Universitat Autònoma de Barcelona

Nebulized anti-coagulants as a therapy for acute lung injury and acute respiratory distress syndrome

Marta Camprubí Rimblas

Doctoral thesis

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Els Doctors **Antonio Artigas Raventós, Raquel Guillamat Prats i Ferrán Segura Porta,** certifiquen:

Que la tesi doctoral titulada **"Nebulized anti-coagulants as a therapy for acute lung injury and acute respiratory distress syndrome"** ha estat realitzada per la doctoranda **Marta Camprubí Rimblas** sota la nostra direcció i supervisió.

Que la tesis en questió és apta per a ser presentada i defensada públicament davant d'un tribunal per optar al grau de Doctora per a la Universitat Autònoma de Barcelona.

Dr. Antonio Artigas Raventós	Dra. Raquel Guillamat Prats	Dr. Ferrán Segura Porta
Director	Directora	Tutor

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A la meva família,

especialment als meus pares i al meu germà

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LIST OF ABBREVIATIONS

AECC	American-European Consensus Conference Committee
ALI	acute lung injury
APC	activated protein C
Arg-1	arginase-1
ATI	alveolar type I
ATII	alveolar type II
ATIII	antithrombin-III
AP-1	activator protein 1
aPTT	activated partial thromboplastin time
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
C5a	complement component 5a fragment
CCL (2,7)	chemokine ligand
CO	carbon monoxide
CPAP	continuous positive airway pressure
Da	dalton
DAD	diffuse alveolar damage
DAMPs	damage-associated molecular patterns
Dan	danaparoid
ENaC	epithelial sodium channels

EPCR	endothelial protein C receptor
E-selectin	endothelial-selectin
FDP	fibrin degradation products
GAPDH	glyceraldehyde-3-phosphatase
GM-CSF	granulocyte macrophage colony-stimulating factor
hAM	human alveolar macrophages
hATII	human alveolar type II
HBSS	hanks balanced salt solution
HCl	hydrochloric acid
hF	human fibroblasts
HMGB1	high mobility group box 1
HSPGs	heparin-like glycosaminoglycans
ICAM-1	intercellular adhesion molecule 1
ICU	intensive care unit
IFN-γ	interferon-γ
IL (1ß,4,6,8,10,12,13)	interleukin
IL-1R	interleukin-1 receptor
iNOS	inducible nitric oxide synthase
IT	intratracheal
IRAK (1,4)	interleukin receptor activating kinase

LMWH	low-molecular-weight heparin
LPS	lipopolysaccharide
LTB4	leukotriene B4
MAPK	mitogen-activation protein kinase
MCP-1	monocyte chemotactic protein-1
MHCII	major histocompatibility complex class II
MIP-1α	macrophage inflammatory protein-1α
MODS	multiple organ dysfunction syndrome
MR	mannose receptor
NaCl	sodium chloride
NETs	neutrophil extracellular traps
NF-κB	nuclear factor-kappa B
NLRs	cytosolic nucleotide-binding oligomerization domain-like receptors
NR	not reported
PA	plasminogen activators
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor-1
PAMPs	pathogen-associated molecular patterns
PaO_2/FiO_2	partial pressure of arterial oxygen to the fraction of inspired oxygen

PAR	protease activated receptors
PAWP	pulmonary-artery wedge pressure
PEEP	positive end-expiratory pressure
PRRs	pattern recognition receptors
qRT-PCR	real time polymerase chain reaction
RAGE	receptor for the advanced glycation end products
RCT	randomized controlled trial
RNS	reactive nitrogen species
ROS	reactive oxygen species
SP	surfactant protein
TATC	thrombin-antithrombin complexes
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF-β	transforming growth factor-β
Th (1,2)	T helper cells
TM	thrombomodulin
TNF-α	tumor necrosis factor-α
TLRs	toll-like receptors
tPA	tissue-type plasminogen activator
TRAF6	tumor necrosis factor-associated factor 6

UFH	unfractioned heparin
uPA	urokinase-type plasminogen activator
VAP	ventilator-associated pneumonia
VCAM-1	vascular cell adhesion molecule-1
VE-cadherin	vascular endothelial cadherin
VILI	ventilator-induced lung injury
ZO-1	zonula occludens-1

INDEX

SUMMARY

Acute respiratory distress syndrome (ARDS) is an acute respiratory failure with a global incidence in Europe of 17.9 per 100,000 person-year. Although significant advances have been performed in supportive care of patients with ARDS, mortality remains high (40%) and survivors present persistent sequelae. An effective pharmacological therapy for this syndrome is not available yet.

ARDS pathophysiology involves pulmonary activated coagulation and inflammation together with the breakdown of the alveolar-capillary barrier. This leads to proteinaceous edema, neutrophils infiltration into the alveolar compartment and the activation of macrophages towards a pro-inflammatory phenotype.

Beneficial effects of anti-coagulants have been proved in pre-clinical models of acute lung injury (ALI) and in ARDS patients, although systemic bleeding offset its positive effects. Anti-coagulants could be effective for their anti-inflammatory activity in addition to their anti-coagulant properties. Moreover, given the cross talk of these pathways and their influence on permeability, anti-coagulants could also restore the alveolar-capillary barrier. Nebulization of anti-coagulants directly into the alveolar compartment might increase local efficacy and decrease the risk of systemic bleeding.

The hypothesis of this thesis is that nebulized heparin and/or antithrombin (ATIII) limit the pro-inflammatory and pro-coagulant response in the lungs after ALI, also promoting the restoration of the alveolar-capillary barrier. The co-administration of both anti-coagulants directly into the lungs via nebulization produces a synergistic effect enhancing the properties of heparin and ATIII, reducing lung injury and avoiding the risk of systemic bleeding.

As part of this thesis we are showing the results of the action of heparin or ATIII in specific primary human injured cell lung populations and the direct administration of heparin and/or ATIII into the lungs by nebulization in a rat model of ALI.

Nebulized heparin and/or ATIII attenuated pulmonary inflammation and coagulation and did not produce systemic bleeding in the model of ALI. Treatment with nebulized heparin modulated alveolar macrophages through reducing TGF-β and NF-κB effectors and the

coagulation pathway and decreased the recruitment of neutrophils into the alveolar space. Local administration of ATIII alone increased beneficial effects in coagulation, while combined ATIII and heparin had a higher impact reducing permeability and decreasing the infiltration of macrophages into the alveolar compartment. The translational action into humans of both anti-coagulants was also studied. In injured human cell lung populations isolated from lung biopsies, heparin diminished the expression of pro-inflammatory markers in alveolar macrophages and deactivated the NF-κB pathway in alveolar type II cells; decreasing the expression of its mediators and effectors. Also, ATIII decreased levels of pro-inflammatory mediators and increased levels of tight junctions in injured alveolar type II cells.

The current studies prove that nebulized heparin and ATIII might be a potential treatment for ARDS, as they act in different pathways and processes of the pathophysiology of this syndrome. Local administration of anti-coagulants attenuates lung injury decreasing inflammation, coagulation and proving ameliorations on permeability without causing systemic bleeding.

RESUM

La síndrome de distrés respiratori agut (ARDS) és una insuficiència respiratòria aguda amb una incidència global a Europa de 17,9 per cada 100.000 persones-any. Tot i els avenços en el tractament de suport dels pacients amb ARDS, la mortalitat continua sent alta (40%) i els pacients que sobreviuen presenten seqüeles persistents. Actualment no existeix un tractament efectiu.

La fisiopatologia de l'ARDS es caracteritza per l'activació de la coagulació i la inflamació a nivell pulmonar, juntament amb el trencament de la barrera alveolar-capil·lar. Això comporta la formació d'edema proteic, la infiltració dels neutròfils cap al compartiment alveolar i l'activació dels macròfags cap a un fenotip pro-inflamatori.

Estudis previs en models pre-clínics de lesió pulmonar aguda (ALI) i en pacients amb ARDS han demostrat els efectes beneficiosos del anti-coagulants, tot i que aquests efectes positius es veuen contrarestats pel risc d'hemorràgia sistèmica. Els anti-coagulants podrien ser efectius gràcies a la seva activitat anti-inflamatòria a més de les seves propietats anti-coagulants. Atesa l'estreta interacció entre aquestes vies i la seva influència en la permeabilitat, els anti-coagulants també podrien restaurar la barrera alveolar-capil·lar. La nebulització dels anti-coagulants directament al compartiment alveolar podria augmentar l'eficàcia local i disminuir el risc d'hemorràgia sistèmica.

La hipòtesi d'aquesta tesi és que l'heparina nebulitzada i/o antitrombina (ATIII) limitaran la resposta pro-inflamatòria i pro-coagulant pulmonar després de la LPA, promovent, també, la restauració de la barrera alveolar-capil·lar. La co-administració dels anti-coagulants directament als pulmons mitjançant nebulització produirà un efecte sinèrgic que potenciarà les propietats de l'heparina i l'ATIII, reduint la lesió pulmonar i evitant el risc d'hemorràgia sistèmica.

Com a part d'aquesta tesi es mostren els resultats de l'acció de l'heparina o l'ATIII específicament en poblacions pulmonars primàries de cèl·lules humanes lesionades i l'administració directa d'heparina i/o ATIII als pulmons per nebulització en un model de rata d'ALI.

La nebulització d'heparina i/o d'ATIII atenuen la inflamació i coagulació pulmonar sense produir hemorràgia sistèmica en el model d'ALI. El tractament amb heparina nebulitzada modula els macròfags alveolars mitjançant la reducció dels efectors de TGF-β i NF-κB i la via de coagulació i disminueix el reclutament de neutròfils a l'espai alveolar. L'administració local d'ATIII augmenta els efectes beneficiosos en la coagulació, mentre que la combinació d'ATIII i heparina tenen un major impacte en la reducció de la permeabilitat i la disminució de la infiltració de macròfags en el compartiment alveolar. En estudiar l'acció translacional en humans d'ambdós anti-coagulants en poblacions cel·lulars humanes lesionades aïllades de biòpsies pulmonars, l'heparina disminueix l'expressió de

marcadors proinflamatoris en els macròfags alveolars i desactiva la via NF-κB en cèl·lules alveolars tipus II; disminuint l'expressió dels seus mediadors i efectors. D'altra banda, l'ATIII redueix els nivells de mediadors proinflamatoris i augmenta les unions estretes en les cèl·lules alveolars tipus II lesionades.

