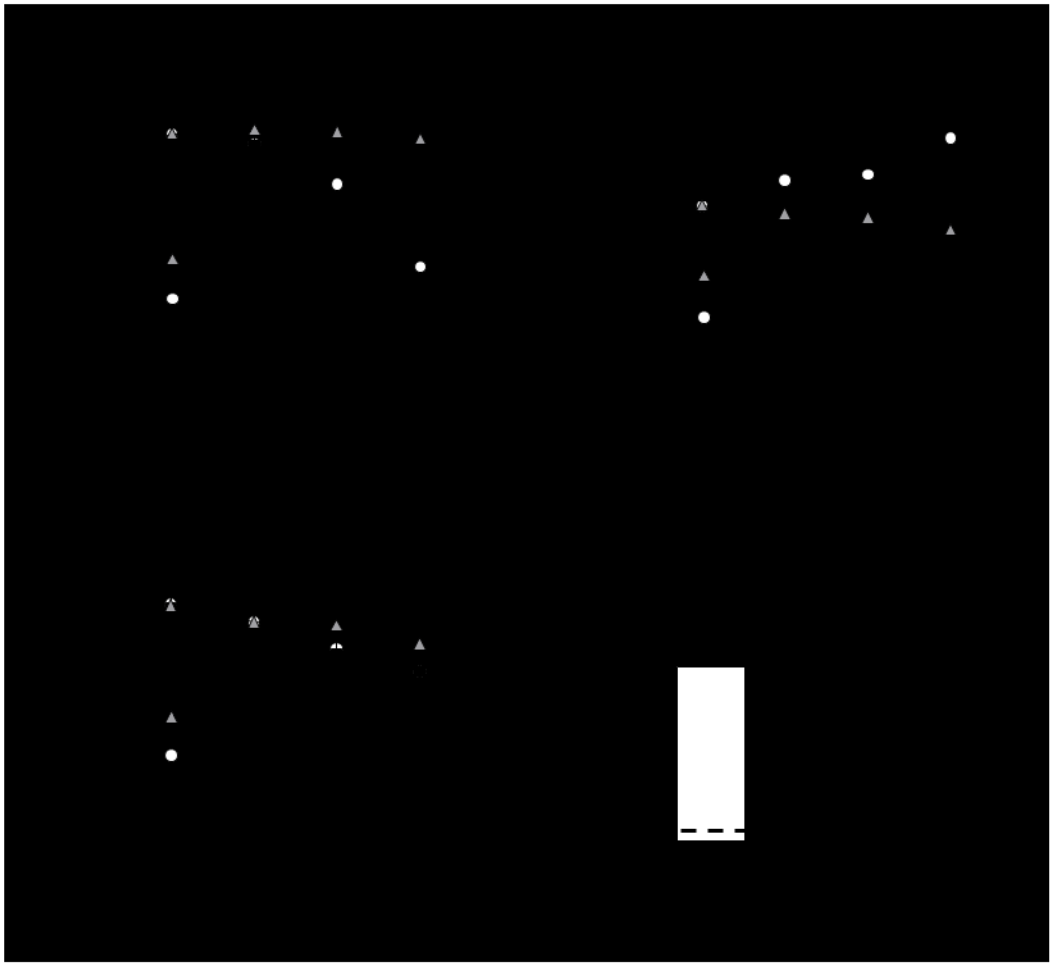


Figure 6.5 - (A) Arterial PO_2, (B) PCO_2, (C) bicarbonate (HCO_3^-) and (D) lavage fluid protein were determined in ventilated animals treated intratracheally with either dummy or p55-specific domain antibody (dAb). While oxygenation was well maintained in mice instilled with i.t. saline, arterial PO_2 significantly deteriorated after acid instillation in animals treated with the dummy dAb but not in mice given the p55-specific dAb, and changes over time were significantly different between these groups. Similarly, PCO_2 did not change significantly in saline controls, while acid-induced changes over time were significantly different between dummy and p55 dAb treated animals (# $P<0.05$ for interaction by 2 way ANOVA). Changes in bicarbonate levels were similar across groups. Acid instillation led to a significant amount of lavage fluid protein in the dummy dAb group, which tended to be lowered by p55-specific dAb treatment. Dotted line represents mean value in saline control animals. N=4-6/group.

Anti-p55 domain antibody attenuates pulmonary inflammation in acid aspiration induced ALI

Having demonstrated that p55-specific dAb can attenuate deterioration in respiratory physiology following acid aspiration, we went on to investigate whether it has beneficial effects on pulmonary inflammation. To address this, we assessed leukocyte margination to the lungs as well as intraalveolar neutrophil influx along with lavage fluid levels of inflammatory chemokines and cytokines.

Leukocyte subsets in lung single cell suspension were identified based on characteristic antigen expression using flow cytometry (Chapter 2) and their number quantitatively assessed, whereas the number of lavage fluid neutrophils was manually counted after differential staining. Acid instillation increased the number of lung-recruited neutrophils as compared to saline control ($2.19\pm0.78x10^6$ vs $1.03\pm0.42x10^6$, respectively), which tended to be lowered by the p55-specific dAb ($1.32\pm0.44x10^6$) (Figure 6.6/A). A similar trend was found with margination of inflammatory subset Gr-1^{high} monocytes, where an acid-induced increase in cell number ($2.74\pm0.98x10^6$ vs $1.77\pm0.65x10^6$ for dummy dAb + acid and dummy dAb + saline, respectively) was significantly attenuated by p55 dAb ($1.25\pm0.79x10^6$, *$p<0.05$ vs dummy dAb + acid) (Figure 6.6/A). Transmigration of neutrophils to the alveolar space was also greatly increased by acid aspiration, and substantially attenuated by p55 dAb treatment (Figure 6.6/B).

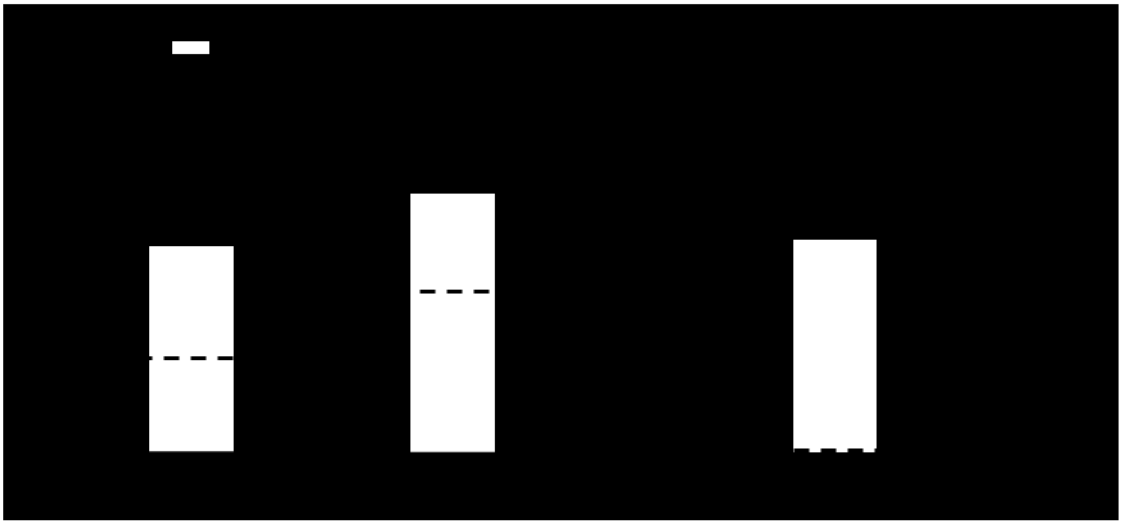


Figure 6.6 – Number of leukocyte subsets in the lungs and lavage fluid of dummy-dAb and p55-dAb treated mice following i.t. acid aspiration. Treatment with p55-specific dAb significantly attenuated the number of (A) lung-marginated Gr-1^{high} monocytes and (B) intraalveolar neutrophils. Dotted line represents mean value in saline control animals. *P<0.05 by unpaired t-test. N=5/group.

Finally, acid aspiration substantially increased the level of inflammatory cytokines/chemokines IL-6, MCP-1, KC and MIP-2 in the alveolar space, which were all significantly attenuated by pre-treatment with the p55 specific dAb (Figure 6.7/A-D).

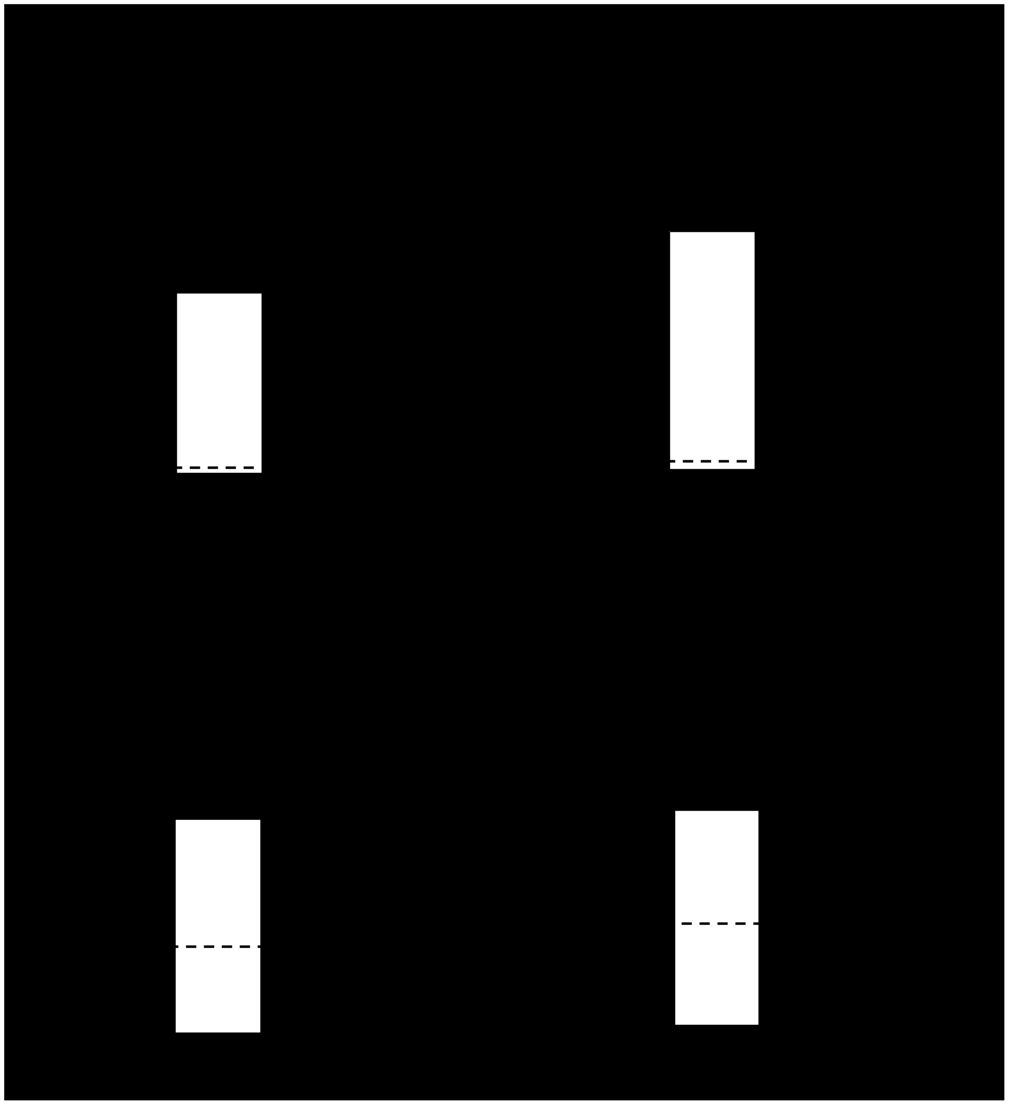


Figure 6.7 - Levels of lavage fluid (A) IL-6, (B) MCP-1, (C) KC and (D) MIP-2 in mice pre-treated intratracheally with either dummy or p55 specific domain antibody followed by intratracheal acid (HCl) instillation. Dotted line represents mean value in saline control animals. *P<0.05, ***P<0.001 by unpaired *t*-test (B, C, D) or Mann-Whitney U-test (A). N=5/group.

6.5 Discussion

TNF signalling in acid aspiration-induced lung injury

Aspiration of gastric contents to the lung is a common complication of general anaesthesia, but other medical scenarios which are associated with a low conscious level, such as drug overdose, seizure or stroke generally present a high risk [882, 894]. The severity of the subsequent lung injury may vary from a mild, subclinical pneumonitis to the more severe picture of ALI/ARDS with associated high mortality [893].

As with VILI, acid aspiration is another situation where the onset of ALI may, in theory, be predicted in some patients, and thus particularly amenable to prophylactic therapy. Tumour necrosis factor alpha seems to be an ideal therapeutic target, as it is expressed early at the course of acid aspiration [686], mediates, at least in part, the production of other cytokines/chemokines in the inflammatory cascade and expression of adhesion molecules on epithelial/endothelial cells and alveolar macrophages (see Chapter 3 and 4), regulates epithelial/endothelial cell death and remodelling [729, 776], and cytoskeleton re-arrangement [776, 778] which is important in the early permeability changes during the first phase of acid aspiration pneumonitis [895].

Indeed, when blockade of TNF signalling by anti-TNF antisera was attempted systemically or intratracheally in rat models of aspiration pneumonitis, it showed mitigation of lung injury by measures of lung oedema and neutrophil sequestration [685, 686]. However, these studies assessed lung injury 3 and 5 hours after acid aspiration, respectively, i.e. in the second phase of the pathogenesis, therefore it is difficult to tease out whether the protective effect of TNF blockade is solely

attributable to the reduction of neutrophil sequestration leading to an attenuation in permeability increase, or it also influenced oedema formation during the early phase in a more direct manner.

In marked contrast to these studies, our group previously showed that genetic inhibition of total TNF receptor mediated signalling in TNF receptor double knockout mice did not attenuate acid aspiration induced pulmonary oedema [814]. In fact, in some respiratory physiology parameters that reflect oedema formation such as respiratory system elastance and peak-inspiratory pressure these animals did worse and diverged from their wild-type controls as soon as 30 minutes after aspiration and maintained this difference throughout the 3 hours protocol [814]. This difference between TNF receptor double knock-out and wild-type mice cannot be ascribed to differential effect on neutrophil accumulation, because this could not happen as early as 30 minutes after acid aspiration, and overall this model did not cause a significant neutrophil sequestration to the alveoli (average total number of PMN <2000 in BALF in wild-types), and in fact it was even lower in DKOs at the end of protocol (average total number of PMN ~100). Therefore, it is possible that in a more prolonged model such as Davidson's [686], where neutrophil accumulation in the alveolar compartment is more prominent, genetic knock-out of both TNF receptors would show similar results to the pharmacological blockade of total TNF signalling seen in their study.

More interestingly, our previous study also found that mice lacking only the p55 TNF receptor were completely protected from acid induced pulmonary oedema and dysfunction, whereas p75 knockout mice behaved as the TNFR DKO mice, i.e. were more susceptible to oedema formation, and were either similar or, at least in some parameters, worse than wild-types. Again, it is worth to note that these

differences could not be ascribed to differential effect on neutrophil sequestration as the number of PMNs in lavage fluid was not different across the receptor knock-out animals, although the number of marginated leukocytes were not investigated. The findings of this study with TNFR knockouts indicated two important things. First, it confirmed that TNF signalling plays an important role in the early, leukocyte-independent permeability changes upon acid aspiration, and second, that its two receptors have differential role mediating this process, i.e. signalling through p55 mediates oedema whereas p75 signalling opposes this, therefore specific targeting of p55 receptor signalling is potentially more beneficial than total TNF signalling blockade.

Effects of i.t. p55 dAb on respiratory physiology

To test whether pharmacological inhibition of p55 receptor signalling is effective to mitigate acid aspiration pneumonitis, in the current study we treated mice intratracheally with p55-specific dAb in a prophylactic manner (i.e. 1 hour prior to acid instillation) and quantified acid aspiration-induced pulmonary responses. We found that inhibition of intraalveolar p55 TNF receptor signalling significantly attenuated pulmonary oedema (by means of elastance and plateau pressure changes) and respiratory dysfunction (by means of gas exchange).

Alveolar epithelial and pulmonary endothelial cells constituting the alveolar-capillary membrane are the main regulators of alveolar fluid homeostasis. Under normal conditions, fluid transport across this barrier is limited and well controlled by adherens and tight junctions between these cells [66]. Although some fluid filtration through the barrier does occur, active cellular transport and lymphatic drainage ensures that the alveoli are not flooded. This active cellular transport is mediated by

ion-channels and is energy consuming for the cells[66]. Pulmonary oedema can develop if any element of this well balanced active system fails, being either a damage/disruption to the cell-cell junctions or paralysis of the ion-channels caused by lack of energy, drugs or cellular damage (or even cell death).