Els estudis actuals demostren que l'heparina nebulitzada i l'ATIII poden ser un tractament potencial per a la ARDS, ja que actuen en diferents vies i processos de la fisiopatologia d'aquesta síndrome. L'administració local d'anti-coagulants atenua la lesió pulmonar disminuint la inflamació, la coagulació i proveeix millores en la permeabilitat sense causar hemorràgia sistèmica.

1. INTRODUCTION

1.1 Acute Respiratory Distress Syndrome (ARDS)

1.1.1 Definition, diagnostic and classification

Acute respiratory distress syndrome (ARDS) is characterized by an acute respiratory failure that develops in patients of all ages with a variety of clinical disorders and that is associated with a high morbidity and mortality [1].

The diagnostic criteria and classification of ARDS have evolved over the years. Ashbaugh *et al.* in 1967 gave the first description of ARDS; it was described as severe dyspnea, tachypnea, cyanosis refractory to oxygen therapy, decreased lung compliance and diffuses alveolar infiltrates on chest radiography [2].

Multiple definitions were proposed until 1994, when the American-European Consensus Conference Committee (AECC) recommended a consensus definition for ARDS [3]. This definition comprised an acute onset with bilateral infiltrates on chest radiography, a pulmonary-artery wedge pressure (PAWP) ≤ 18 mmHg or the absence of clinical evidence of left atrial hypertension. Furthermore, the consensus definition included the degree of hypoxemia based on the ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO_2/FiO_2) < 300 mmHg for acute lung injury (ALI) and < 200 mmHg for ARDS.

In 2012, in Berlin, the European Society of Intensive Care Medicine revised ARDS definition and addressed its limitations, establishing which is today known as the "ARDS Berlin definition" [4]. Three categories of ARDS were defined based on the degree of hypoxemia: mild (200 mmHg < PaO_2/FiO_2 ≤ 300 mmHg), moderate (100 mmHg < PaO_2/FiO_2 ≤ 200 mmHg) and severe (PaO_2/FiO_2 ≤ 100 mmHg). The ALI term was substituted by mild ARDS subgroup in this last definition, although nowadays the term ALI is only used for experimental models of ARDS. Furthermore, as an acute onset it was specified that the timing from the insult to its worsening or the appearance of new symptoms should be within one week. Moreover, the definition recognized that bilateral opacities of chest imaging could be either demonstrated by computed tomography, and clarified the criteria

with example radiographs. At last, the PAWP requirement was removed from the definition as the cardiac failure or fluid overload may coexist with ARDS (Table 1).

Table 1. The Berlin Definition of Acute Respiratory Distress Syndrome

Oxygenation	Mild	200 mmHg< PaO_2/FiO_2≤ 300 mmHg PEEP or CPAP ≥ 5 cm H_2O
	Moderate	100 mmHg< PaO_2/FiO_2≤ 200 mmHg PEEP ≥ 5 cm H_2O
	Severe	PaO_2/FiO_2≤ 100 mmHg PEEP ≥ 5 cm H_2O
Timing	Within 1 week of a known clinical insult or new or worsening respiratory symptoms	
Chest imaging	Bilateral opacities – not fully explained by effusions, lobar/lung collapse, or nodules	
Origin of edema	Respiratory failure not fully explained by cardiac failure or fluid overload Need objective assessment (eg, echocardiography to exclude hydrostatic edema if no risk factor present	

Abbreviations: PEEP: positive end-expiratory pressure, CPAP: continuous positive airway pressure, $PaO_{2/}FiO_2$: ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen

Diffuse alveolar damage (DAD) is widely used as a typical histologic hallmark of ARDS and considers the presence of intraalveolar haemorrhage and alveolar edema, followed by the formation of hyaline membranes, interstitial edema and epithelial-cell hyperplasia [5]. However, the presence of DAD according to the Berlin Definition is not really extended, as in the analysis of autopsies of ARDS patients the degree of DAD was 12% in mild ARDS, 40% in moderate ARDS and 58% in severe ARDS, correlating with the degree of hypoxemia [6].

Moreover, ARDS is a heterogeneous disease concerning its underlying biology (section 1.2 Etiology), severity of illness and duration of disease and this together with individual patient characteristics might affect the course of the disease and treatment response. Studies suggest that the identification of subsets of patients might improve the therapeutic effect of targeted therapies. Calfee *et al.* identified two subphenotypes within ARDS based on different patient histories, clinical characteristics, biomarkers and clinical outcomes [7]. Phenotype 1 was characterized by less severe inflammation and shock, while phenotype 2 was hyperinflammatory, with elevated plasma levels of interleukin-6 (IL-6), IL-8, tumor necrosis factor-α (TNF-α) and plasminogen activator inhibitor-1 (PAI-1), decreased levels of protein C, and presented severe shock and metabolic acidosis. So,

phenotype 2 recognizes patients with worse clinical outcome and increased mortality compared with phenotype 1.

We should also emphasize that nowadays there is neither single biomarker nor biological variable able to identify ARDS or its subphenotype. The Berlin definition does not include a diagnostic accurate biomarker for inflammation nor for alveolar-capillary injury or permeability [8].

1.1.2 Etiology

ARDS may originate from a direct or indirect insult based on the mechanism that develops lung injury [9]. A direct insult means a primary infection or trauma, between others that affects the lung and directly produces an ARDS (Table 2). An indirect insult implies a systemic disease such a sepsis or other pathologies that at the end will require mechanical ventilation and will develop an ARDS as a consequence of this primary disease (Table 2). Sometimes, differentiation of ARDS into direct or indirect lung injury is not so obvious, possibly because of the coexistence of the two insults. Also, host predisposing conditions such as genetic factors might be a first hit in healthy lungs and this, together with a chain reaction of multiple hits like those specified in Table 2 or a non-appropriated treatment will determine the severity and progression of ARDS [10]. So, as it will be further explained in the treatment section, supportive care could produce some complications such as ventilator-induced lung injury (VILI), ventilator-associated pneumonia (VAP) or transfusion-related ALI following blood transfusion; these hits might not produce an ARDS in the absence of host predisposing conditions [10]. The development of ARDS has different stages and involves different pathways depending on ARDS origin that should be taken into account when administering the treatment, as it will be deeply explained in the pathophysiology and treatment sections [11].

Table 2. Causes of the development of ARDS

Direct lung injury	Indirect lung injury
Pneumonia (bacterial, viral, fungal, or opportunistic)	Sepsis
Aspiration of gastric contents	Burns
Pulmonary contusion	Transfusion of blood products
Inhalation injury	Pancreatitis
Reperfusion injury	Severe trauma with multiple contusions
Near drowning	Cardiopulmonary resuscitation
Non-protective ventilation	Drug overdose or toxic ingestion

1.1.3 Epidemiology

1.1.3.1 Prevalence and mortality

The incidence of ARDS has been difficult to predict due to demographic, cultural, genetic economical and healthcare system differences [12]. Different available methodologies for diagnostic, health resources and hospital admission practices cause a variation on the recognition of ARDS [13]. For example, in Kigali, Rwanda, no cases of ARDS where found when the Berlin definition was applied due to the lack of resources to perform the diagnosis. While applying some modifications such as the substitution of the lung ultrasonography for radiography or computed tomography and percutaneous oxygen saturation for blood measurements 4 cases per 1,000 patients were identified [14].

The incidence of ARDS ranges from 10 to 86 cases per 100,000 person-year, with relevant geographic diversity, presenting the highest rates in Australia and the United States [12, 15]. In Europe the global incidence is 17.9 per 100,000 person-year, and in Spain 7.2 cases per 100,000 person-year, being in the range estimates of previous European epidemiological studies [16]. Although ARDS mortality has decreased during the last decades, it remains as high as 40% [15, 17]. In Spain, hospital mortality is 47.8% while intensive care unit (ICU) mortality is 42.7% [16].

In an observational study of 459 ICU in 50 countries, the prevalence of ARDS was 10.4 of total ICU admissions [17]. According to the Berlin definition, 30% of patients presented mild ARDS, 46.6% had moderate ARDS and 23.4% severe ARDS. The mortality in the

hospital was 40%, being 29.7% for mild ARDS, 35% for moderate ARDS and 42.9% for severe ARDS. The mortality in the ICU was 35.3%, being 34.9%, 40.3% and 46.1% for each ARDS category, respectively.

High occurrence of ARDS is linked to various factors such as older age and clinical disorders [1]. The most common causes of ARDS are pneumonia (35-50%), sepsis (30%), aspiration of gastric contents (10%) and trauma (10%) [15–17]. Non-caucasian race, defined genetic variants and ozone exposure are associated with higher ARDS rates [13, 15].

The decrease in the incidence and mortality of ARDS in recent years can be attributed to the use of supportive therapy, which will be explained in the treatment section. Furthermore, treat the underlying cause (or causes) of ARDS such as sepsis, which requires early resuscitation, antibiotic therapy and source control, is either a first priority. Further therapies focused on the pathophysiology of the disease are needed due to high morbidity and mortality underlying ARDS.

1.1.3.2 Survival and sequelae

Survivors of critical illness and ARDS experience an important persistent and prolonged physical, mental and quality-of-life impairment [18]. Functional limitation is mainly due to muscle loss, weakness and fatigue. Patients undergoing extrapulmonary conditions present increased muscle wasting and weakness.

In one study with 109 patients who survived ARDS and were followed up for one year it was observed that some survivors presented persistent pain at sites of insertion of chest tubes, neuropathies, contractured fingers or frozen shoulders [19]. Moreover, survivors improved the distance walked in a six minute-walk test over the 12 months. None were receiving supplemental oxygen and the median carbon monoxide diffusion capacity had improved. At one year, almost 49% of patients returned to its original position at work. The follow up of 109 patients for five years after ARDS found persistent exercise limitation, especially on young patients [20]. Physical functioning domains were also altered. Furthermore, normal to near-normal pulmonary function was presented. During

the first year, 12 patients died and 9 died during the next four years. At five years 77% patients returned to work. From years 3 to 5 average costs were $5,000 - 6,000 per patient.

Sequelae variation on survivors is due to the heterogeneity of ARDS and the long-term effect of an ARDS episode, which is related to the age and the preexisting pulmonary function. Specific medical attention after recovery of ARDS on cognitive abnormality, weakness, depression and / or post-traumatic stress disorders is necessary. Therefore, it is important to highlight the reduced physical and psychological quality-of-life and the necessary resources that should be applied for the planning of ICU, mechanical ventilation, and rehabilitation in the future of these patients [21].

1.2 Pathophysiology

1.2.1 The lung: normal alveolus

The respiratory system is composed by both, the upper and the lower respiratory tract [22] (Figure 1). The upper respiratory tract consists of the nose, mouth, pharynx, and larynx. The lower respiratory tract comprises the trachea, bronchi and lungs. The respiratory system filters and drives the air from the environment to the respiratory surfaces where gas exchange takes place.

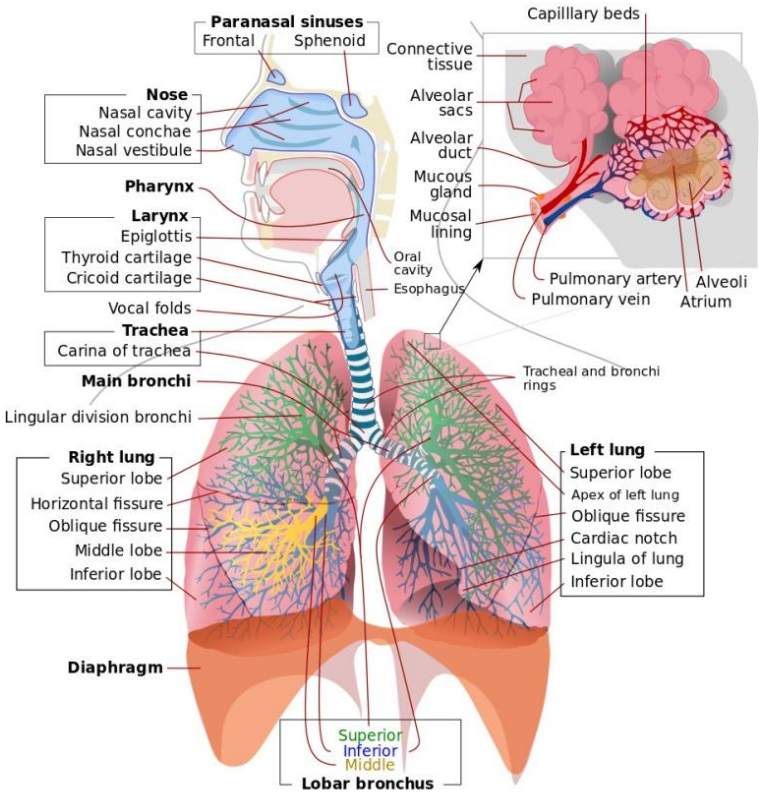

Figure 1. The respiratory system. From Ball *et al.* [22]

The thorax contains two lungs: the right and the left. The right lung is composed of three sections called lobes, while the left lung is formed by two lobes. The trachea is divided in two bronchi, delivering air to both lungs. Each bronchus is divided several times into smaller and smaller bronchi to end branched into bronchioles. The bronchioles become alveolar ducts that end in groups of alveoli surrounded by capillaries, called alveolar sacs.

The alveolar epithelium is formed by alveolar type I (ATI) and alveolar type II (ATII) cells which carry out the gas exchange (Figure 2A). The alveolar epithelial cells lie upon an interstitial matrix that is maintained by these cells, by smooth muscle cells such as fibroblasts and myofibroblasts, and by pulmonary capillary endothelial cells. The epithelial barrier is much tighter than the endothelial barrier. In the alveolar space, alveolar macrophages are found, where they play a critical response in host defense by sensing and ingesting inhaled particulates or pathogens or removing endogenous substances like surfactant or apoptotic or necrotic cells. At the pulmonary interstitium there are the interstitial macrophages, which, compared with alveolar macrophages, have more immunomodulatory functions releasing immunoregulatory cytokines. Moreover, interstitial macrophages have less phagocytic ability than alveolar macrophages. In addition, leukocytes such as neutrophils, monocytes and lymphocytes, in the pulmonary capillaries can be considered part of the overall alveolar unit, being a reservoir for alveolar defense. All these cells work together to facilitate respiration and form a physical barrier between the environment and the organism, forming a complex of multiple interactions. Damage to any single cell type is inevitably accompanied by injury or a change in behaviour to other cell types.

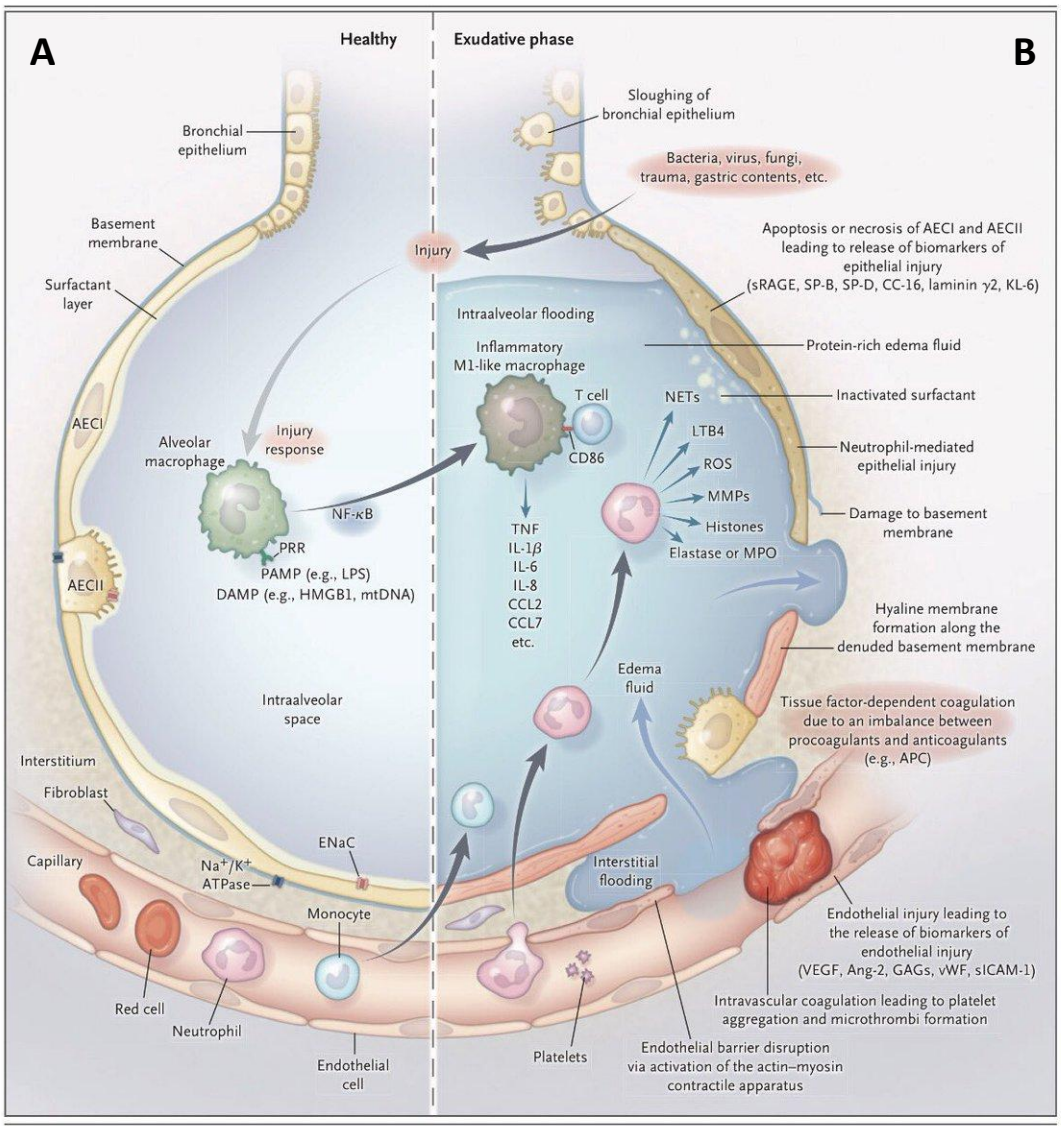

Figure 2. On the right (A) a normal alveolus. On the left (B), an injured alveolus during the acute phase of ARDS. From Thompson *et al.* [5]

1.2.2 Alveolar damage. Mechanisms of lung injury in ARDS

Injury is initiated by both direct and/or indirect insults to the alveolar structure. In direct ARDS, the insult primarily affects the alveolar epithelium, while in indirect ARDS it first affects the vascular endothelium by inflammatory mediators [11].

The acute phase of ARDS (Figure 2B), within 6 days, is characterized by inflammation and increased coagulation factors leading to alveolar damage, which drives the loss of

epithelial and endothelial integrity and results in proteinaceous edema fluid into the alveolar space [23]. This produces the activation of pulmonary macrophages towards a pro-inflammatory phenotype and the recruitment and activation of neutrophils, *de novo* monocytes, platelets and erythrocytes into the alveolar space and the formation of collagen deposition [9].