It is believed that acid aspiration induced permeability oedema is, at least in part, mediated by cytoskeleton re-arrangement in epithelial/endothelial cells of the alveolar-capillary membrane. For example, in a study by Goldman *et al.* intratracheal administration of phalloidin, which facilitates assembly of cytoskeletal proteins [896, 897], to rats 20 minutes after acid instillation attenuated pulmonary oedema [895]. The authors ascribed this to a direct effect on pulmonary endothelial cells, as the number of lung-marginated neutrophils was not attenuated by phalloidin treatment. Importantly, phalloidin treatment neither reduced inflammatory mediators such as leukotriene-B_4 (LTB_4) or thromboxane B_2 (TxB_2), which are known to mediate pulmonary oedema [886, 898, 899] through microfilament disassembly in endothelial cells [900, 901].

TNF is also known to cause cytoskeleton re-arrangements in epithelial/endothelial cells via different mechanisms. For example, Ferrero *et al.* showed *in vitro* that specific activation of the p55 but not the p75 TNF receptor results in cytoskeleton re-organisation in endothelial cells with associated increased permeability in a p38 MAPK dependent manner [813]. On the contrary, Hamm-Alvarez *et al.* demonstrated that p75 receptor associated epithelial/endothelial tyrosine kinase (Etk) activation leads to an increase in transepithelial resistance (TER), a common measure of junctional tightness between epithelial cells, which was accompanied by changes in actin filaments organisation [778]. In another study Pan and co-workers showed that TNF activates the Etk/Bmx pathway in endothelial

cells specifically via the p75 receptor, which then mediates tube formation and angiogenesis [776]. Together these findings suggest that p75 mediated TNF signalling can play an important role in maintaining the barrier function of the alveolar-capillary membrane by strengthening the epithelial/endothelial cytoskeleton. However, it remains unclear whether these mechanisms are involved in the pathogenesis of acid aspiration-induced pulmonary oedema formation.

Direct cellular damage and apoptosis of the cells of the alveolar-capillary membrane may be an alternative mechanism as to how TNF may mediate pulmonary oedema formation in aspiration. A recent study by Maniatis *et al.* showed that acid aspiration in wild-type mice resulted in significant oedema formation associated with increased apoptotic activity in lung cells as measured by caspase-3 staining, and this response was completely abrogated in p55 receptor knockouts [902]. Importantly, genetic knockdown of TNF was without effect and resulted in similar injury as in wild-types.

The findings of these studies may help us to better understand our previous knockout data and that of the current study. For example, the potential importance of p75 signalling in barrier function may explain why p75 knock-outs and mice lacking both of the receptors behaved very similarly upon acid challenge. It also suggests that the positive effects of p55 signalling blockade, both genetically and pharmacologically, may be secondary to a combination of direct and indirect impact on barrier function, i.e. 1) by prevention of TNF to elicit its deleterious effects through p55 receptor (direct), and 2) by directing proportionally more TNF signalling through the p75 receptor, thus improving or helping the maintenance of barrier function.

Effects of i.t. p55 dAb on pulmonary inflammation in acid aspiration-induced lung injury

As described by Kennedy *et al.*, the second phase of lung injury propagation following acid aspiration is characterised by leukocyte infiltration to the lung parenchyma and alveolar space associated with additional increase in alveolar permeability which peaks 3-4 hours after the initial insult [884]. Inflammatory chemokines and cytokines seem to orchestrate this process, not only by trafficking leukocytes to the pulmonary circulation, but also by retaining and activating them in situ via enhancement of cell to cell interactions with pulmonary endothelial and epithelial cells in which expression of adhesion molecules and their counterpart molecules on leukocytes play a pivotal role [903, 904]. Upon activation, these leukocytes release their mediators such as thromboxane, oxygen radicals and proteases that eventually cause a subsequent increase in vascular permeability and leads to pulmonary oedema and hypoxaemia [80, 888, 903].

The inflammatory cytokine/chemokine TNF, IL-6, IL-8 or its murine homologs KC and MIP-2 seem to be key mediators in the pathogenesis of acid aspiration as pharmacological or genetic inhibition of these mediators attenuate neutrophil infiltration, thus the propagation of lung injury, and their level may predict the severity of pulmonary dysfunction [686, 885, 893, 905, 906]. In keeping with this, we observed here that attenuated lung injury in p55 dAb pre-treated mice was associated with a lower level of these mediators in lavage fluid, reduced number of inflammatory leukocytes in the lung tissue and of neutrophils in the alveolar space.

The role of MCP-1 in acid aspiration is, however, a bit more controversial. Similarly to the mediators mentioned above its level is increased in BAL upon acid aspiration [893] and correlates with the degree of lung injury. However, when MCP-1 was targeted with polyclonal antibodies in the same study, it failed to attenuate injury [893]. Moreover, chronic inhibition of MCP-1 in genetically modified mice results in

more profound lung injury by means of increase BAL protein, neutrophil numbers, histology changes and reduced survival [907]. One possible explanation for the neutral or even detrimental effect of MCP-1 inhibition is a compensatory change in other cytokines that produce similar end effects as postulated by Knight *et al.* [893]. Indeed, in the study by Raghavendran and co-workers acid aspiration resulted in significantly higher levels of BAL IL-6 and KC in MCP- 1 knock-out mice compared to wild-type animals [908]. In the current study, however, reduced MCP-1 levels were associated with improved lung function in p55 dAb treated mice, although the treatment also attenuated the levels of all the measured chemokines/cytokines, therefore the result may have been a combinatory and/or synergistic effect.

Neutrophils are clearly primary mediators of lung and multi-organ injury following acid aspiration as demonstrated by depletion studies [79, 80]; however the role of inflammatory monocytes, specifically Gr-1^{high} monocytes to my knowledge has not been investigated yet, although their pathognomic role in other forms of lung injury, such as sepsis and VILI has been well established by our group [114-116]. Although the role of this inflammatory subset of monocytes was not investigated directly in the current model, as acid aspiration led to their increased pulmonary margination and was attenuated by p55 dAb with associated mitigated injury, it is tempting to speculate that Gr-1^{high} monocytes are also key players in the pathogenesis. It is also worth to note, that the substances used to deplete neutrophils in the previous studies by Knight *et al.* and Goldman and colleagues [79, 80] (anti-CD18 and nitrogen mustard), are not neutrophil specific and may have also depleted monocytes, therefore their role may have been overlooked at the time. Depletion studies targeting Gr-1^{high} monocytes will help to dissect out the role of these cells in acid aspiration lung injury.

It was interesting to find that while our previous acid model using TNFR knockout mice resulted in only a small degree of alveolar neutrophil influx (<2000 PMN in wild-type mice upon acid) [814], the current model led to a more than 15-times greater number of alveolar PMNs, despite the same length of post-acid incubation, i.e. injury time. One possible explanation for this discrepancy is the overall length of ventilation time, which was 60 minutes longer due to the pre-incubation period with the dAb. This may have implicated additional inflammatory stimulus to the animals, which is well supported by the fact that the saline control animals had 5-times higher number of alveolar neutrophils on average in the current model (532 vs 100). Another possibility is that the current model had a 2-hit nature, where the initial instillation of dAb primed the lungs for increased inflammatory response to the subsequent acid administration. However, our findings are overall in keeping with that of others showing intraalveolar neutrophil accumulation to manifest as soon as 2 hours post acid [886].

In summary, our results showed that selective targeting of intraalveolar p55 TNF receptor signalling by domain antibodies significantly attenuates lung injury caused by acid aspiration, which may represent a novel preventative measure for patients at high risk of this condition. Further investigations are necessary to better understand the mechanisms by which these positive effects were exerted, to rule out any potential downsides of this treatment, and to explore the possibility of using it as a therapeutic tool for patients in whom aspiration has already occurred.

7. Discussion

TNF signalling represents a complex system influenced by many factors, including distinct signalling pathways associated with each TNFR and modulation of signalling by the change in the relative expression of the receptor subtypes on cells. Increased levels of TNF in the systemic and alveolar compartments have been implicated in clinical and animal models of ALI/VILI. Differential signalling of TNFRs has also been implicated in ALI of different aetiologies, including VILI and acid aspiration-induced lung injury. In this project we investigated expression profile and roles of TNFRs on pulmonary endothelial cells and the effect of specific receptor inhibition on the pathogenesis of VILI and acid-aspiration-induced lung injury. This discussion chapter will summarise our major findings and discuss their implications and possible future directions.

7.1 Background

Despite an undisputable role of TNF in the pathogenesis of ALI/ARDS, clinical trials with anti-TNF therapy have been largely unsuccessful, which could be due in part to a rather complex TNF signalling. This is due to a number of factors, including the presence of cell-surface and soluble forms of TNF and its two receptors, p55 and p75, through which the majority of TNF-induced cellular responses are mediated.

Whether TNFRs signal co-operatively (via either ligand passing from p75 to p55 or by interactions between receptor-associated intracellular pathways) or in a directly opposing manner (via distinct pathways) could play an important role in directing TNF-mediated cellular responses. TNFR p55 and p75 can act in opposition during VILI and acid aspiration-induced lung injury, wherein p55 promotes while p75 protects from the development of pulmonary oedema [639, 814], by as yet unknown mechanisms.

Although these observations suggest that specific inhibition of p55 receptor signalling may confer more benefits than total TNF blockade in attenuating ALI/VILI, it remains unclear as to where these important differential TNFR signalling mechanisms take place, be it intrapulmonary (alveolar) or extra-pulmonary (vasculature), thus where p55 signalling should be targeted.

Also, in such differential signalling the relative degree of TNFR p55 vs. p75 signalling could be key to the overall biological effect of TNF. This could be largely influenced by cell-surface TNFR p55 and p75 expression, directed by the balance between rates of *de novo* cellular receptor production and expression on the cell surface and receptor shedding, producing sTNFRs. However, the expression profile kinetics of cell-surface TNFRs in the lung and their relative roles in the pathogenesis of ALI/ARDS is yet unclear.

Characterisation of TNFR subtype expression and signalling on the pulmonary endothelium and epithelium *in vivo* would help us to identify where TNFRs should be targeted to potentially improve the outcome of ALI/ARDS. Previous work in our group showed expression of both receptors on pulmonary epithelial cells (Dorr – PhD thesis) and that alveolar expression of the p55 receptor is well during lung inflammation, whereas p75 is prone to shedding [819]. However, characterisation of TNFR expression and signalling on the endothelial side remains to be fully elucidated as previous studies rely on semi-quantitative methods [735] or observations in prolonged *in vitro* cell cultures [53] which may not reflect the *in vivo* situation.

This project therefore had two main aims: firstly, to quantitatively characterise the expression profile and roles of TNFRs on pulmonary endothelial cells using a combined *in vivo* and *in vitro* approach, and secondly, to investigate whether specific

targeting of p55 receptor signalling in the intraalveolar or intravascular space confers benefits in ALI of different aetiologies, namely VILI and acid aspiration-induced lung injury, using *in vivo* mouse models.

7.2 Characterisation of TNFR subtype expression and signalling on PECs

In the first aim of this project we investigated the expression profile of TNF receptor p55 and p75 on pulmonary microvascular endothelial cells (PECs) and their relative roles in cell activation using *in vivo* and *in vitro* approaches.

This first required the development of a flow cytometry technique that allows reliable quantitative detection of TNFR expression in lung tissue. We found that TNFRs were extremely sensitive enzymatic digestion and heat during tissue processing therefore had to alter our previously established methodology. Therefore we used protease inhibitors and low working temperatures to preserve TNFR expression on the cells. Using this technique we found expression of both TNFRs on PECs, with dominance of p55. To validate our technique, we assessed receptor expression in TNFR knockout mice, and found no staining for p55 in TNFRp55 knockout animal, whereas TNFRp75 knockout showed a very small degree of non-specific staining for p75. We also assessed TNFR expression on liver endothelial cells to rule out the possibility that the low level of p75 on PECs was not secondary to differential susceptibility to shedding during tissue processing. Liver endothelial cells had higher p75 expression than that of p55, indicating that the relative expression of TNFRs on PECs is unlikely to be an artefact of tissue processing. It also points out the phenotypical heterogeneity of endothelial cells and the danger of extrapolating findings in one vascular bed to another.

We then assessed the role of individual TNFRs in mediating TNF-induced adhesion molecule expression on PECs, as a surrogate marker of cell activation. The *in vivo* experiments using TNFR knockout mice indicated that p55 is the major regulator of TNF-induced expression of VCAM-1, ICAM-1 and E-selectin, whereas p75 played a similar role, although to a lesser extent, and did not seem to influence ICAM-1 expression. Complementary *in vitro* studies in primary mouse PEC culture utilising pharmacological inhibition of individual TNFRs confirmed the *in vivo* findings, indicating that they were specific to TNFR subtype signalling on PECs without the influence of TNFR signalling on other cells, such as circulating leukocytes. These findings indicating a minor role for p75 in TNF-mediated adhesion molecule expression may be consistent with ligand passing, or alternatively simply reflects the relative expression of the two receptors on PECs.

We then investigated whether inflammation has an influence on TNFR expression on PECs. We found that during the early stages of endotoxaemia, expression of p55 dramatically decreased and did not recover completely within 24 hours post-LPS, whereas p75 expression remained grossly unchanged, resulting in a significant change in p55:p75 ratio. The physiological significance of this is not clear from this study. It is possible that this rapid shedding of p55 from the cell surface has anti-inflammatory role, as it prevents further signalling through this receptor [850, 852]. With regard to adhesion molecule expression, the relative increase in p75 expression may not be significant as it played similar role to p55. However, may have a significant influence on other cellular responses in which the two receptor play opposing role, for example in regulating cell death [853], which will ultimately determine cell function.

In summary, we developed a new flow cytometry technique to quantitatively assess TNFR subtype expression profile on pulmonary endothelial cells *in vivo* for the first time, and demonstrated expression of both receptors with dominance of p55. The role of TNFRs in mediating TNF-induced adhesion molecule expression seemed to reflect their expression level on PECs, but their overall roles may vary at different stages of pulmonary microvascular inflammation following changes in their relative expression. Further investigations are indicated to elucidate this possibility.

7.3 Selective targeting of intraalveolar p55 TNFR signalling during VILI

As our previous work in TNFR knockout mice indicated a deleterious role for p55 and a protective role for p75 signalling in VILI [639] and acid aspiration-induced lung injury [814], the second aim of this project was to investigate whether pharmacological inhibition of p55 receptor specific signalling would attenuate the development of lung injury in these models. We initially wanted to test this in a VILI model, but from the knockout data it was unclear whether p55 signalling should be targeted in the intraalveolar compartment or the intravascular space.

Findings of Chapter 3 indicated that although p55 signalling is crucial in the activation of pulmonary endothelium, its expression dramatically decreased in inflammatory condition, and previous work from our group (Dorr – PhD thesis) showed similar findings with high-stretch ventilation. On the contrary, it was also suggested that surface expression of p55 in the alveolar compartment is well preserved during pulmonary inflammation [819]. Moreover, TNF is up-regulated in the alveolar space during high-stretch ventilation. Therefore, it seemed logical to first target p55 receptor signalling in the alveolar space. As monoclonal antibodies are

not optimal for this purpose mainly due to high risk of receptor cross-linking and activation as well as poor biodistribution, we used a novel p55-specific domain antibody (dAb) that does not have such limitations.

As high-stretch was standardised based on respiratory mechanics, and lung injury development was mainly monitored by these parameters, we first had to develop a VILI model that eliminates the impact of intratracheal fluid instillation, therefore allow to reveal an even a potentially subtle effect of the p55 dAb. After investing a lot of time and effort, we finally established a robust and reproducible VILI model with low variability in respiratory mechanics and using this model managed to produce some very exciting results.

We showed that intratracheal administration of p55 targeting dAb at the beginning of high-stretch ventilation significantly attenuated pulmonary oedema formation, and reduced pulmonary inflammation in a clinically more relevant LPS+VILI model. Interestingly, we found that targeting total TNF-signalling by an intratracheally delivered monoclonal anti-TNF antibody did not attenuate pulmonary oedema in the pure VILI model, despite the fact that the same antibody at the same dose and route of delivery successfully attenuated high-stretch-induced alveolar neutrophil infiltration in one of our previous study [109]. Our findings therefore are in keeping with an opposing role for the two TNFRs during the pathogenesis of VILI. Future investigations comparing the effect of p55 dAb to anti-p75 and/or anti-TNF domain antibodies (once developed) could lend further support to this.