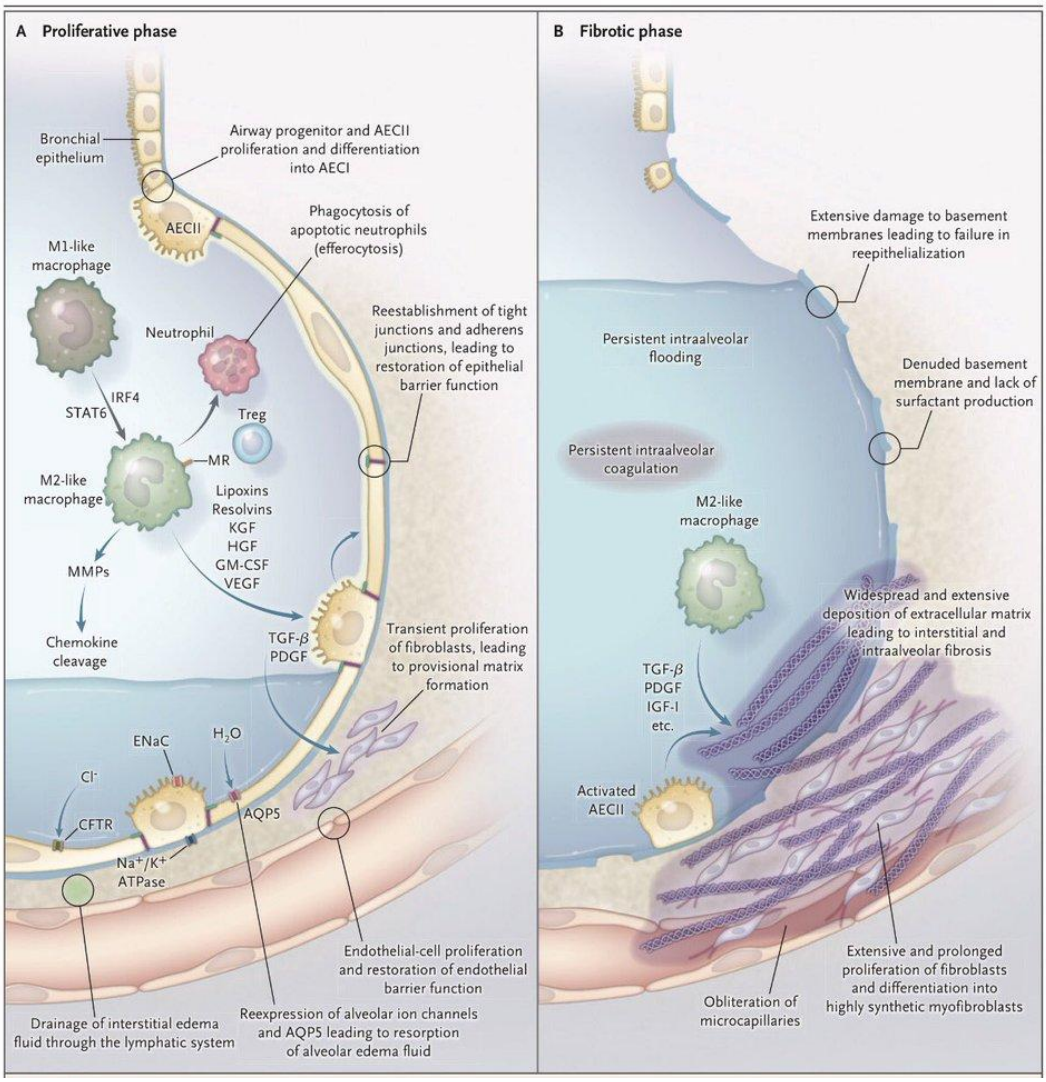

Figure 3. On the right (A) the proliferative and on the right (B) the fibrotic phase of ARDS. From Thompson *et al.* [5]

Within 7-14 days, proliferative phase takes place with the aim to restore tissue homeostasis (Figure 3A). This phase is characterized by the proliferation of ATII cells, which differentiate to ATI cells to reepithelize the alveolar membrane, and by the

proliferation and expansion of fibroblasts to form a provisional matrix. Furthermore, edema fluid is gradually cleared.

The fibrotic phase will take place in the following 14 days (Figure 3B). In case ATII cells are also injured the production of surfactant will be compromised inducing alveolar collapse, and damaged ATII cells will not be able to reepithelize the alveolar membrane [24]. All this will lead to disorganized and fibrotic reparation [25]. Bellow, the pathophysiology of ARDS is further reviewed, deeply explaining the molecular and cellular mechanisms of this disease: inflammation, alveolar macrophages, alveolar-capillary barrier, alveolar epithelial cells, endothelial cells, neutrophils, coagulation and pulmonary inflammation and coagulation cross talk in ARDS.

1.2.2.1 Inflammation

Excessive lung inflammation is a main characteristic of ARDS. The inflammatory response begins with the recognition of foreign pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) such as transmembrane toll-like receptors (TLRs), IL-1 receptor (IL-1R), receptor for the advanced glycation end products (RAGE) or cytosolic nucleotide-binding oligomerization domain-like receptors (NLRs) [26]. Alveolar cells express PRRs that recognize PAMPs such as lipopolysaccharide (LPS), double-stranded RNA or lipoproteins after an infection, or DAMPs released after a trauma, non-infectious ARDS etiologies or through an effector triggered by original infectious ARDS.

Binding of PAMPs and DAMPS to the different receptors like TLR, IL-1R or RAGE, promotes the initiation of several pro-inflammatory pathways (Figure 4). Independently of the cause, the transcription of nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) are activated in ARDS, inducing pro-inflammatory mediators that raise the inflammatory response [27]. While TLRs can be activated by both PAMPs and DAMPs, RAGE can only be stimulated by DAMPs [28]. TLR2 is a major receptor that recognizes Gram-positive cell-wall components, while TLR4 identifies LPS, an endotoxin found in the outer membrane of

Gram-negative bacteria. In the lung, TLR2, TLR4, IL-1R and RAGE are expressed by alveolar macrophages, alveolar epithelial cells and vascular endothelial cells [28, 29].

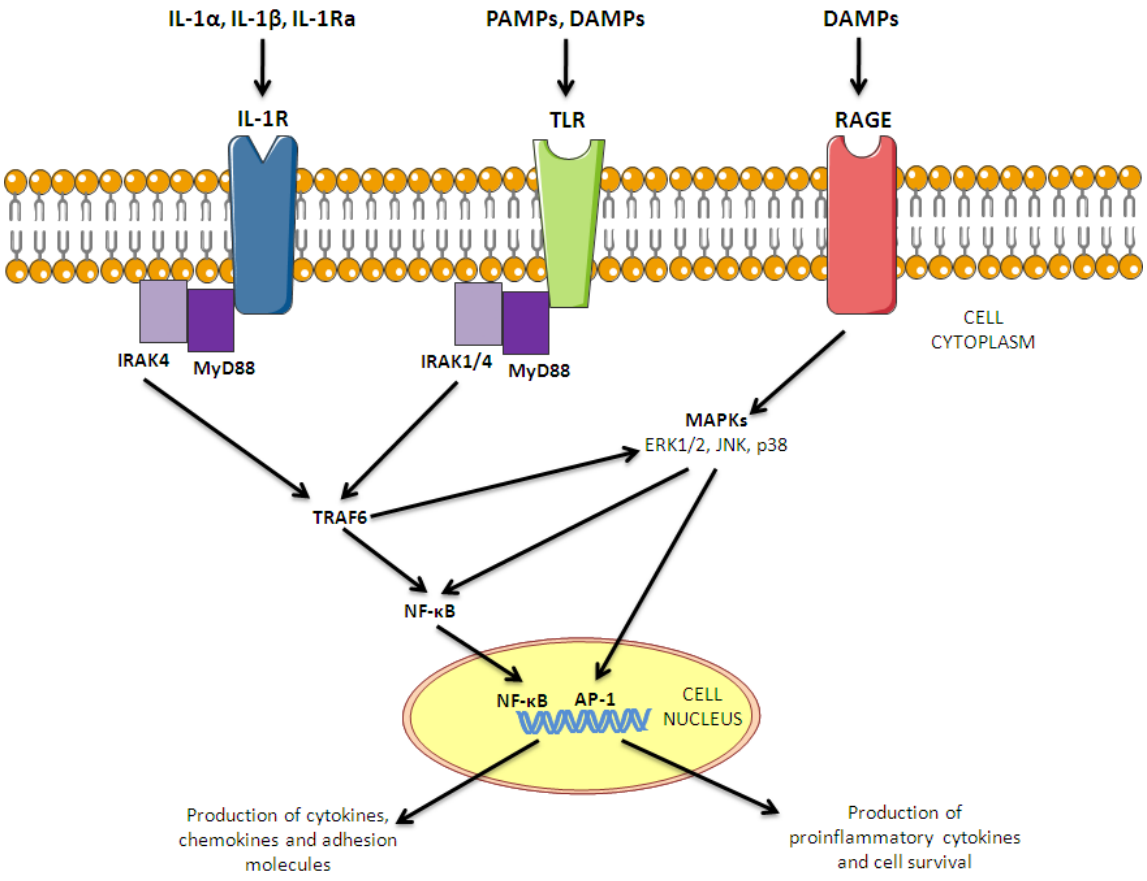

Figure 4. Pro-inflammatory pathways in ARDS.

When a TLR is activated, it activates its adaptor, MyD88, which, in turn, promotes association with IL-1 receptor activating kinase (IRAK1) and IRAK4 and recruits tumor necrosis factor-associated factor 6 (TRAF6) [30, 31]. These lead to NF-κB and mitogen-activation protein kinase (MAPK) activation. Regarding NF-κB, this molecule is liberated from the complex after IκB is phosphorilated by IKKα/β and ubiquitinazed. The liberation of NF-κB promotes its activation, entering to the nucleus where regulates the transcription of pro-inflammatory mediators, adhesion molecules and chemokines like intercellular adhesion molecule 1 (ICAM-1), TNF-α, IL-1ß, IL-6 and IL-8, among others [30]. Concerning MAPK, three different pathways are activated: ERK1/2, JNK and p38, leading to the activation of AP-1 and, as a result, the production of pro-inflammatory cytokines,

cell proliferation and survival. The activation of IL-1R and RAGE also promote NF-κB and MAPKs activation, leading to the expression of pro-inflammatory cytokines [28].

The mediators released during the injury promote the recruitment of neutrophils, monocytes, lymphocytes and platelets into the alveolar space [24], which contribute to the amplification and modulation of a complex network of cytokines together with lung cell types, such as epithelial and endothelial cells, alveolar macrophages and fibroblasts [31]. The exaggerated pro-inflammatory environment causes epithelial and endothelial dysfunction, which not only potentiates inflammation but also activates coagulation, as it will be explained in the following sections.

The balance of pro-inflammatory (TNF-α, IL-1ß, IL-6, IL-8, high mobility group box 1 (HMGB1)) and anti-inflammatory (IL-10, IL-13, IL4, IL-1Ra) mediators might determine the global inflammatory response [23, 32]. In ARDS, TNF-α, IL-1ß, IL-6 and IL-8 are usually increased, while IL-10 is decreased [33].

In the early phase of ARDS, TNF-α and IL-1ß are the most secreted cytokines by activated alveolar macrophages. They promote the release of pro-inflammatory chemokines: monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), IL-6 and IL-8.

Macrophages, monocytes, endothelial cells and fibroblast induce the expression of IL-6. This cytokine activates pro-inflammatory and anti-inflammatory mechanisms, although in early ARDS it induces a pro-inflammatory profile. Also, in early ARDS increased levels of IL-8 are found. This cytokine promotes neutrophil and monocyte chemotaxis, together with neutrophil apoptosis inhibition, enhancing their survival.