The current study did not investigate what mechanisms are involved in TNF-mediated lung injury during high-stretch ventilation, and how p55 dAb influenced them, but we propose that differential regulation of epithelial/endothelial integrity by the two receptors may be involved. For example, TNF has been implicated in

mediating increased endothelial cell permeability and pulmonary oedema formation [687, 689], which is associated with increases in actin stress fibres, formation of intercellular gaps and compromised vascular endothelial (VE)-cadherin-based cell-cell junctions [690]. Ferrero *et al.* showed that specific activation of the p55 but not the p75 TNF receptor results in cytoskeleton reorganisation with associated increased endothelial cell permeability *in vitro* [813]. In contrast, activation of the p75 receptor-associated endothelial/epithelial tyrosine kinase (Etk/Bmx) pathway [776] results in increased transepithelial resistance thus barrier function *in vitro* [778]. The two receptors also have opposing roles in regulating endothelial/epithelial cell death, whereby p55 signalling is pro-apoptotic and p75 is anti-apoptotic [777], therefore could significantly influence cell function. In view of these studies, it is possible that the positive effects of p55 signalling blockade by dAb may be secondary to a combination of direct and indirect impact on barrier function, i.e. 1) by prevention of TNF to elicit its deleterious effects through p55 receptor (direct), and 2) by directing proportionally more TNF signalling through the p75 receptor, thus improving or helping the maintenance of barrier function. Future studies comparing the effects of p55 and p75 dAb could help us to explore these possible mechanisms.

Attenuation of pulmonary inflammation by p55 dAb may also have additional benefits that are not clear from this study, because it is associated with attenuated organ injury and improved survival in clinical ARDS. Future studies therefore could be dedicated to investigate the effects of intrapulmonary delivered p55 dAb on high-stretch related extra-pulmonary injury.

Anti-TNF treatment in clinical studies is associated with increased susceptibility to infections and flare up of occult Mycobacterial infection. Therefore it is important to rule out that such long-term complications also apply to p55 dAb

administration, as p55 signalling is involved in controlling phagocytic function of leukocytes thus immune defence against air-borne pathogens [751, 752]. However, the pulmonary delivery of the dAb as opposed to systemic delivery of anti-TNF drugs, the rapid clearance from the lung and its selectivity to p55 signalling which may enhance anti-inflammatory effects of TNF through p75, may reduce the chance of such complications. Future studies in more chronic lung injury models are required to elucidate this.

In summary, specific targeting of intraalveolar p55 receptor signalling by domain antibodies attenuate pulmonary oedema and inflammation in VILI and thus may open a new therapeutic approach for ventilated patients with ALI/ARDS.

7.4 Selective targeting of p55 TNFR signalling during VILI by systemically delivered dAb

Having demonstrated that specific inhibition of intraalveolar p55 receptor signalling attenuates VILI, in Chapter 5 we investigated whether systemic delivery of the p55 dAb would be similarly effective. This model was designed to target p55 receptor signalling both in the intravascular and the intraalveolar compartment, as p55 dAb can pass the alveolar-capillary membrane and showed increasing concentrations in the alveolar space during preliminary pharmacokinetic studies. By doing this, we were aiming to elucidate whether targeting p55 signalling in the pulmonary microvasculature would confer additional benefits to intraalveolar p55 receptor blockade.

We found that in contrast to the i.t. p55-dAb+VILI model, systemic administration of p55 dAb did not attenuate pulmonary oedema formation or the plasma and BALF levels of inflammatory cytokines/chemokines, despite evidence of

p55 dAb accumulation in the alveolar space measured at the end of the ventilation protocol.

The exact reasons behind this lack of efficacy are not fully understood, but are likely to be related to inadequate dosing and/or timing issues. For example, the concentration of p55 dAb in the BALF by the end of experiments was ~500ng/ml, but we do not know how this compares to the intratracheal VILI model, as antibody concentrations were not measured in that model, therefore could be insufficiently low in the current model. Another possibility is that TNF already signalled through the p55 receptor before sufficient concentration of intraalveolar p55 dAb was achieved. Further pharmacokinetic studies are therefore needed to solve these technical issues.

The current data also suggest that p55 signalling in the pulmonary microvasculature is unlikely to have a significant contribution to the development of high-stretch induced lung injury, despite a role in mediating endothelial permeability *in vitro* [813], and adhesion molecule expression *in vivo* (Chapter 3). This could be secondary to a rapid shedding of p55 receptor from PECs (Chapter 3 and Dorr – PhD thesis), which may represent a protective mechanism against further p55-mediated cellular response, and suggests that receptor inhibition on these cells is unlikely to have further benefits in VILI.

However, targeting p55 receptor signalling systemically may have benefits that are not clear from the current study. For example, our group has previously demonstrated that high-stretch ventilation leads to margination of inflammatory leucocytes to the liver and kidney [363], where they can potentially cause organ injury. This is important because patients with ALI or ARDS die from MOF rather than respiratory failure [324]. Margination of neutrophils to the liver, kidney and lung

in response to systemic TNF seems to be p55-mediated as p55 receptor knockout mice have an abrogated response [695].

TNF related systemic organ injury in response to high-stretch mechanical ventilation may not only be mediated by indirect mechanisms such as through upregulation of other circulating inflammatory cytokines/chemokines, endothelial adhesion molecules and consequent leucocyte infiltration to end-organs, but by more direct cellular effects. For instance, Al-Lamki and co-workers showed in rejecting and ischaemic renal allografts, that injured tubular cells display down-regulation of TNFRp55 and activation of the pro-apoptotic apoptosis-signalling regulating kinase-1 (ASK1) [777]. In the same study they also demonstrated in a kidney organ culture that activation of TNFR p55 with receptor-specific mutein results in activation of ASK1 and triggers apoptosis in tubular cells, whereas TNFRp75 mutein led to activation of epithelial/endothelial tyrosine kinase (Etk), tubular and endothelial cell proliferation and down-regulation of TNFRp55 on peritubular endothelial cells. Similarly to these findings the same group later showed that such differential role of TNFRs also exists in the pathogenesis of cardiac allograft rejection [834], i.e. p55 signalling mediates apoptosis whereas p75 signalling triggers cell survival/proliferation in cardiomyocytes. All these findings suggest that targeting p55 receptor signalling by domain antibodies systemically may prevent or attenuate VILI-related end-organ injury and thus reduce mortality. Further investigations are required to explore these possible benefits.

In summary, specific inhibition of p55 receptor signalling in the pulmonary microvasculature does not attenuate high-stretch induced lung injury, however systemic administration of p55 dAb may still have benefits in attenuating end-organ

damage. Further investigations are needed to explore the potential of the systemic delivery route to target intraalveolar p55 signalling in VILI.

7.5 Selective targeting of intraalveolar p55 TNFR signalling during acid aspiration-induced lung injury

Having successfully demonstrated the beneficial effects of targeting intraalveolar p55 receptor signalling in VILI, we turned to investigate if this approach was similarly effective in acid aspiration-induced lung injury.

We showed that intratracheal administration of p55 targeting dAb one hour before acid instillation significantly attenuated pulmonary oedema formation, and reduced pulmonary inflammation. This timely separation of agent administration was necessary because the acid could potentially damage the structure thus function of p55 dAb. From the current data it is unclear whether giving the p55 dAb after the acid could be similarly effective. This would potentially depend on the kinetics of TNF production in the alveolar space upon acid aspiration. Although we did not measure TNF kinetics in our model, previous literature suggests that TNF is produced within 1 hour with a peak at 2 hours after acid aspiration [686], indicating that early intervention may be necessary to attenuate the deleterious effects of acid. Moreover, the respiratory mechanics in our study showed a trend for separation of p55 dAb group from dummy dAb treated mice by 2 hours post-acid, suggesting that TNF signalling via p55 receptor likely have occurred before this time-point.

This relatively early separation of the groups is unlikely to be secondary to an effect of p55 signalling inhibition on leukocyte infiltration, because this happens at later time-points [884]. It is more likely that pulmonary oedema formation in the early

stage of acid injury is secondary to TNF-induced alveolar epithelial cell apoptosis and dysfunction mediated via p55, as suggested by recent studies (since the completion of this PhD project) from our group and others [815, 902]. Our group showed that, acid aspiration up-regulated TUNEL positive cells and lung homogenate caspase-8 activity in wild-type mice, both of which were significantly attenuated in p55 knockout mice. Pharmacological caspase-8 inhibition in wild-type mice significantly improved pulmonary oxygenation and alveolar fluid clearance in this model, suggesting that epithelial cell apoptosis mediated by p55 signalling is the major determinant of lung injury.

Acid-induced production of inflammatory cytokines/chemokines was also significantly reduced by intratracheal p55 dAb treatment, which may have contributed to attenuation of leukocyte margination to the lung. Alternatively, this reduction in the number of lung sequestered leukocytes may be due to attenuated adhesion molecule expression on alveolar epithelial and/or pulmonary endothelial cells (Chapter 3), but this remains just a speculation. It is also unclear from the current model whether this reduced pulmonary inflammation leads to attenuated end-organ damage in this model. These aspects will need to be further investigated.

In summary, we have demonstrated that inhibition of intraalveolar p55 receptor signalling by a domain antibody significantly attenuated pulmonary oedema and inflammation in acid-induced lung injury, and may offer a prophylactic approach for patients who are at high risk of aspiration. Further investigations are needed to clarify the therapeutic potential of i.t. p55 dAb in situations where the treatment cannot be given in a prophylactic manner.

7.6 Future directions

The findings of our characterisation study suggest that the two TNFRs may play different roles, particularly when their relative expression on cells changes, such as that observed during endotoxaemia. A two-hit model, whereby an i.v. LPS challenge is followed by i.v. TNF - 2 hours post-LPS, when the p55 expression was found to be lowest on PECs – could potentially explore this possibility. In particular, the main interest of such model would be to assess wheher this results in an altered expression profile of adhesion molecules, and consequently in a differential pattern of leukocyte subset margination (measured by flow cytometry). This is important, because it is recognised that while ICAM-1 is important in neutrophil margination/sequestration, VCAM-1 preferentially controls monocyte and lymphocyte margination in the microvasculature [909], which may have differential impact on lung injury pathogenesis and recovery. Should the in vivo studies prove to be difficult due to complexity, or the findings be inconclusive, an in vitro adhesion assay could be set up, where different leukocyte subsets are added to cultured PECs, and the percentage of adhered cells would be measured by flow cytometry.

Both of our VILI and acid model showed a beneficial effect of intratracheally administered p55 targeting dAb on pulmonary oedema formation. While the mechanisms by which these positive effects were achieved were not investigated in this PhD project, more recent findings from our lab (since the completion of this PhD study) suggest that it might be related to prevention of alveolar epithelial cell apoptosis and consequently attenuated dysfunction (alveolar fluid clearance) [910]. Also, it is tempting to speculate that blockade of p55-mediated TNF signalling by dAb could potentially result in proportionally enhanced TNF signalling via the p75 receptor, which may have beneficial effects on stabilising epithelial/endothelial

barrier function by the activation of Etk/Bmx pathway [776, 778]. By measuring the level of phosphorylated Etk/Bmx in AECs and PECs in our VILI and acid models using flow cytometry, this potential mechanism could be further investigated. As p75 antagonist dAb is now available, proof of concept studies could be also carried out, whereby blocking both of the receptors using dAbs we could model blockade of total TNF signalling. From these experiments we would expect less or no attenuation of lung injury, as seen in our previous knock-out model [639].

We also found in both the VILI and the acid model, that blockade of intraalveolar p55 signalling by dAb attenuated pulmonary inflammation and lung injury. According to the biotrauma hypothesis of VILI/ALI, this would result in attenuation of extrapulmonary organ dysfunction, which is the main cause of mortality in patients with ALI. Future experiments should be dedicated to investigate this potential benefit of p55 dAb, e.g. by measuring liver enzymes and creatinine in the serum at the end of experiments, and by assessing the number of marginated leukocytes to the liver and kidney using flow cytometry.

As p55 signalling is important in pathogen killing and clearance, it is crucial to investigate any potential increase in susceptibility to infections. In order to test this, we will use our recently developed mouse model of resolving acute lung injury [268]. In this model, spontaneously breathing mice are administered hydrochloric acid intratracheally under anaesthesia and then supported by oxygen in a recovery chamber. Acute lung injury develops by day 1, which then peaks by day 2 and 3 but almost completely resolves by day 10, athough with evidence of lung fibrosis. Animals could be intratracheally instilled with p55 dAb prior to acid insult, and then be intranasally inoculated with live bacteria or LPS/LTA during the recovery period to see if they are more prone to infection/inflammation compared to those animals not

receiving p55 dAb. Using this model of resolving lung injury it could be also tested whether the p55 dAb has any positive effects on the resolution period, such as lung fibrosis.

7.7 Concluding remarks

This project has addressed a wide array of aspects of TNFR biology in ALI of different aetiologies. Firstly, we have developed a new flow cytometry technique and quantitatively characterised the expression profile and roles of TNFRs on pulmonary endothelial cells *in vivo* for the first time. We showed that TNFR p55 is the dominantly expressed receptor on PECs and demonstrated a crucial in vivo role of p55 and an auxiliary role of p75 in TNF-mediated adhesion molecule expression. We also demonstrated that the relative expression of the receptors change during inflammatory conditions, which may influence their overall role in TNF-mediated responses, but further investigations are needed to explore this possibility. Our data also highlight the importance of endothelial cell heterogeneity and thus the danger of extrapolating findings in one vascular bed to another.

Secondly, we have investigated the effect of inhibiting intraalveolar TNFR p55 signalling with a domain antibody in a mouse model of VILI. For this, we have first developed a robust model that eliminated the influence of intratracheal fluid instillation on respiratory mechanics and thus standardisation of high-stretch. Using this model we found that selective targeting of intraalveolar p55 signalling significantly reduced pulmonary oedema and inflammation during VILI, and this effect did not seem to require significant pre-incubation with the antibody. Importantly, anti-TNF treatment with a monoclonal antibody did not attenuate

pulmonary oedema. The data suggest that selective targeting of intraalveolar p55 signalling with domain antibodies may be beneficial for ventilated patients with ALI.

Thirdly, we assessed if systemically delivered p55 domain antibody would be similarly effective in attenuating VILI. We found no attenuation of lung injury (measured by pulmonary oedema and inflammatory cytokines/chemokine levels) with this delivery method despite evidence of antibody accumulation in the alveolar space. This lack of benefit may be related to inadequate antibody dosing or timing issues, as intraalveolar delivery of p55 dAb attenuated lung injury in the previous model. This issue will need to be investigated further, before this route of delivery is completely rejected. Our findings, also suggest that targeting p55 in the intravascular space has no obvious benefits in attenuating high stretch-induced lung injury. However, whether it has benefits in VILI-induced extra-pulmonary organ injury is unclear and warrants further investigations.

Fourthly, we showed that selective inhibition of intraalveolar p55 signalling also attenuates ALI of a different aetiology, namely acid aspiration-induced lung injury. Intratracheally delivered p55 specific dAb significantly attenuated pulmonary oedema and pulmonary inflammation. In this set of experiments the p55 dAb was administered one hour prior the acid challenge, therefore from the current data it is unclear whether it would have been similarly effective had been given after the initial insult. Although further investigations are required to elucidate this, intrapulmonary delivered p55 dAb may be still beneficial after acid aspiration by attenuating VILI in the already injured and ventilated lung.

This project adds a large quantity of interesting data to our knowledge in the fields of ALI and TNFR signalling. In particular, the data from p55 receptor targeting studies using domain antibodies are potentially very clinically applicable, and

requires further investigation. We have also developed a novel methodology that enables quantitative investigation of pulmonary TNFR expression *in vivo*. Moreover, our model development of intrapulmonary dAb delivery in high-stretch ventilation could be very useful for future research investigating the roles of other intraalveolar molecules in the pathogenesis of VILI.

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Appendix I

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Characterization of TNF receptor subtype expression and signaling on pulmonary endothelial cells in mice

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Bertok S, Wilson MR, Dorr AD, Dokpesi JO, O'Dea KP, Marczin N, Takata M. Characterization of TNF receptor subtype expression and signaling on pulmonary endothelial cells in mice. *Am J Physiol Lung Cell Mol Physiol* 300: L781–L789, 2011. First published March 4, 2011; doi:10.1152/ajplung.00326.2010.—TNF plays a crucial role in the pathogenesis of acute lung injury. However, the expression profile of its two receptors, p55 and p75, on pulmonary endothelium and their influence on TNF signaling during lung microvascular inflammation remain uncertain. Using flow cytometry, we characterized the expression profile of TNF receptors on the surface of freshly harvested pulmonary endothelial cells (PECs) from mice and found expression of both receptors with dominance of p55. To investigate the impact of stimulating individual TNF receptors, we treated wild-type and TNF receptor knockout mice with intravenous TNF and determined surface expression of adhesion molecules (E-selectin, VCAM-1, ICAM-1) on PECs by flow cytometry. TNF-induced upregulation of all adhesion molecules was substantially attenuated by absence of p55, whereas lack of p75 had a similar but smaller effect that varied between adhesion molecules. Selective blockade of individual TNF receptors by specific antibodies in wild-type primary PEC culture confirmed that the in vivo findings were due to direct effects of TNF receptor inhibition on endothelium and not other cells (e.g., circulating leukocytes). Finally, we found that PEC surface expression of p55 dramatically decreased in the early stages of endotoxemia following intravenous LPS, while no change in p75 expression was detected. These data demonstrate a crucial in vivo role of p55 and an auxiliary role of p75 in TNF-mediated adhesion molecule upregulation on PECs. It is possible that the importance of the individual receptors varies at different stages of pulmonary microvascular inflammation following changes in their relative expression.

cytokine; adhesion molecule; inflammation; flow cytometry; lipopolysaccharide

PULMONARY MICROVASCULAR INFLAMMATION is one of the first manifestations during the pathogenesis of acute lung injury (ALI), involving production of cytokines and upregulation of adhesion molecules on endothelial cells. Together these orchestrate the margination of inflammatory leukocytes within the microvasculature and their subsequent migration into lung parenchyma (42). The proinflammatory cytokine TNF has been implicated as a key player in regulating this process (12, 42, 50, 63).

Cellular effects of TNF are mediated via two cell surface receptors, p55 and p75 (also known as receptor type I and II, CD120a and CD120b, or TNF receptor superfamily 1a and 1b, respectively) (62). Historically, p55 has been considered as the major effector of TNF-mediated responses, with p75 proposed merely to enhance p55 signaling via "ligand-passing." This dogma may need reevaluation, as it is becoming clear that p75 can mediate cellular responses partly or completely independently from p55 (6, 31, 36, 62). Moreover, it has been reported that the two receptors can even play directly opposing roles in some acute inflammatory conditions, including sepsis, retinal ischemia, and lung injury induced by high-stretch mechanical ventilation (17, 18, 22, 64). Thus tissue-specific expression of each individual TNF receptor would play a major role in determining the inflammatory response of an organ to TNF stimulation. Indeed, cell-specific differential expression of TNF receptors has been proposed as being important in determining the outcome of heart transplant rejection (5).

The majority of previous studies on the roles of each TNF receptor in endothelial cells have indicated that p55 receptor almost exclusively regulates TNF-induced inflammatory gene activation, such as adhesion molecule expression (35, 37, 47, 59, 67), although such studies primarily utilized human umbilical vein endothelial cells (HUVEC) cultured in vitro. In direct contrast, Chandrasekharan et al. (10) observed an essential role for p75 in regulating TNF-induced expression of adhesion molecules in aortic endothelial cells. Such inconsistencies reflect the crucial importance of endothelial cell heterogeneity across the different vascular beds (4), highlighting the danger of extrapolating findings obtained in HUVECs to endothelial cells in other tissues. The lung microvasculature, in particular, is known to be functionally and phenotypically distinct from most other vascular beds, in terms of the site and adhesion molecule requirements of leukocyte extravasation, for example (14). While a small number of studies have attempted to investigate the specific role of pulmonary endothelial TNF receptors, they may be considered somewhat limited in terms of using either semiquantitative methodologies (20, 44) or cultured lung cells (26). Cell culture often results in changes in the phenotype of primary cells, and, therefore, reconciliation of in vitro results with in vivo findings is essential to define the role of each TNF receptor during the activation of pulmonary endothelium. Thus the relative expression of each TNF receptor within the pulmonary circulation and their precise roles in pulmonary microvascular inflammation in vivo still remain unclear.

In this study, we established a flow cytometry-based technique to quantitatively evaluate for the first time in situ expression of TNF receptors and cell adhesion molecules on pulmonary endothelial cells (PECs), in lung single-cell suspensions freshly harvested from in vivo treated mice. These data

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show that p55 is dominant over p75 in terms of both expression and influence on TNF-induced adhesion molecule upregulation on PECs. However, the importance of individual receptors may vary during different stages of pulmonary microvascular inflammation following changes in their relative expression.

MATERIALS AND METHODS

Animals. All experimental protocols involving animals were approved by the Ethical Review Board of Imperial College London and carried out under the authority of the UK Home Office, in accordance with the Animals (Scientific Procedures) Act 1986. Experiments were performed using male wild-type C57/Bl6 mice, aged 9–12 wk, or age- and sex-matched mice lacking p55 [p55 knockout (p55KO)], p75 (p75KO), or both [double knockout (DKO)] TNF receptors (generous gifts from Amgen, Thousand Oaks, CA) (50).

Adhesion molecule expression on PECs in response to TNF in vivo. Wild-type or TNF receptor knockout mice were injected with 500 ng recombinant mouse TNF (R&D Systems Europe, Abingdon, UK) via tail vein. Two or four hours later, animals were killed by isoflurane overdose and exsanguinated by cardiac puncture, the lungs were excised, and a single-cell suspension was prepared for flow cytometric analysis of adhesion molecules. Untreated animals served as controls.

TNF receptor expression on PECs in vivo. Wild-type mice were killed, and 1 ml of ice-cold inhibitor cocktail was instilled through tracheostomy into the lungs to minimize TNF receptors shedding during lung tissue processing. The cocktail consists of serine/cysteine protease inhibitor (1 mM PMSF, Sigma-Aldrich, Poole, UK) and metalloprotease inhibitor (10 μM BB94, British Biotech, Abingdon, UK) in PBS with 2% FCS, 1% sodium azide, and 5 mM EDTA. Lungs were then excised to prepare a single-cell suspension, and TNF receptor expression on PECs was assessed by flow cytometry. In a separate series of experiments, mice were injected with 20 μg LPS (Ultra-pure LPS from *E. coli* O111:B4, Source BioScience UK, Nottingham, UK) via the tail vein. After 2, 4, 8, or 24 h, animals were killed, and the lungs processed as described.

Preparation of lung single-cell suspensions. Lung single-cell suspensions were prepared slightly differently, depending on whether adhesion molecule or TNF receptor expression was to be studied. For adhesion molecule expression, mechanical disruption and enzymatic digestion of excised lungs was carried out using an established technique, described and validated by our laboratory previously (46). Briefly, after harvest, the lung was rinsed in saline and finely minced in a petri dish by manual chopping using a surgical blade. The tissue was resuspended in saline and incubated in 0.5 mg/ml collagenase (type IV, Sigma) at 37°C for 30 min. The resultant digest was then pushed through a 40-μm nylon mesh sieve (BD Falcon, Oxford, UK) with a syringe plunger and flushed through using FACS wash buffer (2% FCS, 1% sodium azide, and 5 mM EDTA), to produce the single-cell suspension.

The tissue processing method was adapted slightly for assessment of TNF receptors. These molecules are highly sensitive to protease-induced shedding (much more so than adhesion molecules), and pilot experiments indicated that even a very short enzymatic digestion step had a major adverse effect on surface expression of the receptors. Therefore, to maximize cell yield while maintaining receptor expression, lungs were chopped (without collagenase, in the inhibitor cocktail described above) using a gentleMACS tissue dissociator (Miltenyi Biotec, Bisley, UK), after which they were also filtered and flushed through a sieve.

The lung single-cell suspension was finally centrifuged at 1,300 rpm for 7 min, the supernatant discarded, and the pellet resuspended. The samples were kept on ice throughout the procedure (except the short collagenase incubation period where utilized) until analyzed by flow cytometry.

Isolation and culture of primary PECs. Wild-type mice were killed, and 1 ml of 1 mg/ml dispase (Gibco, Invitrogen, Paisley, UK) was instilled through tracheostomy into the lungs and left for 5 min, after which lungs were excised and washed in HBSS (Sigma-Aldrich) containing 0.5% FCS. Samples were finely minced, digested in 1 mg/ml dispase at 37°C for 45 min, and then mechanically dissociated by triturating and filtered through a 40-μm nylon mesh to form a single-cell suspension. PECs were isolated from the suspension by a magnetic labeling method, using the midiMACS separation unit (Miltenyi), according to the manufacturer's instructions. In brief, depletion of $CD45^+$ (pan-leukocyte marker) cells was followed by two repeated steps of enrichment of $CD31^+$ (PECAM-1, endothelial cell marker) cells. By this method, we could obtain 6 million PECs on average per mouse [determined by flow cytometry using microsphere counting beads (Caltag Medsystems, Towcester, UK)]. Cells were then cultured at 37°C in 5% CO_2 in DMEM supplemented with 1% nonessential amino acids (Sigma), 1% sodium pyruvate (Sigma), 12 U/ml heparin (Leo Laboratories, Princes Risborough, UK), 25 mM HEPES (VWR, Lutterworth, UK), 10% heat-inactivated FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all from Gibco), and 50 μg/ml endothelial cell growth supplement (Sigma) in flasks coated with 1% type B gelatin (Sigma). After overnight culture, cells were washed to remove nonadherent cells, and fresh medium was added. Once the cells reached confluence, they were replated using trypsin-EDTA (Sigma) and used for experiments between *passages 3* and *8*.

Adhesion molecule expression on PECs in response to TNF in vitro. Cultured PECs were seeded at a density of 10^5 cells/well in a 24-well plate coated with 1% type B gelatin. After overnight culture, the medium was replaced with RPMI containing 10% FCS, and triplicate wells were allocated into one of four groups: no treatment (control); 10 ng/ml recombinant mouse TNF for 4 h; and preincubation with anti-p55 (90 μg/ml; 55R-170, BD Biosciences) or anti-p75 (20 μg/ml; TR75-32, BD Biosciences) neutralizing antibody for 1 h before TNF treatment. The anti-TNF receptor antibodies are validated by the manufacturer as having no cross-reactivity between receptor subtypes. Cells were recovered/detached with 1% trypsin-EDTA at 37°C for 1 min and processed for flow cytometry.

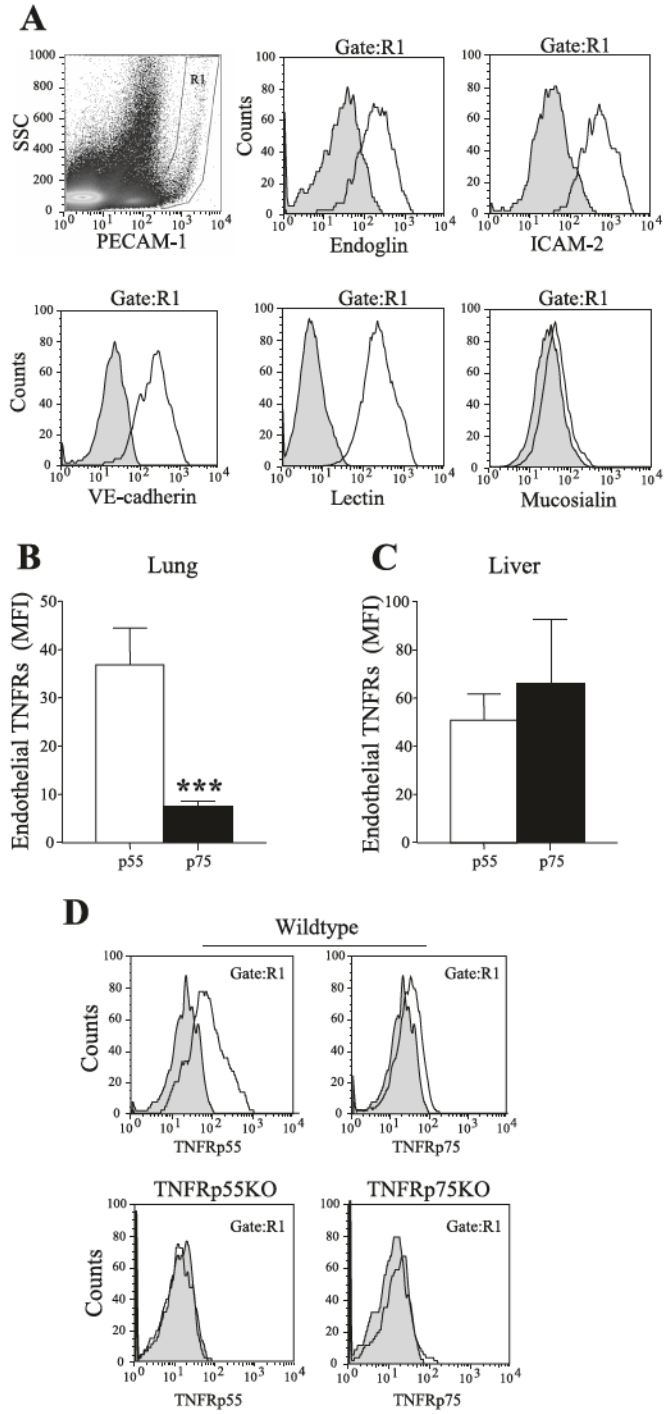
Flow cytometry. In both the in vivo and in vitro studies, experiments were first carried out to characterize phenotype of PECs. Cell samples were stained in the dark on ice for 30 min with fluorophore-conjugated anti-mouse antibodies for general endothelial cell markers PECAM-1 (CD31, clone MEC13.3), Endoglin (CD105, MJ7/18), VE-cadherin (CD144, BV13), and ICAM-2 (CD102, 3C4), capillary/microvascular markers Mucosialin (CD34, RAM34) and *Griffonia simplicifolia* lectin, and their appropriate controls (i.e., isotype or unstained). Antibodies were all from BD Biosciences, apart from *Griffonia simplicifolia* lectin (Sigma). For TNF receptor expression, samples were stained for PECAM-1 and for TNF receptor p55 (55R-286), p75 (TR75-89), and their isotype controls (AbD Serotec, Kidlington, UK). For adhesion molecule expression, samples were stained for PECAM-1 and for VCAM-1 (CD106, 429), ICAM-1 (CD54, 3E2), and E-selectin (CD62E, 10E9.6) and their isotype controls (antibodies all from BD Biosciences). Samples were analyzed using a FACSCalibur flow cytometer (BD) and FlowJo software (Tree Star, Ashland, OR). Expression levels of TNF receptors and adhesion molecules were assessed on PECs (defined as the high $PECAM^+$ cells, at least 2,000 gated events in each analysis), expressed as mean fluorescence intensity after subtraction of isotype control values.

Statistical analysis. Data are expressed as means ± SD. Statistical comparisons were made by ANOVA with Bonferroni tests or by *t*-tests. A value of $P < 0.05$ was considered as significant.

RESULTS

Basal expression of p55 and p75 receptors on PECs. To clarify the expression of individual TNF receptors on PECs in vivo, we developed a quantitative flow cytometry technique to assess the expression of the two receptor subtypes in single-cell

suspensions freshly prepared from the excised lungs of mice. Endothelial cells were identified as PECAM-1 high events on the dot plot, which consistently displayed positive expression of endoglin (CD105), VE-cadherin (CD144), and ICAM-2 (CD102), confirming the endothelial origin of these cells (Fig. 1*A*). Additionally, cells were stained for mucosialin (CD34) and the lectin *Griffonia simplicifolia*. Positive staining for both of these markers suggests that the vast majority of endothelial cells analyzed were microvascular in origin (32, 43, 56), although some contamination by macrovascular cells cannot be entirely excluded.



Under resting conditions, both the p55 and p75 receptors were expressed on PECs, with significantly greater expression for p55 compared with p75 (Fig. 1*B*). To confirm that the relative level of expression of the individual receptors was not due to differential susceptibility to shedding during cell harvest and processing, we determined TNF receptor levels on endothelial cells harvested from the liver using the same tissue processing methodologies. On these cells, we found that p55 expression was similar to that found on pulmonary endothelia, but p75 expression was much greater, indicating that tissue processing artifacts did not explain the relative expression levels (Fig. 1*C*). As a final validation of the flow cytometry technique, TNF receptor expression was also assessed on PECs from genetically modified animals lacking TNF receptors (Fig. 1*D*). The p55 signal was completely absent in the p55KO mice, while the p75 signal was effectively nil in the p75KO animals with a very small degree of nonspecific staining with this antibody, demonstrating that low-level p75 expression on wild-type PECs was indeed an authentic signal. The histograms of p55 or p75 staining in wild-type animals are consistent with the pattern of normal distribution, suggesting that all PECs are expressing a moderate (p55) or low (p75) level of receptors, with no evidence of subpopulations of positive and negative cells.

p55 and p75 TNF receptors both influence upregulation of adhesion molecules on PECs in vivo. Having identified both p55 and p75 receptors on the surface of PECs, we then attempted to dissect the specific roles of the receptors in pulmonary endothelial activation. Wild-type and TNF receptor knockout mice were treated with intravenous (iv) TNF, and the surface expression of leukocyte adhesion molecules VCAM-1, E-selectin, and ICAM-1 was quantitatively assessed on PECs by flow cytometry. Although these cells were harvested using a slightly modified technique compared with the TNF receptor expression studies (i.e., inclusion of a short collagenase digestion step), we confirmed that they had the same phenotypic characteristics, i.e., positive expression of ICAM-2, VE-cadherin, and endoglin (not shown). Although it is possible that such a short warming period during the cell isolation procedure (i.e., collagenase digestion) could induce nonspecific effects on PECs, pilot studies indicated that these were negligible, at least with regards to adhesion molecule expression (not shown). In preliminary time course experiments using wild-type mice, E-selectin showed peak expression on PECs at 2 h after TNF treatment, while VCAM-1 and ICAM-1 expression was greater

Fig. 1. Flow cytometric analysis of TNF receptor (TNFR) expression on pulmonary endothelial cells (PECs). *A*: in characterization studies, PECs were identified as PECAM-1$^+$ events (R1), and their endothelial phenotype was confirmed by positive staining for endothelial cell markers, i.e., endoglin (CD105), VE-cadherin (CD144), ICAM-2 (CD102), mucosialin (CD34), and *Griffonia simplicifolia* lectin. Staining with the respective antibody (solid line) and isotype control (unstained in the case of lectin; shaded fill) is shown on histograms. *B*: in control condition, PECs showed greater expression of p55 (open bar) than p75 (solid bar). Values are means + SD; $N = 4$. ***$P < 0.001$ by paired *t*-test. *C*: liver endothelial cells expressed similar amount of the p55 (open bar) and p75 (solid bar) receptors. Values are means + SD; $N = 3$. *D*: validation of flow cytometry technique using wild-type (WT) and p55 and p75 knockout animals (TNFRp55KO and TNFRp75KO, respectively) indicated small nonspecific binding of the p75 antibody. Staining with the respective antibody (solid line) and isotype control (shaded fill) is shown on histograms. MFI, mean fluorescence intensity; SSC, side scatter.

at 4 h (data not shown). Subsequent experiments compared E-selectin expression between strains at 2 h, and VCAM-1 and ICAM-1 expression at 4 h.