Monocytes and alveolar macrophages also express HMGB1 in the early phase of ARDS, which is a DNA nuclear binding protein that activates TLR4 and RAGE [34].

Regarding anti-inflammatory cytokines, IL-10 is produced by lymphocytes, monocytes and alveolar macrophages and inhibits the production of IL-1ß and TNF-α and the release of pro-inflammatory mediators by alveolar macrophages.

Activated cells further release reactive oxygen species (ROS) and reactive nitrogen species (RNS) promoting increased inflammation and damage [32].

1.2.2.2 Alveolar macrophages

Alveolar macrophages are monocytes derived from the yolk sac and fetal liver that shortly after birth migrate to the alveolar space where mature as macrophages in a granulocyte macrophage colony-stimulating factor (GM-CSF)-dependent process [35]. They are self-renewed in normal conditions but if a profound depletion occurs, circulating monocytes originated in the bone marrow or in the spleen can repopulate the macrophage niche [35].

These cells are the first line of defense against external agents and play a critical role in alveolar homeostasis [36]. They are exposed to the external environment, being in contact with the air and the blood materials [37]. Macrophages are the effector cells of the innate response and are involved in the regulation of adaptive responses through antigenic presentation, expression of co-stimulatory molecules and production of cytokines [37].

Alveolar macrophages present a high functional plasticity which allows them to become pro-inflammatory or anti-inflammatory activated, depending on the environmental signals; being a heterogeneous population which can present different phenotypes. They are classified into classically activated (M1) or alternatively activated (M2) macrophages, based on patterns of gene expression, protein secretion and activity in host defense (Figure 5) [38].

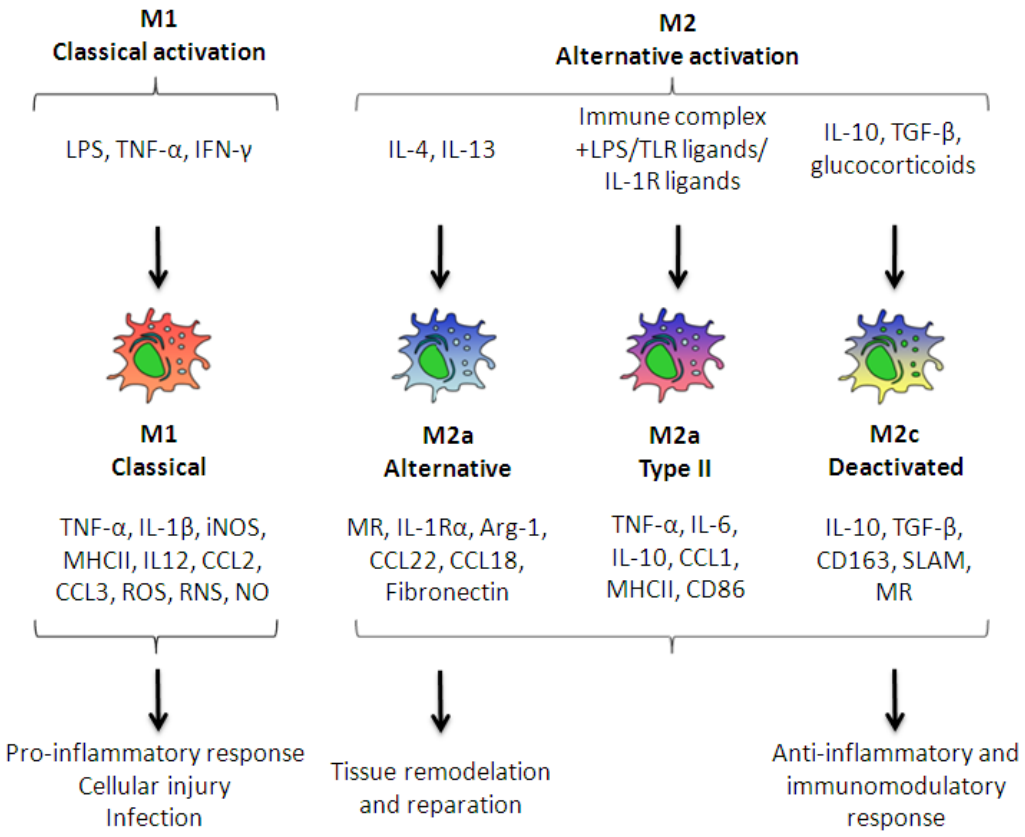

Figure 5. Classical and alternative activation of macrophages

Classically activated (M1) macrophages were first introduced by Mackanes in 1960s as inflammatory cells capable of secreting inflammatory mediators, phagocyte and act as antigen presenting cells [39, 40]. They produce pro-inflammatory cytokines like TNF-α, IL-1β, IL-6 or inducible nitric oxide synthase (iNOS) in response to LPS and in response to T helper 1 (Th1) produced cytokines such as TNF-α and interferon-γ (IFN-γ). Moreover, their activation induces the production of ROS and RNS.

Subsequently, Mills *et al.* proposed a type of macrophage induced by cytokines from Th2 lymphocytes (IL-4, IL-13 and IL-10) which were called alternative activated (M2) macrophages [41]. They were involved in the resolution of inflammation and tissue repair. Alternative activated macrophages (M2) include at least three different overlapping phenotypes (M2a, M2b, M2c) [42–45].

The phenotype M2a is induced by Th2 lymphocytes through IL-4 and IL-13 cytokines. M2a macrophages express Arginase-1 (Arg-1) and mannose receptor (MR) and are linked to

tissue reparation. M2b cells are activated by TLR and LPS. They produce high levels of IL-10 and major histocompatibility complex class II (MHCII), low levels of IL-12 and do not express Arg-1, promoting Th2 differentiation. This phenotype also contributes in asthma and allergy pathologies. M2c macrophages are induced by IL-10, transforming growth factor-β (TGF-β) and glucocorticoids and are associated with immuneregulatory mechanisms. As their antigen presentation is attenuated, M2c present low levels of MHCII. Furthermore, M2c produce high levels of CD163, remarking its anti-inflammatory function.

In ARDS, alveolar macrophages contribute in both the induction and resolution of the injury. At the initial acute phase of ARDS, activated M1 macrophages release early response cytokines such as TNF-α IL-1β, IL-6, IL-8 and iNOS which stimulate the cells of the alveoli and promote the recruitment of neutrophils, monocytes and lymphocytes into the alveolar compartment, promoting and amplifying the inflammatory response. Injured alveolar cells further release vesicles that activate M1-phenotype. When the reparative process starts, the switching capacity of macrophages confer a pivotal role of these cells in the resolution of ARDS and polarized M2 macrophages are activated. Anti-inflammatory cytokines such as IL-10 or Arg-1 are released promoting tissue remodeling and the phagocytosis of death cells [37, 46, 47].

1.2.2.3 Alveolar-capillary barrier

Alveolar damage drives the loss of epithelial and endothelial barrier integrity, favouring extravasation of protein-rich edema and leukocytes into the alveolar compartment [48, 49].

1.2.2.3.1 Alveolar Epithelial cells

Alveolar epithelial injury has a key role in ARDS severity [23]. As explained before, the alveolar epithelium is composed by ATI and ATII cells. ATI cells are flat and cover 95% of the alveolar surface, being the major responsible for gas exchange. These cells are active players in the host defense mechanisms in the lung [30]. ATII cells are cuboidal and cover the remaining 5% of the alveolar surface. ATII cells are the progenitor cells of the alveolar

epithelium, proliferating and differentiating into ATI cells. Moreover, ATII cells synthesize, secrete and recycle surfactant, they are critical in ion transport and present immunologic functions releasing cytokines and chemokines that modulate macrophages, neutrophils and lymphocytes [36, 50, 51]. In order to form the alveolar epithelial barrier, ATI and ATII cells present tight junctions; cell-cell interactions produced by the fusion of the outer layer of adjacent cells. The permeability of tight junction depends on the protein composition. So, the specific tight junctions that compose the alveolar epithelial barrier are occludins and various claudins [52]. The protein family of claudins consists of 27 members and the combination of claudins that form the tight junction determines its permeability. Lateral contacts between adjacent ATI cells are better sealed by tight junction complexes than lateral contacts between adjacent ATI and ATII cells.

In ARDS, increased TNF-α activates NF-κB and produces a negative regulation in tight junction proteins, decreasing the expression of claudins and occludins such as zonula occludens-1 (ZO-1), which conducts to an increased permeability. Moreover, cytokines induced by NF-κB activation further interfere with tight junction integrity. In addition to the gaps between alveolar epithelial cells, injury can also lead to apoptosis and necrosis of alveolar epithelial cells. Macrophages clearance apoptotic cells in inflammation after the recognition of membrane molecules (CD44). Caspase-cleaved cytokeratin-18, a marker for alveolar epithelial cell apoptosis is increased in bronchoalveolar lavage (BAL) of patients with ARDS.

Damage on ATI cells induces the release of RAGE, activating the transcription of NF-κB and the production of pro-inflammatory cytokines, which further increase injury [53]. Increased RAGE levels have been correlated with severity of ARDS [54]. Moreover, ATI cells also produce ROS and proteases.

Surfactant maintains the tension of the alveolar surface avoiding the alveolar collapse and has immune properties. Furthermore, surfactant protein A and D (SPA and SPD) present antimicrobial properties, as they act as opsonins in the surface of pathogens, facilitating their elimination by alveolar macrophages. ATII cells damage has a negative connotation on surfactant synthesis and turnover, producing abnormalities on its lipid and protein

components [9]. In this line, the presence of proteins and enzymes in the edema further increases surfactant dysfunction [55]. As a result, there is less quantity of surfactant and it presents reduced properties, being unable to maintain the superficial alveolar tension and losing host defense functions. In addition, damage affects transepithelial ion transport of ATII cells, impairing excess liquid removal from the alveolar space. Half of the patients have decreased alveolar liquid clearance. Another biomarker of ATII cells injury is a membrane glycoprotein Krebs von den Lungen 6, which is increased in both plasma and BAL of patients with ARDS [56].

In response to injury both cellular types will produce coagulation and inflammatory mediators. Additionally, given the damage produced to ATII cells severe epithelial injury will lead to an inadequate epithelial repair and fibrosis [57].

1.2.2.3.2 Endothelial cells

The alveolar endothelium is different from the systemic vascular endothelium, as it is exposed to higher oxygen tensions while maintaining low-pressure blood flow. The integrity of the endothelial barrier is determined by tight junctions and adherent junctions between neighbouring cells [48]. Tight junctions in the endothelium comprise claudins, occludins and junctional adhesion molecules while adherent junctions include cadherins and vascular endothelial cadherin (VE-cadherin).

Endothelial injury promotes the destruction of the vascular bed, which can be evaluated by the pulmonary dead-space fraction [9]. In addition, endothelial damage induces the expression of adhesion molecules such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and endothelial-selectin (E-selectin), which are involved in the rolling, adhesion and extravasation of circulating leukocytes [58]. Moreover, inflamed activated cells produce ROS and RNS, which directly downregulate VE-cadherin, which induce the formation of gaps between endothelial cells leading to increased permeability and neutrophils entrance to the alveolar space, together with edema.

As we will see next, a part of its importance on inflammation endothelial injury also has a direct influence on coagulation and fibrinolysis [23]. Endothelial damage and coagulation

activation promote pulmonary vascular thrombosis and obstruction by clots formation, leading to pulmonary hypertension and an increase in pulmonary functional dead space.

Biomarkers of endothelial injury have been studied in patients with ARDS, such as von Willebrand factor, angiotensin converting enzyme, tissue factor pathway inhibitor (TFPI), angiopoietin-1 and angiopoietin-2 or E-selectin [23, 32].

1.2.2.4 Neutrophils

An influx of neutrophils into the alveolar compartment is a defining hallmark of ARDS [57, 59]. Pulmonary edema and BAL from ARDS patients present high levels of neutrophils [60, 61]. Neutrophilia duration and severity are predictors of mortality of this disease [57, 61]. As previously explained, epithelial and endothelial damage together with the pro-inflammatory activation of pulmonary macrophages induces neutrophils recruitment into the lung. One of the main chemotactic factors for neutrophils is IL-8, together with other chemokines such as CXCL15, leukotriene B4 (LTB4) and complement component 5a fragment (C5a). Furthermore, MCP-1 and chemokine ligand 7 (CCL7), classically chemotactic factors for monocytes, also contribute to neutrophil recruitment [59, 62].

Activated neutrophils release proteolytic enzymes, ROS, RNS, cytokines, pro-coagulant molecules and growth factors [24, 62, 63]. Furthermore they produce neutrophil extracellular traps (NETs) through the release of their nucleus and proteins of granular contents, which have antimicrobial properties. In ARDS, NETs formation causes excessive inflammatory response and also primes an increase of permeability. Neutrophils and their toxic mediators cause tissue injury and induce epithelial and endothelial breakage, promoting permeability and the influx of protein edema and arterial hypoxemia [59]. In order to facilitate neutrophils entrance into the airspace, proteases injure the extracellular matrix of the lung [64]. Collagenase, gelatinase A and B and other products such as platelet activating factor (PAF) and leukotrienes are also released by neutrophils [9]. Injury mediated by neutrophils is modulated by natural inhibitors of neutrophils function like CC16, which has been found in the BAL of ARDS patients [65]. Neutrophil mediated inflammation is ended by apoptosis of neutrophils, which normally are removed by

alveolar macrophages. In early ARDS, the neutrophil clearance mechanism is disrupted due to the presence of several cytokines such as IL-8, which promote the accumulation of neutrophils into the lungs. In patients with ARDS lower levels of apoptotic neutrophils are found [66].

1.2.2.5 Coagulation

Activated coagulation and reduced fibrinolysis promote pulmonary coagulopathy in ARDS [67, 68], similar to the altered coagulation found systemically in septic patients. Different pathways of the coagulation cascade are involved in the pathophysiology of ARDS: tissue factor (TF) pathway, protein C pathway and the regulation of fibrinolysis by the plasminogen activator and inhibitor pathway (Figure 6).

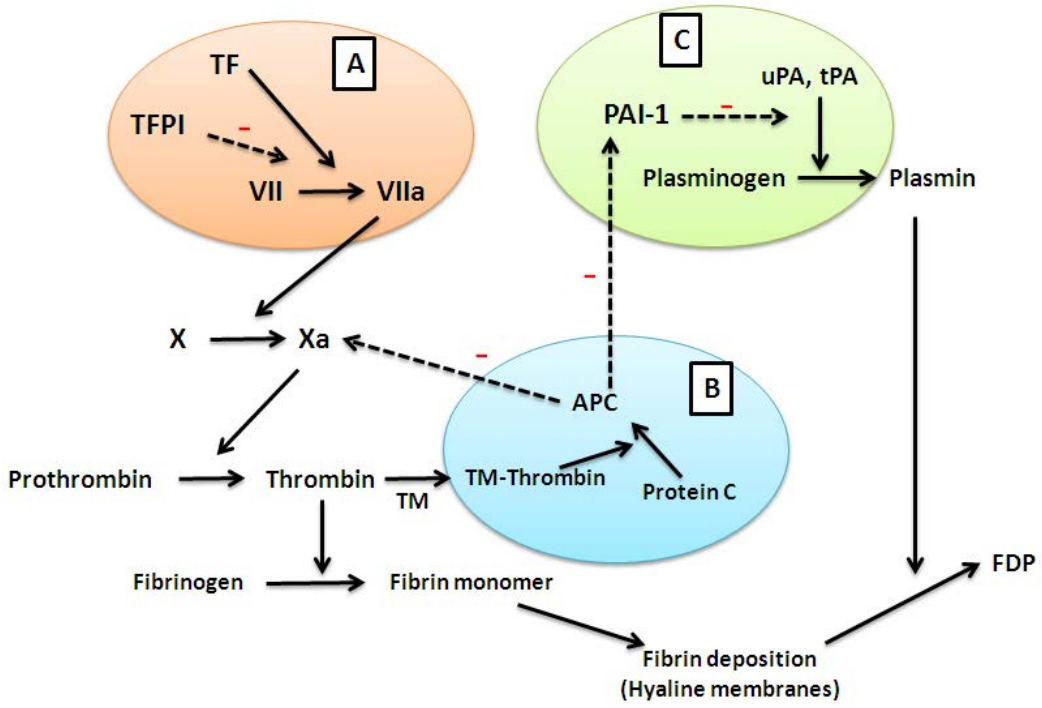

Figure 6. Coagulation pathway. A) Tissue factor pathway, B) Protein C pathway C) Plasminogen activator and inhibitor pathway

1.2.2.5.1 Tissue factor pathway

TF is a transmembrane protein that drives the extrinsic coagulation cascade and is activated by the binding of factor VIIa on the cell surface. This complex cleaves factor X producing its activated form, Xa, promoting the generation of thrombin and the formation of fibrin. Factor Xa generates thrombin by the cleavage of prothrombin, which is produced in the liver, and in the presence of calcium. In normal conditions, there is a balance between TF and its natural anti-coagulant inhibitor: TFPI, which modulates the initiation of extrinsic coagulation cascade via TF pathway. TFPI is produced in the vascular endothelium and on the surface of platelets [69]. This inhibitor interferes with the complex TF:VIIa:X inhibiting thrombin production and fibrin deposition. In order to become active, TFPI must bind to factor Xa, which means that this inhibition process just takes place after the initiation of the coagulation pathway [69].

In human lung tissue from ARDS patients [70] and in mice that received LPS directly into the lungs [71] TF is found in alveolar epithelial cells and alveolar macrophages. Increased expression of TF in the endothelium and by leukocytes is also detected [72]. In this line, increased TF pro-coagulant activity is found in the BAL of patients with ARDS or pneumonia without ARDS [67, 73, 74] and in plasma of septic patients [75]. Elevated concentrations of TF in the plasma of ARDS patients are associated with poor clinical outcomes and indicate a common coagulation mechanism in different ARDS etiologies [69]**.** Pulmonary edema fluid of patients with ARDS presents 100-fold higher levels of TF protein than in plasma [70]. The alveolar epithelium is the major source of TF in ALI, promoting coagulation and the deposition of fibrin, which produces a barrier and reduces the leakage of the alveolar-capillary barrier, being protective during ARDS [76]. Moreover, intratracheal administration of TF in a model lacking murine TF reestablished local coagulant activity and reduced haemorrhage and permeability in an ALI model of LPS [77].

Regarding TFPI, higher levels are found in the alveolar compartment of patients with ARDS, although it does not compensate TF increment, maintaining a predominant pro-coagulant activity in the lungs. Levels of TFPI are 7-fold greater in patients at risk and 20-

fold more elevated in established ARDS patients [78], and no differences are found in plasma.

Furthermore, increased levels of factors VIIa and thrombin are found in the injured lungs of patients with ALI/ARDS [79, 80].

1.2.2.5.2 Protein C pathway

Coagulation and fibrinolysis are also regulated by protein C pathway. Produced by the liver, protein C is a vitamin K-dependent glycoprotein that circulates as a zymogen. Thrombomodulin (TM) is a thrombin receptor that together with thrombin creates a complex that cleaves protein C producing activated protein C (APC). This complex also prevents thrombin from activating coagulation and platelets. As a result, anti-coagulant and fibrinolytic effects are produced [81]. Endothelial cells were first described to produce TM [82], although lately other cell types like alveolar epithelial cells were also found to liberate TM [83]. The endothelial protein C receptor (EPCR) is another cell surface protein that potentiates activation of protein C while binding to TM-thrombin complex in a concentration dependent manner.

APC induces its anti-coagulant properties when proteolytically inactivating factors Va and VIIIa, which suppress thrombin formation, and promotes fibrinolysis by neutralizing PAI-1.

APC, TM and EPCR are produced by alveolar epithelial cells in normal conditions. In response to an injurious stimulus, alveolar epithelial cells release TM and EPCR from the cell surface, due to a metalloproteolytic process, reducing the ability of these cells to activate protein C and promoting a pro-coagulant state and the inhibition of fibrinolysis [84].

ARDS patients present lower levels of protein C in plasma and have reduced levels in the alveolar compartment [83], especially those patients presenting phenotype 2 [7]. Lower levels of protein C are associated with increased mortality [83]. In pulmonary edema from patients with ARDS, TM concentration is 2-fold higher than in plasma of patients with ARDS, and more than 10-fold higher than in normal plasma [9]. Besides, raised levels of

TM in plasma are related with increased mortality in ARDS [85] together with genetic variants in TM and EPCR genes [86].