VCAM-1 had a moderate basal expression on PECs from all mouse strains, which was somewhat variable, although no strain was significantly different compared with wild types (Fig. 2*A*). Surface expression was substantially upregulated upon TNF treatment in wild-type mice. Expression of VCAM-1 following stimulation was reduced in p75KO animals compared with wild-type mice and decreased to an even greater extent in p55KO and DKO mice. Unlike VCAM-1, E-selectin was not expressed under resting conditions (Fig. 2*B*), but it was induced in wild-type mice upon iv TNF treatment. E-selectin expression tended to be reduced (although not significantly) in p75KO animals compared with wild types, whereas both strains of mice lacking the p55 receptor (i.e., p55KO and DKO) showed effectively zero expression. In contrast to VCAM-1 and E-selectin, ICAM-1 is expressed on PECs at a relatively high basal level in all strains (Fig. 2*C*) and was upregulated further after TNF treatment in wild-type mice. While absence of the p75 receptor caused some tendency to decreased ICAM-1 expression, this was not significant. In contrast, mice lacking the p55 receptor (p55KO and DKO animals) expressed significantly less ICAM-1 after TNF stimulation compared with wild types.

Differences in adhesion molecule expression are due to TNF receptor signaling specifically on PECs. The in vivo data demonstrated that lack of p55 receptor signaling influenced TNF-induced adhesion molecule upregulation on PECs to a much greater extent than that of p75. To clarify that our in vivo findings reflect the importance of TNF receptors on "endothelial cells" per se, rather than an indirect consequence of TNF receptors signaling on other cells [e.g., leukocytes, which are known to activate PECs in a TNF-dependent manner (46)], we took an in vitro approach utilizing acute receptor blockade. For this purpose, PECs were isolated from wild-type mice and cultured and then treated with TNF in the presence or absence of a vast excess of neutralizing antibody for p55 or p75 receptors, and surface expression of adhesion molecules was quantified by flow cytometry as in vivo. Isolation of PECs by this method resulted in 95% purity of PECAM-1$^+$ cells (Fig. 3*A*). Before each set of experiments, cells were stained for various endothelial cell markers, to confirm both that they still retained their phenotype across passages, and that measurement of adhesion molecule expression did not include the presence of any contaminating nonendothelial cells (Fig. 3*B*).

Consistent with the in vivo findings, p75 blockade resulted in significant reduction of TNF-induced VCAM-1 and E-selectin expression by 28 and 23%, respectively ($P < 0.05$), but had no significant effect on ICAM-1 (Fig. 4*A*). On the other hand, as with in vivo experiments, p55 blockade significantly decreased the expression of all adhesion molecules ($P < 0.01$) (Fig. 4*B*).

p55 Receptor expression decreases, while p75 remains stable, during acute endotoxemia. Finally, we determined the influence of acute inflammation on the relative levels of TNF receptor expression. Historically, it is reported that p75 is more readily upregulated than p55 on stimulation (20, 26, 27), while both receptors also show a propensity to be shed on cellular activation (15, 54, 61). However, such data almost exclusively relate to primary leukocytes or leukocytic cell lines. Following

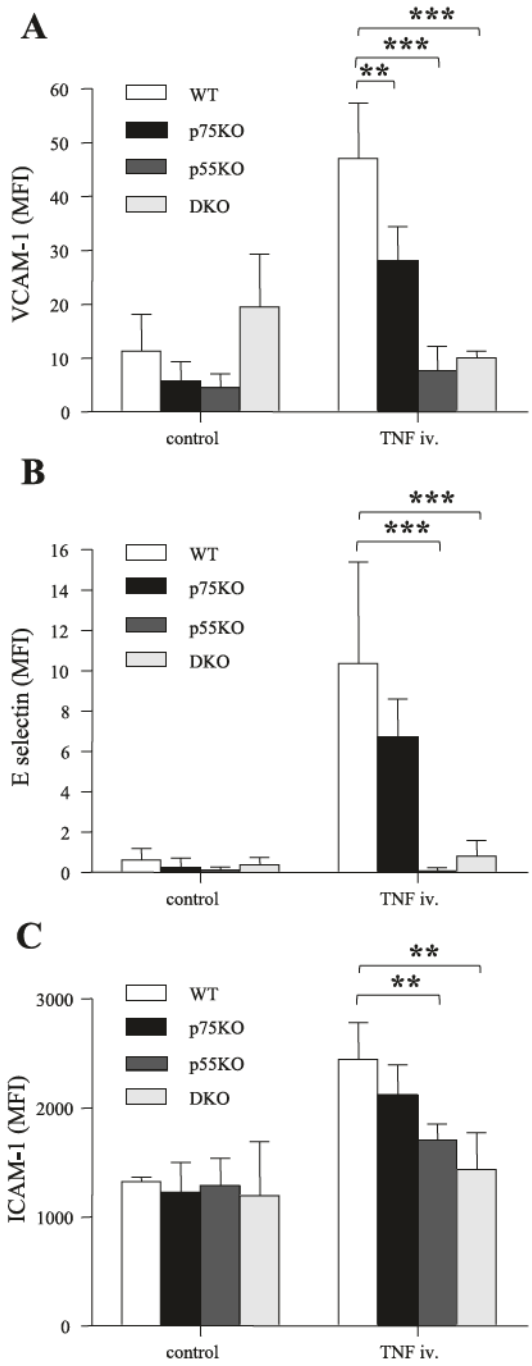


Fig. 2. Effects of chronic absence of TNFR subtype signaling on TNF-induced expression of adhesion molecules on PECs in vivo. Surface expression of VCAM-1 (*A*), E-selectin (*B*), and ICAM-1 (*C*) was assessed by flow cytometry on PECs in different mouse strains either 2 (E-selectin) or 4 h (VCAM-1, ICAM-1) after intravenous (iv) TNF injection (500 ng). p75KO, TNFRp75 knockout; p55KO, TNFRp55 knockout; DKO, TNFRp55/p75 double knockout. Values are means + SD of MFI; $N = 3$–6/group. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. TNF-treated WT by one-way ANOVA with Bonferroni posttests.

iv LPS administration, we found that p75 expression on PECs did not change substantially over the following 24 h. In marked contrast, there was a substantial decrease of p55 expression at 2 h after LPS, which started to recover by 4 h and reached ~80% of baseline levels by 24 h (Fig. 5).

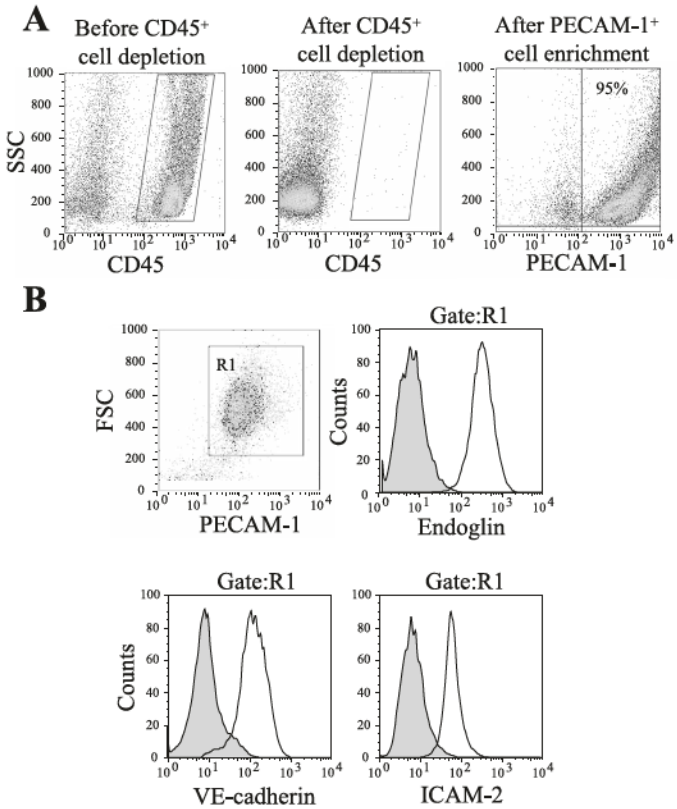


Fig. 3. Analysis of purification and characterization of mouse primary PECs by flow cytometry. *A*: primary PECs were isolated from WT mice by enzymatic digestion and mechanical dissociation, followed by depletion of CD45$^+$ cells and enrichment of PECAM-1$^+$ cells by a magnetic labeling method, using the midiMACS separation unit. This provided a 95% purity of PECs for culture. Dot plots represent composition of cell suspension before and after CD45$^+$ cell depletion and after PECAM-1$^+$ cell enrichment. *B*: before experiments (as well as at time of purification), PECAM-1$^+$ cells were characterized by staining for endoglin, VE-cadherin, and ICAM-2 to ensure their endothelial phenotype was maintained from passage to passage. Staining with the respective antibody (solid line) and isotype control (shaded fill) is shown on histograms in *passage 6* cells. FSC, forward scatter.

DISCUSSION

In this study we investigated for the first time the quantitative expression profile of TNF receptors on the surface of freshly harvested mouse PECs using flow cytometry and elucidated their roles in mediating TNF-induced expression of adhesion molecules as surrogate markers of endothelial activation. We found that, under resting conditions, both receptors are expressed on PECs, with p55 expressed at substantially higher levels than p75. Both receptors played some role in mediating TNF-induced expression of adhesion molecules, although the p55 receptor had a much greater impact than the p75 receptor in vivo. Finally, we demonstrated that acute endotoxemia induced a rapid downregulation of p55 expression on PECs, but no change in p75, which may have important consequences for TNF signaling during pulmonary microvascular inflammation.

Although TNF plays an indisputable role in sepsis/ALI (9, 25, 40, 41, 48), blockade of total TNF signaling using anti-TNF antibodies is clinically not effective (1–3, 11, 21), suggesting more complex mechanisms in TNF-mediated responses during sepsis/ALI than originally considered. Our laboratory previously demonstrated a novel role for differential TNF receptor signaling in ALI induced by mechanical stretch (64) and acid aspiration (49). Using TNF receptor knockout mice, we found that signaling through p55 receptor promoted the development of pulmonary edema and respiratory failure, whereas signaling through TNF receptor p75 opposed this. Such differential signaling through the two TNF receptors has been observed in other acute inflammatory conditions (17, 18, 22, 29), indicating that the relative expression of the two receptor subtypes within the lung is critically important in determining the consequences of TNF signaling during the course of ALI.

Most of our current understanding of TNF receptor expression on endothelial cells comes from HUVEC data, where TNF binding studies suggest that p55 is marginally the dominantly expressed receptor (47, 53, 60), while flow cytometric analysis showed similar or slightly higher expression of p75 (37, 59). The expression on endothelial cells in the pulmonary microvasculature is not clear. Previous studies suggested relatively similar expression of p55 compared with p75 on PECs of rats

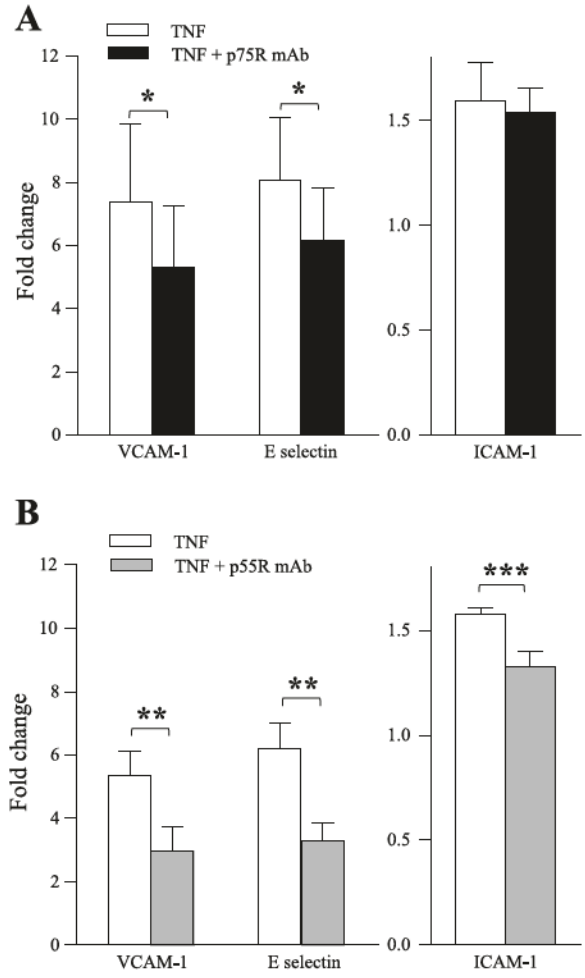


Fig. 4. Effects of acute TNFR inhibition on TNF-induced expression of adhesion molecules in primary PEC culture. Fold changes (compared with untreated cells in the same experiment) are shown of adhesion molecule expressions on WT primary mouse lung endothelial cells following 4-h TNF treatment (10 ng/ml) in the presence or absence of p75 (20 μg/ml; *A*) or p55 (90 μg/ml; *B*) receptor neutralizing monoclonal antibody (MAb). p55 and p75 blockade experiments were conducted separately. Values are means + SD; $N = 3–4$. $*P < 0.05$, $**P < 0.01$, $***P = 0.001$ vs. TNF by paired *t*-test.

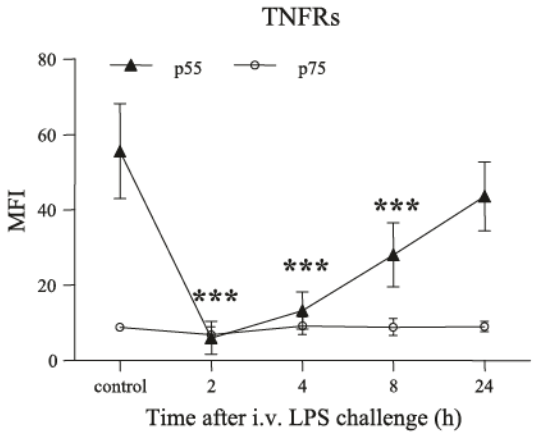


Fig. 5. Changes in TNFR expression on PECs during endotoxemia in vivo. Mice were injected with iv LPS (20 μg), and after 2, 4, 8, and 24 h, the lungs were processed for flow cytometric analysis. Untreated animals served as controls. Surface expression of TNFR p55 and p75 was quantified on PECAM-1$^+$ events, as described before. Values are means ± SD of MFI; N = 4–5/each time point. ***$P < 0.001$ vs. control for p55 expression by one-way ANOVA with Bonferroni posttests.

and humans, although these findings rely on data from prolonged in vitro cultures (26) and semiquantitative methods, such as immunohistochemistry (20). Therefore, quantitative in vivo analysis of TNF receptors on PECs has been lacking.

To address this, we established a flow cytometry method that enabled us to quantitatively evaluate the receptor expression on PECs in a single-cell suspension. Positive cell staining for a panel of endothelial cell markers, including mucosialin and *Griffonia simplicifolia* lectin, suggest that the PECs analyzed were primarily microvascular in origin. This distinction is potentially important, as phenotypic and functional heterogeneity has been described among PECs of microvascular and macrovascular origin (24, 32, 65). Using a mixture of protease inhibitors and keeping the working temperature close to 4°C, we attempted to minimize loss/shedding of the receptors during the lung processing and analysis procedures. In untreated mice, we found that both TNF receptors were expressed on PECs, with p55 expression substantially higher than that of p75. By the use of knockout mice, we were able to demonstrate that the antibody binding was indeed specific to each receptor, with only a small nonspecific signal for p75. We also measured the relative expression of TNF receptors on endothelial cells harvested from the liver using the same techniques and found that, while p55 expression was similar to that found in PECs, p75 expression was greater, exceeding the levels of p55. These data strongly support that the low signal of p75 on PECs is not due to processing artifacts and, more importantly, highlight the potential risks of extrapolating between endothelial cells from different sources.