1.2.2.5.3 Plasminogen activator and inhibitor pathway

Coagulation activation and fibrinolysis lead to fibrin deposition into the lung. Plasminogen activators (PA), which can be urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA), drive the conversion of plasminogen to plasmin, a fibrinolytic enzyme. This conversion is neutralized by PAI-1.

Alveolar macrophages, endothelial cells and alveolar epithelial cells are sources of PA and PAI-1. When boost with a pro-inflammatory stimulus, alveolar macrophages and alveolar epithelial cells express higher levels of PAI-1, and endothelial cells express less tPA, resulting in increased fibrinolysis inhibition [87]. In patients with ARDS, PAI-1 levels are increased in both plasma and edema fluid, proving diminished fibrinolysis and presenting a correlation with mortality [73, 88].

1.2.2.6 Pulmonary inflammation and coagulation cross talk in ARDS

There is extensive cross-talk between the coagulation and the inflammation system, as inflammation contributes to coagulation activation and activated coagulation extensively affects inflammation (Figure 7) [89].

Independently of ARDS etiology, pro-inflammatory cytokines like TNF-α, IL-1β, IFN-γ and LPS of Gram-negative bacteria regulate the expression of TF [90], the main activator of the extrinsic coagulation cascade. After administrating a pro-inflammatory stimulus that activates the transcription of NF-κB, alveolar macrophages, alveolar epithelial cells and endothelial cells have been found to over express TF [49, 69]. This activation of the coagulant pathway is due to a protective mechanism that pretends to limit blood loss and avoid the spreading of foreign particles from the initial point of infection.

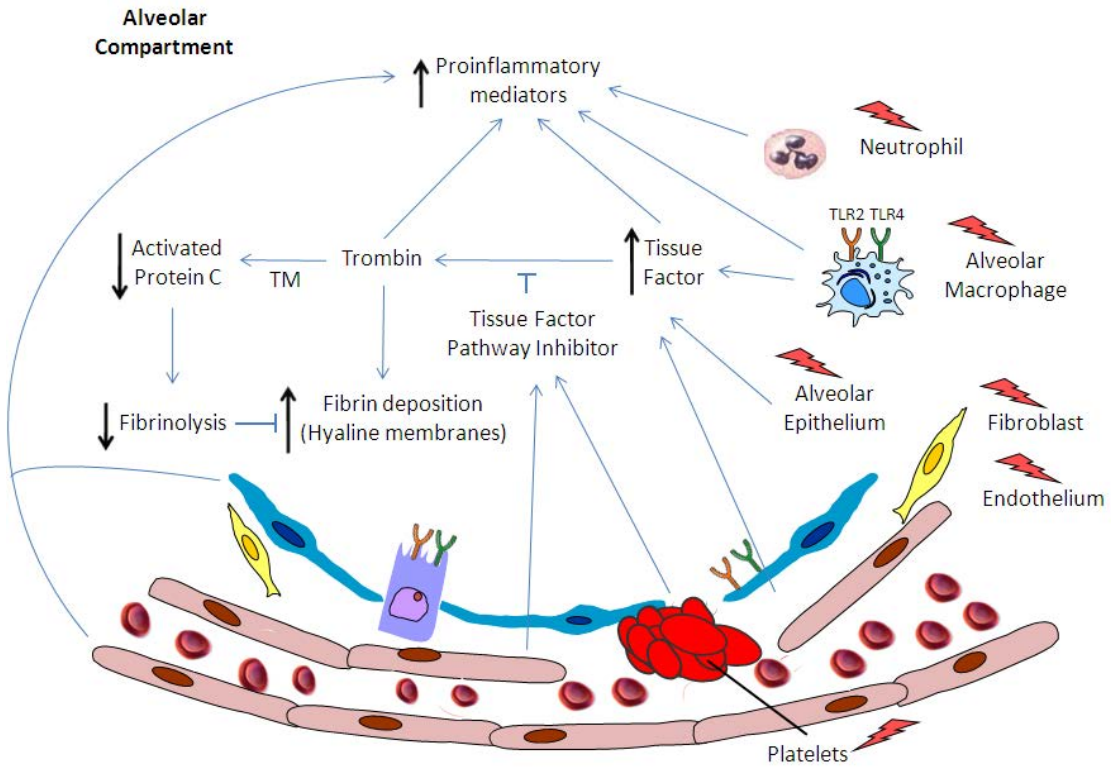

Figure 7. Pulmonary inflammation and coagulation cross talk

However, activated coagulation pathway further increases the damage, as it intensifies the inflammatory response. The major link between inflammation and coagulation are protease activated receptors (PAR) (Figure 8). These receptors are a family of G protein – coupled receptors and are found on the cell surface of the immune cells, platelets and endothelial cells, and, when activated, induce the expression of molecules of adhesion and inflammatory genes in the lung, aggravating the inflammatory process [49]. Factor Xa is the activator of PAR-1, 2 and 4, while thrombin activates PAR-1, 3 and 4. Activation of PAR-1, 3 and 4 on platelet's membrane further promotes platelets activation and recruitment. Activation of PAR-1 by high levels of thrombin might disrupt endothelial barrier function. In contrast, APC has been shown to use EPCR as a co-receptor to cleave PAR-1 on endothelial cells mediating protective and opposite effects than thrombin while activating the same receptor PAR-1 [91]. However, thrombin activates PAR1 proteolitically much more efficiently than APC.

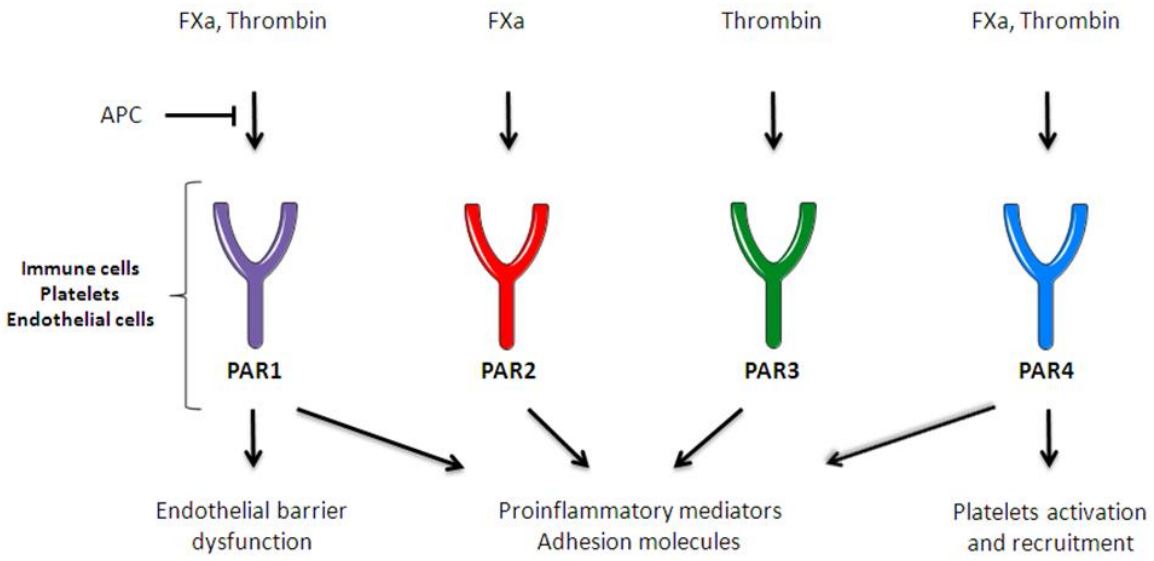

Figure 8. Protease activated receptors link inflammation and coagulation

Thus by suppressing thrombin through APC, anti-inflammatory effects are produced in addition to the anti-coagulant repercussion. Moreover, by inhibiting PAI-1, APC limits the pro-inflammatory effects of fibrin and the binding of neutrophils to the endothelium [92, 93]. Direct anti-inflammatory effects of APC are produced through its binding to EPCR, inhibiting neutrophils chemotaxis. Also, APC blocks NF-κB translocation in LPS-stimulated monocytes, inhibiting the expression of pro-inflammatory cytokines [94]. Further, APC has anti-apoptotic functions through p53 inhibition [87]. It is well known that thrombin is involved in the regulation of tight junctions (occludin and ZO-1) and gap formation, leading to the dysfunction of the alveolar-capillary barrier [93]. So, while suppressing thrombin, APC also ameliorates permeability.

Both inflammatory and coagulant pathways play a key role in ARDS development. The exaggerated coagulation activation in response to the inflammatory injury leads to a positive feedback between both systems and its uninterrupted activation in the alveolar compartment.

1.3 Treatment

1.3.1 Current strategies

Even with significant advances in the understanding and management of ARDS patients, morbidity and mortality remain high (40%). Treatment mainly consists of measures to avoid worsening lung injury and improve outcomes in ARDS (Figure 9), such as mechanical ventilation [95], prone positioning [96] and neuromuscular blockers [97].