A previous in vivo study by Neumann et al. (44) using knockout mice suggested a crucial role of p55 in TNF-induced expression of E-selectin and VCAM-1 (measured by immunohistochemistry) and recruitment of leukocytes within the lung, although this study lacked investigation into any involvement of p75. To clearly dissect out the roles of the receptor subtypes, we injected TNF iv to wild-type mice or mice lacking one or both of the TNF receptors and quantitatively evaluated E-selectin, VCAM-1, and ICAM-1 expression on PECs using flow cytometry. We found that p55 receptor signaling was necessary for each adhesion molecule upregulation (in agreement with the study by Neumann et al.), while p75 signaling contributed to VCAM-1 upregulation, but any effects on E-selectin and ICAM-1 expression were less clear. To further clarify our observations from genetically modified mice, we used in vitro receptor blockade experiments. We found that the consequences of individual TNF receptor signaling observed in vivo were very likely due to direct effects on PECs and confirmed that p75 signaling influenced VCAM-1 and E-selectin expression. The influence of p75 signaling on ICAM-1 was less clear, which may indicate some differential effect of p75 on the different adhesion molecules, or reflect a relative lack of sensitivity as a result of the much higher basal expression of ICAM-1.

Our findings indicating a minor role for p75 in TNF-mediated adhesion molecule expression may be consistent with the ligand-passing theory of TNF signaling on PECs, whereby p75 functions primarily to modulate signaling through p55. Alternatively, it could be a reflection of the relative level of receptor expression (i.e., p55 > p75) on PECs, and p75 may be more important in other organ beds (e.g., liver) or conditions, where the relative expression of the receptors are changed. For example, our finding that the p75 receptor does not contribute much to ICAM-1 upregulation contrasts with a previous report by Chandrasekharan et al. (10). Using aortic endothelial cells from TNF receptor knockout animals, they reported that p75 receptor signaling was greatly responsible for ICAM-1 expression, although they did not assess the relative levels of TNF receptor expression on their cells. This suggests that the role of p75 in signaling varies between vascular beds, which would tend to argue against a generalized ligand-passing concept for this receptor.

Expression of the TNF receptors is regulated by protein synthesis, both at the transcriptional/translational level, by the action of sheddases, such as TNF-α converting enzyme (33, 57, 62) and by internalization mechanisms (28). Both p55 and p75 may be shed or internalized from the surface of cells, and under certain circumstances both can be upregulated, p75 more dynamically than p55 (13, 15, 16, 28, 30, 61). However, the vast bulk of this knowledge comes from hematopoietic cells and cancer cell lines, which may not be applicable to endothelial cells. To assess the changes in TNF receptor expression on PECs following an inflammatory stimulus, we treated mice with LPS and determined the expression of the two receptors over 24 h. For the first time in freshly harvested PECs, we showed that surface p75 expression was effectively unchanged, while expression of the p55 receptor decreased greatly following LPS. Such a loss of surface p55 is likely to represent a regulatory mechanism to limit ongoing stimulation of the receptors following the initial ligand-receptor interaction. Indeed, TNF-induced TNF receptor shedding leading to deactivation of cells has been observed and investigated extensively in leukocytes (15, 55, 58), and impaired shedding of p55 leads to an exaggerated inflammatory state in humans and mice (39, 66). Although mice lacking the p55 receptor show attenuation in pulmonary neutrophil accumulation following systemic LPS challenge (8), suggesting that TNF likely signals through the p55 receptor before shedding occurs, the subsequent down-regulation of p55 receptors on endothelial cells may have significant long-term consequences (e.g., endothelial hypore-

sponsiveness to further TNF stimuli) beyond the effect on adhesion molecules that we observed here.

Our findings regarding the changes in TNF receptor expression during inflammation are apparently inconsistent with those reported by Grau et al. (26) investigating the functional characteristics of PECs isolated from lungs of patients who died with acute respiratory distress syndrome. They demonstrated greater p75 expression on PECs from the patients than controls, which appeared to positively correlate with the duration of the disease, while no difference in p55 was observed. However, their study investigated TNF receptor expression on PECs in vitro after 2- to 3-wk culture, which may not reflect the phenotype at the time of isolation. Both our study and that by Grau et al. do, however, indicate a change in the ratio of p55 to p75 receptors on PECs during ALI/inflammation. The downstream consequences of such changes in the p55-to-p75 expression ratio remain unclear. As the present data indicate that p75 plays a similar role to p55 signaling in regards to adhesion molecule upregulation (albeit of lesser magnitude), it may be concluded that a relative shift in the contribution of signaling through p75 vs. p55 would have little impact. However, adhesion molecule upregulation is but one consequence of TNF receptor activation, and it is now clear that the two TNF receptors are able to signal independently. For example, TNF has been shown in neurons to mediate proapoptotic signaling through p55, but antiapoptotic signaling through p75 (38). Changes in the relative levels of TNF receptor expression may, therefore, have substantial consequences not apparent from the present observations.

In more complicated models than used currently, our laboratory previously found that p75 signaling opposed, rather than complemented, that of p55 during the development of high stretch-induced pulmonary edema (64). We consider that any apparent discrepancy between the present study and our laboratory's previous findings is likely to be a reflection of the relative complexity of the models and the parameters being investigated. The present study evaluated a single TNF-mediated response in a specific cell type (i.e., pulmonary endothelial adhesion molecule expression in response to iv TNF). In contrast, the previous study represented a much more complex effect of TNF receptor signaling on various pathophysiological processes during full-blown ALI models, involving endothelial/epithelial cell permeability (34, 45, 51, 52) and fluid reabsorption (7, 19, 23). One possible interpretation is that the "protective" role of p75 signaling observed in our laboratory's previous study is not related to endothelial cell adhesion molecule upregulation (as we found p75 signaling plays a similar "injurious" role to that of p55, albeit less potent), although it is not certain that various ALI models cause injury in similar ways.

In summary, the present study provides invaluable new data of individual TNF receptor expression and signaling on pulmonary endothelium in vivo, by quantitative assessment of TNF receptors and TNF-induced expression of adhesion molecules on freshly harvested PECs using flow cytometry. The results highlight the potential risks in extrapolating data obtained in cell lines/endothelial cells from other vascular beds or using semiquantitative methods. Our findings add crucial pieces of information to our knowledge base of endothelial cell biology within the lung, which would lead to novel insights into TNF-mediated pathophysiology within the pulmonary microvasculature. Further work needs to be carried out to elucidate the functional consequences of individual TNF receptor signaling during pulmonary inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

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Appendix II

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ORIGINAL ARTICLE

Selective inhibition of intra-alveolar p55 TNF receptor attenuates ventilator-induced lung injury

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ABSTRACT

Background Tumour necrosis factor (TNF) is upregulated in the alveolar space early in the course of ventilator-induced lung injury (VILI). Studies in genetically modified mice indicate that the two TNF receptors play opposing roles during injurious high-stretch mechanical ventilation, with p55 promoting but p75 preventing pulmonary oedema.

Aim To investigate the effects of selective inhibition of intra-alveolar p55 TNF receptor on pulmonary oedema and inflammation during ventilator-induced lung injury using a newly developed domain antibody.

Methods Anaesthetised mice were ventilated with high tidal volume and given an intratracheal bolus of p55-specific domain antibody or anti-TNF monoclonal antibody ('pure' VILI model). As a model of enhanced inflammation, a subclinical dose of lipopolysaccharide (LPS) was included in the intratracheal antibody bolus (LPS+VILI model). Development of lung injury was assessed by respiratory mechanics and blood gases and protein levels in lavage fluid. Flow cytometry was used to determine leucocyte recruitment and alveolar macrophage activation, while lavage fluid cytokines were assessed by ELISA.

Results The ventilation protocol produced deteriorations in respiratory mechanics and gas exchange with increased lavage fluid protein levels in the two models. The p55-specific domain antibody substantially attenuated all of these changes in the 'pure' VILI model, while anti-TNF antibody was ineffective. In the LPS+VILI model, p55 blockade prevented deteriorations in respiratory mechanics and oxygenation and significantly decreased neutrophil recruitment, expression of intercellular adhesion molecule 1 on alveolar macrophages, and interleukin 6 and monocyte chemotactic protein 1 levels in lavage fluid.

Conclusions Selective inhibition of intra-alveolar p55 TNF receptor signalling by domain antibodies may open new therapeutic approaches for ventilated patients with acute lung injury.

Key messages

What is the key question?

- Does selective inhibition of tumour necrosis factor (TNF) receptor p55 signalling attenuate ventilator-induced lung injury?

What is the bottom line?

- Selective inhibition of the p55 TNF receptor within the alveolar space using a domain antibody attenuated pulmonary oedema and inflammation in mouse models of ventilator-induced lung injury, whereas total TNF blockade using a traditional monoclonal antibody was without effect.

Why read on?

- This paper reports for the first time that more specific pharmacological modulation of TNF signalling is potentially effective in the treatment of ventilator-induced lung injury.

INTRODUCTION

Mechanical ventilation is essential for the treatment of patients with acute lung injury/acute respiratory distress syndrome (ALI/ARDS), although this frequently results in the development of ventilator-induced lung injury (VILI).[1] While the initiating trigger is unique, the final pathophysiology of VILI is indistinguishable from that of ALI of other aetiologies—that is, characterised by pulmonary oedema and inflammation.[2 3] VILI may be particularly amenable to early pharmacological intervention as its iatrogenic nature means that onset occurs in a predictable manner and within the hospital setting.

The proinflammatory cytokine tumour necrosis factor (TNF) has been consistently implicated in the pathogenesis of ALI/VILI, both clinically and in experimental models.[3–6] Clinical trials of anti-TNF therapy in patients with sepsis/ALI[7–10] have unfortunately shown little or no benefits, and in some cases may have led to increased morbidity. The reasons behind such a lack of benefit are unclear, but are likely to include issues relating to the design of the agents used and also to the complicated biology of TNF signalling. TNF signals through two cell surface receptors, p55 and p75, and recent evidence suggests that signalling through these individual receptors may have differential—even opposing—roles in a number of pathological conditions.[11–13] We previously found that biologically active TNF is upregulated within the alveolar space early during the development of VILI.[14] Moreover, using TNF receptor knock-out mice, we found that the two receptors of TNF play opposing roles during VILI: p55 receptor knock-out mice were protected while p75 receptor knock-out animals were more susceptible to pulmonary oedema.[15] In light of these data, we propose that 'selective' inhibition of 'p55 TNF receptor'

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signalling, specifically within the alveolar space, would be clinically much more effective than total TNF inhibition to ameliorate the deleterious effects of mechanical ventilation.

Conventional antibody technologies are not optimal for this purpose for a variety of reasons, perhaps the most important of which being that monoclonal antibodies to this and, in general, other cell surface receptors have a tendency to induce receptor cross-linking, thereby activating rather than inhibiting signalling.[16 17] To investigate the possibility of pharmacological inhibition of p55 signalling, we therefore used a novel IgG fragment known as a domain antibody (GlaxoSmithKline, Stevenage, UK) that was selected to specifically bind and inhibit mouse p55 TNF receptor. Domain antibodies (dAbs) comprise the variable domains of either the heavy or light chain, and are thus the smallest functional antigen-binding portions of the IgG molecule.[18 19] Domain antibodies offer multiple advantages over conventional antibody technology: they bind monovalently to their targets, avoiding the potential for receptor cross-linking; they lack the Fc portions and do not induce Fc effector responses such as complement-dependent cytotoxicity or antibody-dependent cytotoxicity; their small size (~12 kD) enables better tissue penetration, delivery at much higher concentrations per unit mass and potentially fewer long-term adverse effects (eg, immunosuppression) due to more rapid excretion; and they can be manufactured to be suitable for local delivery such as inhalation.

In this study we investigated the effects of an intratracheally delivered p55 TNF receptor-targeting dAb in in vivo mouse models of VILI. We found that selective blockade of intra-alveolar p55 signalling reduced alveolar-epithelial barrier permeability and pulmonary inflammation, which suggests potential for new treatments of VILI and ALI.

METHODS

Ventilation protocol

Male C57BL6 mice (Charles River, Margate, UK) aged 8–12 weeks were anaesthetised (intraperitoneal ketamine 80 mg/kg and xylazine 8 mg/kg), tracheostomised and ventilated using a custom-made jet ventilator.[14 20 21] Airway flow was monitored by a miniature pneumotach in the ventilator system and tidal volume (V_T) was calculated by integrating flow during inspiration. The left carotid artery was cannulated for blood pressure (BP) and arterial blood gas (ABG) measurements. Ventilation was then set to produce VILI ('pure' VILI model), standardised by inspiratory plateau pressure ($P_{plateau}$) of 14 cm H_2O equating to V_T of ~22 ml/kg with positive end-expiratory pressure 3 cm H_2O, respiratory rate 90/min and oxygen+4% carbon dioxide (to prevent hypocapnia). Immediately afterwards, mice received an intratracheal bolus of either dummy (non-targeting) dAb or p55-specific blocking dAb (25 μg), or either mouse anti-TNF monoclonal antibody (50 μg; TN3-19.12; BD Pharmingen, Oxford, UK) or its isotype control (hamster IgG, BD Pharmingen) in a total volume of 50 μl. We previously demonstrated the efficacy of the monoclonal anti-TNF used (with the same route and dose) to reduce alveolar neutrophil infiltration associated with VILI.[22] In a separate series of experiments, a subclinical dose of lipopolysaccharide (LPS, 20 ng; *Escherichia coli* O111:B4, Source Bioscience, Nottingham, UK) was co-administered intratracheally with the dAb bolus to enhance the inflammatory component of the model. In this LPS+VILI model, slightly lower $P_{plateau}$ (13 cm H_2O) and V_T (~20 ml/kg) were used as described previously[21] because LPS was expected to enhance the degree of VILI.

Ventilation using the same constant high V_T was continued in all groups for 240 min or until the mean BP dropped below 45 mm Hg.[14] Anaesthesia was maintained by bolus administrations of intraperitoneal ketamine:xylazine every 20–25 min. A small number of animals showed very rapid haemodynamic deterioration: in the 'pure VILI' model, three mice treated with dummy dAb, one treated with p55-specific dAb, one treated with isotype control and two mice treated with anti-TNF; in the LPS +VILI model, one mouse treated with dummy dAb. These prematurely terminated experiments were excluded from the analyses. $P_{plateau}$, respiratory system elastance (E_{rs}) and resistance (R_{rs}) were determined by the end-inflation occlusion technique[20] every 20 min, followed each time by sustained inflation (35 cm H_2O, 5 s) to avoid the development of atelectasis. ABG were assessed at predetermined points throughout the protocol (figure 1).

Lung lavage

Following termination, lung lavage was performed[14] and lavage fluid was analysed for protein concentration (Bio-Rad Laboratories, Hemel Hempstead, UK) and, in some cases, for TNF levels by ELISA (R&D Systems, Abingdon, UK). In the LPS +VILI model, levels of interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1), keratinocyte chemoattractant (KC) and macrophage inflammatory protein 2 (MIP-2) in the lavage fluid were evaluated by ELISA (R&D) and cell pellets were used for flow cytometry. Cytokine levels are expressed as total amount (pg) recovered by lavage.

Lung histology

In some experiments, lungs were removed and instilled with 1% low melting point agarose in 4% paraformaldehyde and processed for histological evaluation by H&E staining.

Flow cytometry

In the LPS+VILI model, lung single cell suspensions were prepared from excised lungs by mechanical disruption for flow cytometry analysis, as described previously.[23–25] Lung and lavage cells were stained with fluorophore-conjugated anti-mouse antibodies for CD11b, CD11c, Gr-1 (Ly6C/G), F4/80 and intercellular adhesion molecule 1 (ICAM-1) or appropriate isotype-matched controls, and analysed using a FACSCalibur flow cytometer with CellQuest (Becton Dickinson, Oxford, UK) and Flowjo (Tree Star, Ashland, OR, USA) software. Cells were quantified using microsphere counting beads (Caltag Medsystems, Towcester, UK) added to the samples. Activation state of alveolar macrophages was evaluated based on surface ICAM-1 expression.

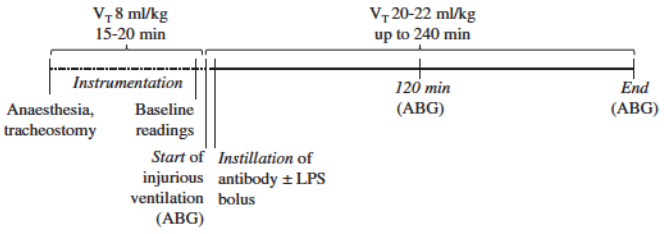


Figure 1 Scheme of ventilation protocol. Mice were anaesthetised, tracheostomised and ventilated with non-injurious ventilation for approximately 15–20 min during instrumentation (dotted line). Injurious ventilation parameters using high tidal volumes (V_T) were then introduced, followed immediately by instillation of the antibody of interest. As a model of ventilation-induced lung injury with enhanced inflammation, a subclinical dose of lipopolysaccharide (LPS, 20 ng) was co-administered with the domain antibody bolus. After instillation of the reagents, four sustained inflations (35 cm H_2O, 5 s) were applied to help the fluid distribute within the lungs. Respiratory mechanics were assessed every 20 min, followed each time by sustained inflation. Arterial blood gases (ABG) were assessed at predetermined time points (start, 120 min, end).

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Statistical analysis

Data are expressed as mean±SD. Statistical comparisons were made by two-way repeated measures ANOVA for time course data or unpaired t tests for end point data using Prism software Version 5.0. A value of $p<0.05$ was considered significant. For some end point analyses, data were compared with those of non-ventilated mice for qualitative purposes.

RESULTS

Effects of anti-p55 domain antibody on respiratory physiology in the 'pure' VILI model

Mechanical ventilation with the high stretch protocol caused a substantial deterioration in lung function in dummy dAb-treated animals, consistent with pulmonary oedema formation. This was represented by a rapidly increasing $P_{plateau}$, E_{rs} and R_{rs} that occurred towards the end of the 4 h ventilation protocol (figure 2A–C). In marked contrast, no deteriorations were seen in respiratory mechanics in the p55 dAb group.

Arterial oxygen and carbon dioxide tensions (PO_2 and PCO_2) were similar between the two groups at the start and 120 min after drug instillation (figure 2E,F). By the end of the protocol, oxygenation tended to deteriorate in dummy dAb-treated animals (365±146 mm Hg) while PCO_2 increased. In contrast, PO_2 was better maintained in the p55 dAb group (440±41 mm Hg) and PCO_2 was unchanged from the 120 min level. To assess alveolar epithelial permeability we measured the protein content in lavage fluid. VILI resulted in substantial protein levels in the dummy dAb group compared with non-ventilated controls (2.82±1.73 vs 0.21±0.03 mg/ml), and this was significantly attenuated in mice given the p55 dAb (1.34±0.4 mg/ml, figure 2H). TNF levels in bronchoalveolar lavage fluid evaluated at the end of the ventilation protocol in the dummy dAb group were found to be upregulated (147±144 pg, n=11) compared with untreated mice (less than the assay detection limit of 15 pg/ml).

Lung histology indicated a moderate degree of injury after VILI with dummy dAb treatment, characterised by neutrophil infiltration and proteinaceous material in the alveolar space (figure 3A,B) that was substantially reduced following p55 dAb (figure 3C,D).

Effects of anti-TNF monoclonal antibody on respiratory physiology during VILI

Having found that blockade of p55 TNF receptor signalling attenuated VILI, we investigated whether total TNF blockade would be similarly efficacious. To address this, we treated mice with anti-TNF monoclonal antibody (or isotype control) using the same instillation strategy and ventilation protocol as for the dAb experiments.

We found that changes in respiratory mechanics in the isotype control-treated group followed a very similar pattern as was observed for the dummy dAb group (figure 4A–C). In marked contrast to the beneficial effect of the p55-specific dAb, however, the anti-TNF monoclonal antibody did not attenuate the changes seen in isotype-treated animals. Similarly, anti-TNF treatment had no beneficial effect on blood gases (figure 4E–G) or alveolar epithelial permeability (figure 4H).

Effects of anti-p55 domain antibody on inflammation following LPS+VILI

Finally, we evaluated whether the p55-specific dAb would also have a beneficial effect on pulmonary inflammation during VILI. Our group and others[26] have shown that pure mechanical

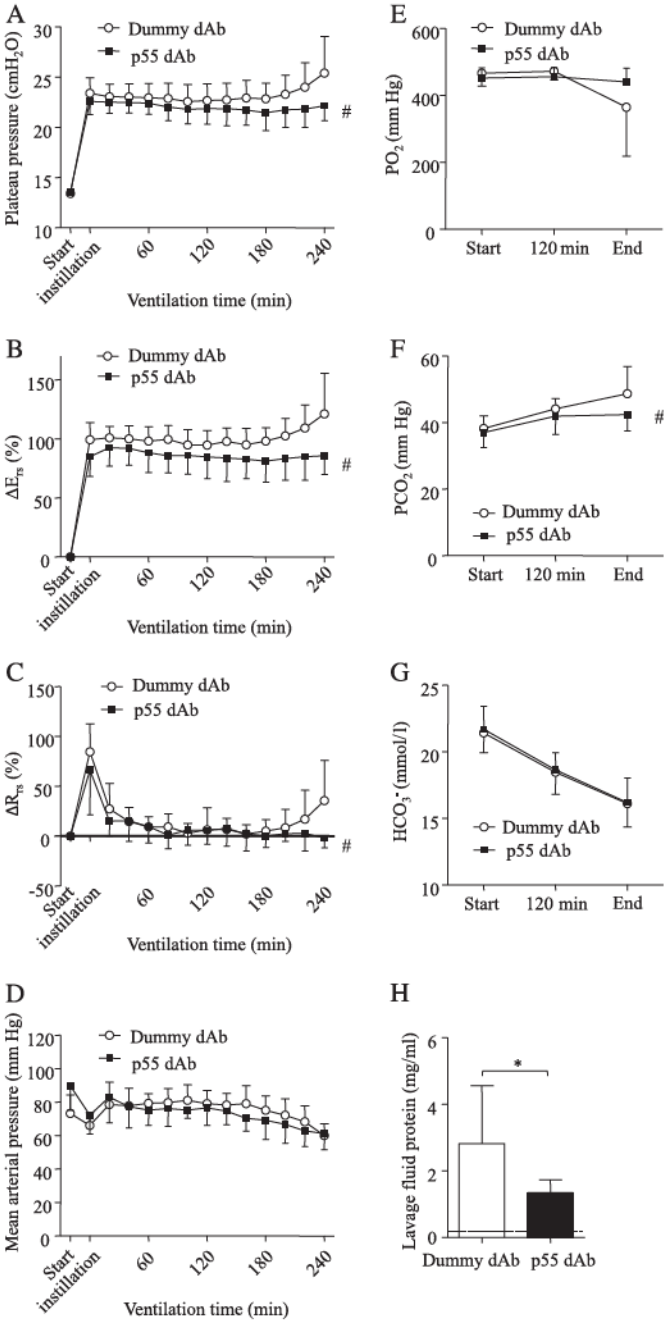


Figure 2 (A) Plateau pressure ($P_{plateau}$), (B) respiratory system elastance (E_{rs}), (C) respiratory system resistance (R_{rs}), (D) mean arterial pressure, (E) arterial oxygen tension (PO_2), (F) arterial carbon dioxide tension (PCO_2), (G) bicarbonate (HCO_3^-) and (H) lavage fluid protein were determined in ventilated animals treated intratracheally with either dummy or p55-specific domain antibody (dAb). Changes in E_{rs} and R_{rs} are expressed as percentage increase in relation to Start (before instillation) values. $P_{plateau}$, E_{rs} and R_{rs} changes over time during ventilation were significantly different between treatment groups (#$p<0.05$ for interaction between treatment and ventilation time by two-way ANOVA). Arterial PO_2 showed a tendency to deteriorate in animals treated with the dummy dAb but not in mice given the p55-specific dAb. Changes in PCO_2 over time were significantly different between treatment groups (#$p<0.05$ for interaction by two-way ANOVA), while changes in bicarbonate levels were similar across groups. Lavage fluid protein was significantly attenuated in the p55-specific dAb group (*$p<0.05$ by unpaired t tests). The dotted line represents the mean value in non-ventilated control animals (N=8–11 per group).

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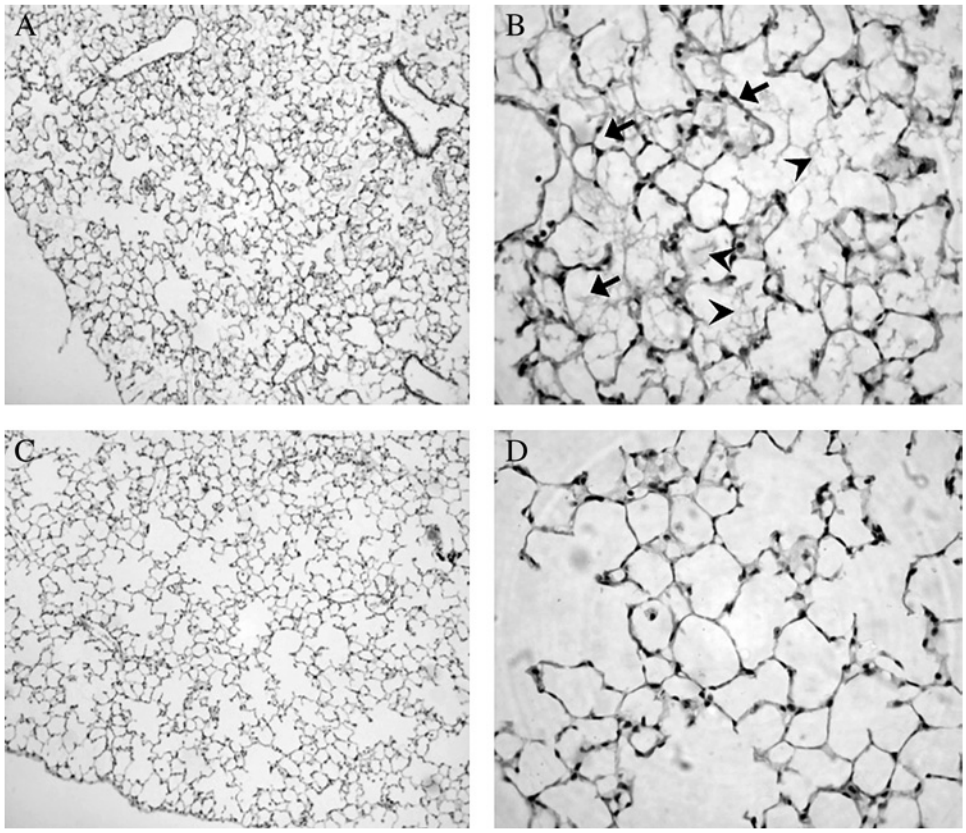


Figure 3 Photomicrographs of lung tissue. Sample from dummy domain antibody (dAb)-treated mouse (A, B) showed marked cellularity, alveolar wall thickening (arrow), hyaline membrane formation and intra-alveolar debris (arrowhead) whereas minimal changes were seen in p55 dAb-treated mouse (C, D). H&E stain. Magnification ×100 (A and C), ×400 (B and D).

ventilation induces a relatively limited degree of alveolar neutrophil infiltration (within the time frame of ventilation used currently), one of the hallmarks of clinical ALI. We therefore included a 'subclinical' dose of LPS (20 ng) mixed with the dummy or p55-specific dAbs at the start of mechanical ventilation to enhance the degree of inflammation within the model. Preliminary studies demonstrated that this dose of LPS did not induce changes in respiratory mechanics with low tidal volume ventilation.

As with the 'pure' VILI model, LPS+VILI induced substantial changes in respiratory mechanics which were significantly attenuated by treatment with p55-specific dAb (figure 5A–C). Similarly, administration of the p55 dAb prevented the deterioration of oxygenation (figure 5E) and tended to attenuate the rise in PCO_2 (figure 5F). p55 dAb also tended to decrease protein levels in lavage fluid (2.94±1.62 vs 2.06±1.45 mg/ml for dummy and p55 dAb, respectively), although this did not reach statistical significance (figure 5H).

Leucocyte subsets in lung single cell suspension and lavage fluid were identified based on characteristic antigen expression using flow cytometry (figure 6A–C) and their number or activation state was quantitatively assessed. LPS+VILI increased the number of lung-recruited neutrophils compared with non-ventilated controls ($2.32\pm0.57\times10^6$ vs $0.13\pm0.05\times10^6$), which was significantly attenuated by the p55-specific dAb ($1.25\pm0.31\times10^6$, $p<0.01$ vs dummy; figure 6A). A similar trend was found with margination of inflammatory subset Gr-1^{high} monocytes, although this was not significant (figure 6A). Transmigration of neutrophils to the alveolar space was also greatly increased by LPS+VILI and substantially attenuated by p55 dAb treatment (figure 6B). Alveolar macrophages in lavage fluid of dummy dAb-treated mice showed increased expression of ICAM-1 compared with non-ventilated controls, indicative of activation,[27] which was also reduced in the p55 dAb group (figure 6C). Finally, treatment with p55 dAb significantly decreased IL-6 and MCP-1 in lavage fluid, with similar trends for KC and MIP-2 (figure 7).

DISCUSSION

ALI and ARDS are characterised by pulmonary oedema, hypoxaemia and inflammation. While mechanical ventilation is an essential aspect of treatment, this can exacerbate underlying lung injury and cause VILI. It has been suggested that this can progress to systemic inflammation and multiple system organ failure, which is a major cause of death among patients with ARDS.[28 29] Although mortality is improved by the use of low tidal volume ventilation in conjunction with other interventions, it is not possible to eliminate completely the consequences of VILI in this manner. An ideal therapeutic agent would therefore attenuate both the respiratory dysfunction which drives the requirement for ventilation and the inflammatory response which may ultimately lead to death. In this study we investigated the use of a novel therapeutic technology—domain antibodies—for the first time in the area of ALI, and showed that intratracheal administration of dAb targeting the p55 TNF receptor attenuated the development of VILI.

Using a mouse model of 'pure' VILI we found that deteriorations in respiratory function and alveolar-epithelial barrier permeability were substantially improved when p55-specific blocking dAb was given intratracheally at the start of the ventilation protocol. In complete contrast, intratracheal administration of a monoclonal anti-TNF antibody did not attenuate stretch-induced respiratory dysfunction. In addition, in an LPS+VILI model incorporating a greater degree of pulmonary inflammation, administration of the p55-specific dAb led to decreased levels of the proinflammatory cytokine IL-6 and the monocyte/neutrophil chemoattractant MCP-1 in lavage fluid, and showed strong trends towards decreases in the

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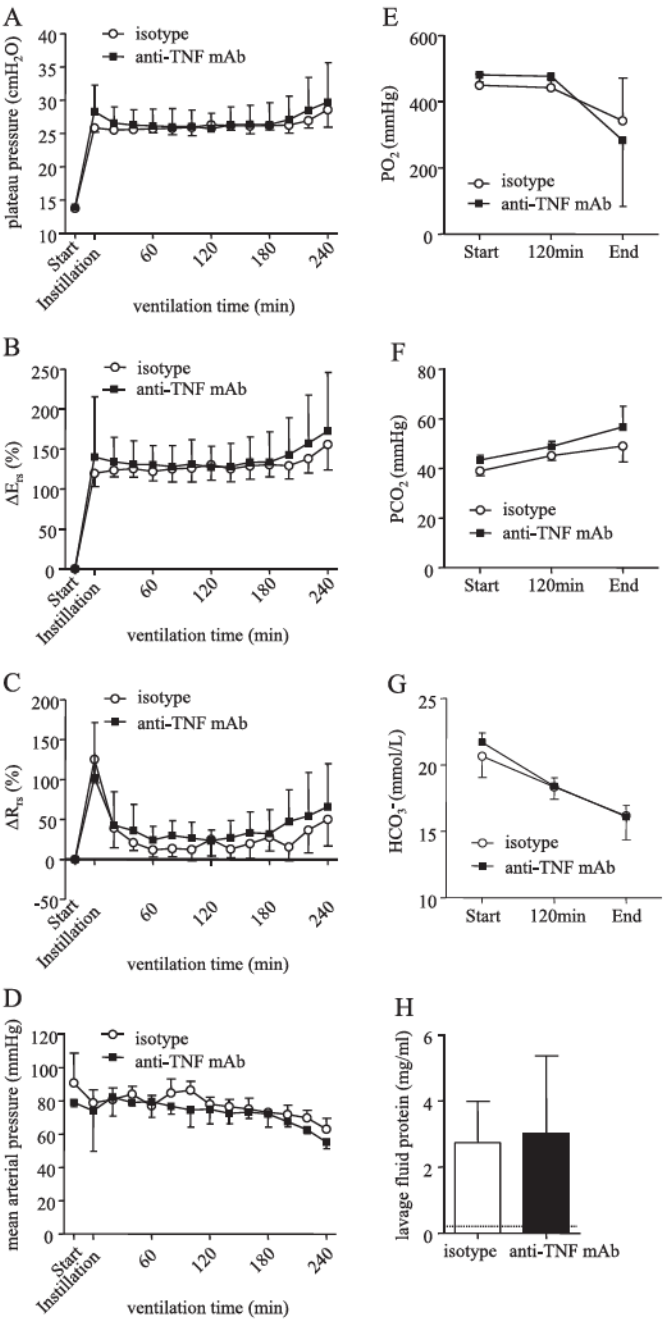


Figure 4 (A) Plateau pressure ($P_{plateau}$), (B) respiratory system elastance (E_{rs}), (C) respiratory system resistance (R_{rs}), (D) mean arterial pressure, (E) arterial oxygen tension (PO_2), (F) arterial carbon dioxide tension (PCO_2), (G) bicarbonate (HCO_3^-) and (H) lavage fluid protein were not significantly different between animals treated intratracheally with either isotype or mouse anti-tumour necrosis factor (TNF) monoclonal antibody (mAb). Dotted line represents mean value in non-ventilated control animals (N=6 per group).

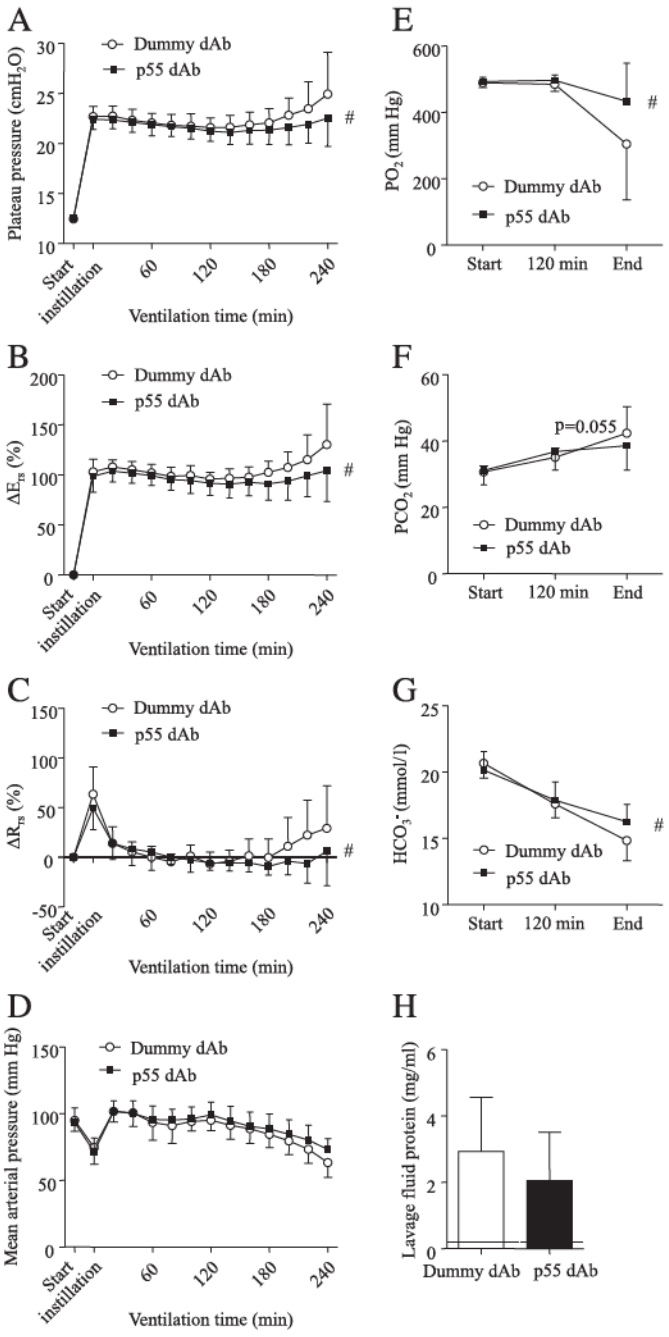


Figure 5 (A) Plateau pressure ($P_{plateau}$), (B) respiratory system elastance (E_{rs}) and (C) resistance (R_{rs}), (D) mean arterial pressure, (E) arterial oxygen tension (PO_2), (F) arterial carbon dioxide tension (PCO_2), (G) bicarbonate (HCO_3^-) and (H) lavage fluid protein of dummy domain antibody (dAb)-treated and p55 dAb-treated mice in the lipopolysaccharide + ventilation-induced lung injury (LPS+VILI) model. Changes in E_{rs} and R_{rs} are expressed as percentage increase in relation to Start values. Changes in $P_{plateau}$, E_{rs}, R_{rs}, arterial PO_2 and bicarbonate over time were significantly different between treatment groups (#$p<0.05$ for interaction between treatment and ventilation time by two-way ANOVA). The p55-specific dAb tended to attenuate the rise in PCO_2, but this did not reach statistical significance ($p=0.055$ for interaction by two-way ANOVA). Lavage fluid protein levels tended to be reduced in p55-specific dAb-treated animals, but not significantly. The dotted line represents the mean value in non-ventilated controls (N=8–11 per group).

chemokines MIP-2 and KC. While the precise sources of these inflammatory mediators in VILI remain uncertain, alveolar macrophages are likely contributors and, indeed, we observed that alveolar macrophage activation (surface ICAM-1 expression[27]) was significantly attenuated by dAb treatment. Presumably as a consequence of this reduced intra-alveolar inflammation, margination and migration of neutrophils within the lung was also attenuated. These data demonstrate clearly that specific blockade of p55 TNF-receptor signalling within the alveolar space attenuates multiple markers of VILI development.

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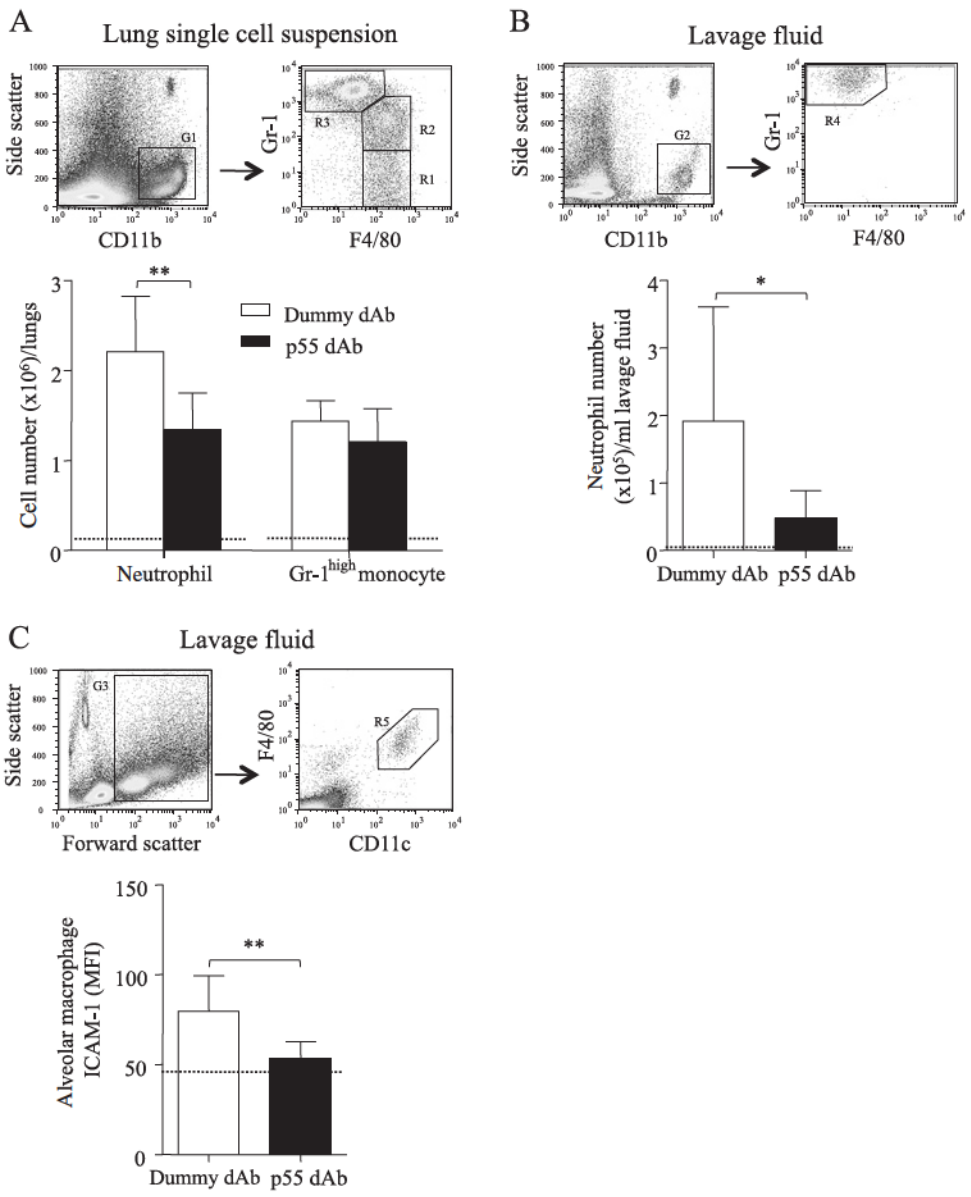


Figure 6 Flow cytometric analysis of leucocyte subsets in the lungs and lavage fluid of dummy domain antibody (dAb)-treated and p55 dAb-treated mice in the lipopolysaccharide + ventilation-induced lung injury (LPS +VILI) model. (A) In lung single-cell suspensions, monocytes were identified as $CD11b^+$, $F4/80^+$ events, and their subsets were defined as either $Gr\text{-}1^{low}$ (R1) or $Gr\text{-}1^{high}$ (R2), differentiated from $CD11b^+$, $F4/80^-$, $Gr\text{-}1^{very\ high}$ neutrophils (R3). (B) Neutrophils in lavage fluid were similarly identified as $CD11b^+$, $F4/80^-$, $Gr\text{-}1^{very\ high}$ events (R4), whereas monocytes were not present. (C) Alveolar macrophages in lavage fluid were recognised as forward/side $scatter^{high}$, $F4/80^+$, $CD11c^+$ events (R5) and their intercellular adhesion molecule (ICAM-1) expression was quantitatively assessed (mean fluorescence intensity (MFI) after isotype subtraction). The dotted line represents the mean value in non-ventilated control animals. *$p<0.05$, **$p<0.01$ by unpaired t test (N=8–11 per group).

The ventilation parameters used within this study (V_T 20–22 ml/kg), although higher than used clinically in humans, would have induced only a moderately high degree of lung stretch in untreated healthy mice as mouse lungs are much more compliant than those of humans.[30] However, due to the impact of the intratracheal fluid instillation, such settings were sufficient to induce injury within 4 h of ventilation. While the timeframe of the current experiment is much shorter than patients with ALI would be ventilated, it is comparable with the majority of published rodent studies of VILI and ALI. Sustained inflations and post end-expiratory pressure were used throughout the ventilation protocols as mouse lungs have a very high tendency for atelectasis even at such V_T, which could have substantial confounding effects. Since atelectasis was minimised by these recruitment measures, the major component of the observed physiological changes can be ascribed to the development of stretch-induced pulmonary oedema. The dAb was given immediately after setting ventilation parameters in order to allow robust standardisation of the VILI protocol, avoiding the unpredictable influence of instillation on respiratory mechanics (upon which the degree of VILI was standardised). The finding that pulmonary oedema and inflammation during VILI were attenuated under these conditions would indicate that a substantial prophylactic period was unnecessary for dAb efficacy.

From the current data it is not possible to determine precisely why the p55-specific dAb was clearly more effective in attenuating VILI than the anti-TNF monoclonal antibody. One possibility is that greater efficacy of the dAb was related to its biophysical properties—its small size (~12 kDa compared to ~150 kDa for IgG) and monovalent target antigen binding allows more potency per unit mass, greater tissue penetration and better biodistribution, and reduced risk of receptor cross-linking and activation compared with conventional antibody technology. Thus, despite the fact that we used a 2× larger amount (in terms of weight) of the monoclonal antibody, the dAb could be delivered at an approximately 6× greater dose than the monoclonal antibody which may simply induce more effective inhibition of TNF receptor binding. This lack of efficacy of anti-TNF treatment to attenuate the physiological manifestations of VILI are somewhat at odds with a previous study showing a positive effect of anti-TNF in a rabbit model of saline

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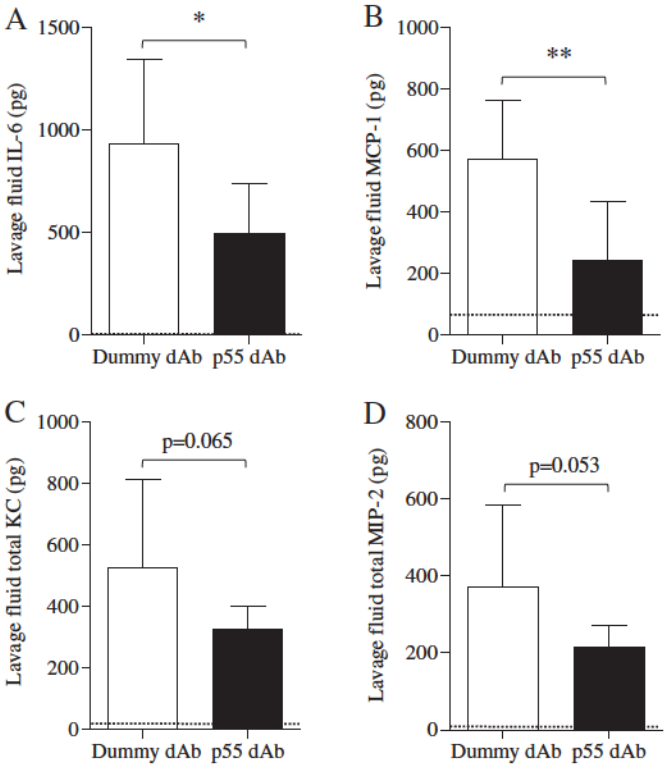


Figure 7 Levels of (A) interleukin 6 (IL-6), (B) monocyte chemotactic protein 1 (MCP-1), (C) keratinocyte chemoattractant (KC) and (D) macrophage inflammatory protein 2 (MIP-2) in lavage fluid of dummy domain antibody (dAb)-treated and p55 dAb-treated mice in the lipopolysaccharide + ventilation-induced lung injury (LPS+VILI) model. The dotted line represents the mean value in non-ventilated control animals (undetectable levels for IL-6). *p<0.05, **p<0.01 by unpaired t test (N=8–11 per group).

lavage followed by injurious ventilation,[4] but do reflect the disappointing results from clinical trials in patients with sepsis/ARDS.[7–10 31]

In addition to the biophysical properties of the respective antibodies, we believe target antigen specificity is vitally important. Despite the large number of studies investigating TNF as a potential therapeutic agent, the complexity of TNF biology remains rather poorly appreciated. It has been assumed that the majority of TNF signalling occurs through the p55 receptor, with p75 playing only an auxiliary role, but it is becoming clearer that the two receptors can signal independently and, in some cases, may exert directly opposite effects to each other.[11 15] For example, we previously showed using knockout mice that signalling through p55 promoted the development of high stretch-induced pulmonary oedema, while signalling through p75 protected against oedema formation during VILI, and that these opposing effects seemed to be independent of TNF-mediated leucocyte recruitment.[15] Such was the nature of this opposing signalling that mice devoid of all TNF receptors showed no protection from oedema (a finding consistent with others[32]). These data from genetically modified animals suggest that non-selective anti-TNF treatment would not be effective however high a dose of antibody we may be able to deliver. Interestingly, in an earlier study we showed that the monoclonal anti-TNF antibody used currently attenuated neutrophilic alveolitis in VILI.[22] Similarly, Wolthuis *et al* found that total blockade of TNF signalling by etanercept, a p75 receptor-Fc fusion protein, failed to attenuate high stretch-induced pulmonary oedema but had some positive effects on inflammation.[33] Taken together, these data suggest that, while treatment designed to block TNF per se may be effective in reducing pulmonary inflammation associated with VILI, it has little impact on pulmonary oedema, presumably as any beneficial effects of signalling through the p75 receptor on pulmonary fluid balance are inhibited. The nature of these beneficial effects of p75 signalling remains unclear, but we speculate that it may involve actin cytoskeleton rearrangements[34 35] or prevention of epithelial/endothelial apoptosis.[12]

In conclusion, we have shown for the first time that specific and selective pharmacological inhibition of intra-alveolar TNF receptor p55 signalling by a dAb can ameliorate pulmonary oedema and inflammation during VILI in mice. Targeting intra-alveolar TNF receptor p55 signalling may open new therapeutic approaches for ventilated patients with ALI.

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Competing interests PJM, RdW and AB are employed by and hold stock in GSK. SB, MW and MT have no competing interests.

Ethics approval All protocols were approved by the ethical review board of Imperial College London as well as the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and carried out under the authority of the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986.

Contributors Conception and design: SB, MRW, PJM, RdeW, AB, MT. Analysis and interpretation: SB, MRW, MT. Drafting of manuscript: SB, MRW, MT, AB.

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