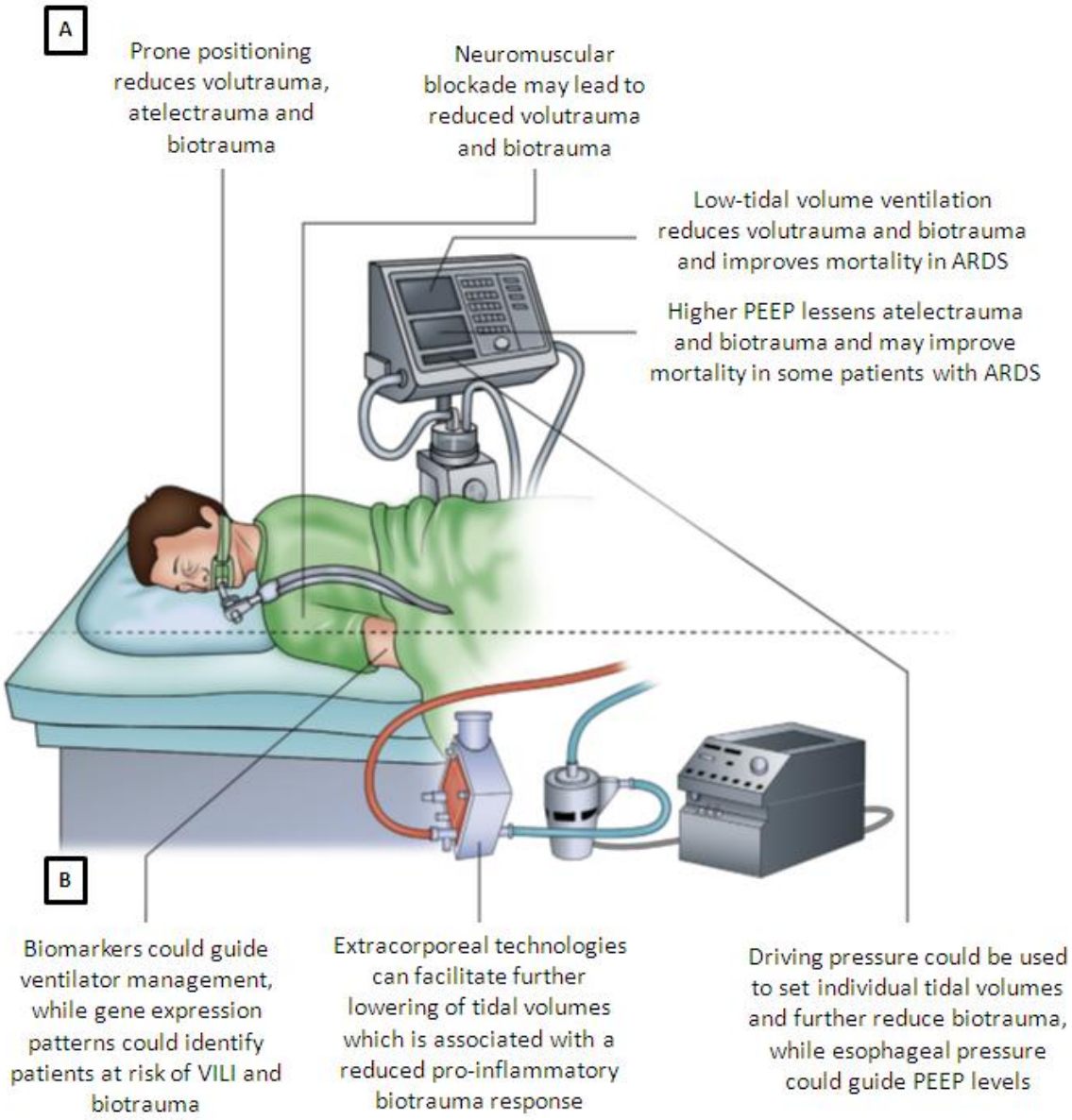

Figure 9. A) Protective ventilation: low-tidal volume, prone positioning, neuromuscular blocking agent, positive end-expiratory pressure (PEEP). B) Adjusted tidal volume and PEEP thanks to individualized approach. From Curley *et at.* [99]

1.3.1.1 Mechanical ventilation

An adequate gas exchange is ensured by mechanical ventilation after respiratory failure. A mechanical ventilator is a machine connected to the patient through a tube located into the mouth or nose that arrives to the windpipe, in order to help people breathe. The ventilator blows air plus oxygen (according to patient's needs), assisting the person to breath or doing all of the breathing. Moreover, the ventilator can provide positive-end expiratory pressure (PEEP), which holds the lungs open avoiding the collapse of the alveolar sacs. How long a person might need a ventilator depends on many factors.

Although this supportive care is potentially lifesaving, its use is associated to some complications, which can cause injurious effects contributing to the worsening of lung injury and developing what is called VILI [98]. These complications are linked to the lung heterogeneity distribution of injury in ARDS patients, presenting both completely aerated and non-aerated respiratory units which coexist in close proximity [99]. This heterogeneity causes a different effect of the administered ventilation according to the area. Next, some mechanisms linked to VILI are described:

- Barotrauma and volutrauma: produced by an excessive pressure or volume delivery into the airway, which causes overdistention, injures the epithelium and increases inflammation. It can also induce the rupture of the alveolar epithelial and endothelial barrier, leading to air leakage outside the respiratory tract which can produce interstitial emphysema or pneumothorax, between others. Furthermore, a part of increased permeability, these complications are either associated to pulmonary edema, inflammatory cells infiltration into the lung and surfactant degradation (Figure 10).
- Atelectrauma: is the result of alveolar collapse due to cyclic opening during inspiration altered with closing during expiration of alveoli. This leads to the wear and tear of the alveolar wall because of the stress forces produced between distended and collapsed alveoli. Endothelial injury, increased permeability and edema and surfactant alteration are also produced (Figure 10).

- Biotrauma: lung and systemic damage linked to the release of inflammatory mediators by epithelial and endothelial injured cells because of inadequate ventilation. Translocation of inflammatory mediators and bacterial products to the systemic circulation might promote multiple organ dysfunction syndrome (MODS) (Figure 10).

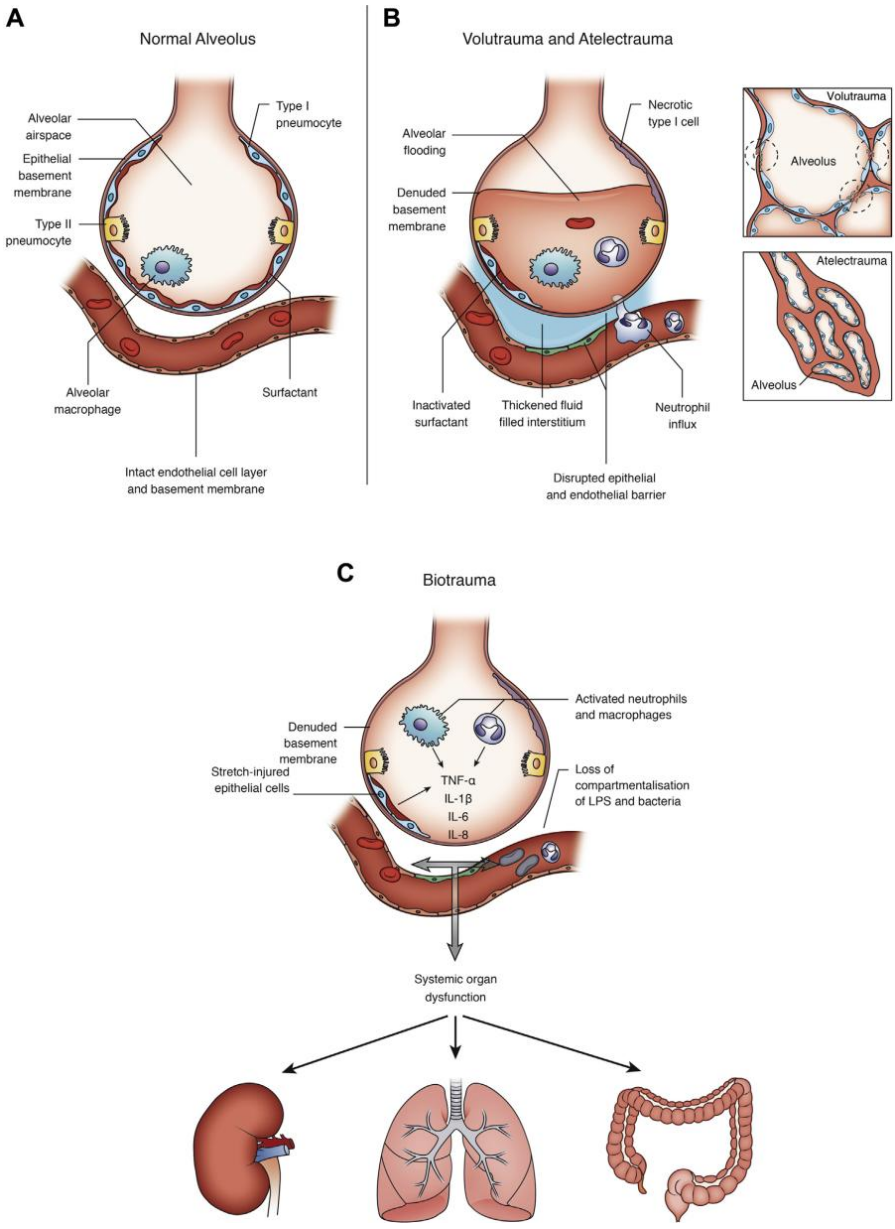

Figure 10. A) The normal alveolus. B) Volutrauma and atelectrauma during mechanical ventilation. Disruption of alveolar-capillary barrier, protein-rich pulmonary edema into the airspace, activated macrophages and neutrophils. C) Biotrauma during mechanical ventilation. Increased inflammatory mediators due to mechanical forces, and release of theses mediators into the systemic circulation, leading to organ dysfunction. From Curley *et at.* [99]

Moreover, the use of a mechanical ventilator can develop other problems such as VAP, delirium, myopathy, neuropathy or inability of weaning [100].

Smaller tidal volumes (6 ml/kg) and moderate PEEP have proved a reduction of 10% mortality in ARDS patients [101, 102]. Villar *et al.* observed MODS reduction and a 21% mortality decrease with low tidal volumes and increased PEEP [103]. Although high PEEP reduces alveolar stress and improves gas exchange, it also produces lung overdistention [99]. Also, it has been demonstrated that decreases in driving pressure, which refers to a ratio that indicates the functional size of the lung, are strongly associated with increased survival [104]. Nowadays, optimal lung-protective ventilation is unknown; at the moment, the best protective strategy option is to adjust the ventilator parameters individually, attending specifically each patient. In this line, combine lung-protective ventilation, to avoid VILI and conservative fluid therapy to prevent lung edema further reduces lung injury [5].

1.3.1.2 Prone positioning

In supine positioning (person lying face-up), the pressure of the dorsal pleura is elevated by the weight of the ventral lungs, heart and abdominal viscera. Moreover, the edematous ARDS lung further increases the ventral-dorsal pleural pressure. All this contributes to decreased regional ventilation of dependent dorsal regions. Furthermore, underlying gravity, these regions are highly perfused. All this is translated as hypoxemia.

In prone positioning (person lying face-down) pleural pressure is reduced because of the movement of the lung weight towards the chest cavity and gravitational effects. This promotes a uniform pulmonary aeration, favoring a lung aeration distribution. These improvements have been supported by physiological studies [105, 106].

Underlying advantages of prone positioning are relief of severe hypoxemia and prevention of VILI and lung compression of adjacent lung areas to mediastinum [96]. Also, prone positioning avoids cardiac compression of pericardial segments. In moderate-to-severe ARDS mechanical ventilation while the patient is in the prone position is recommended, as it further reduces mortality. There is a unique clinical trial of prone positioning that proved

reduced mortality and a reduction of ventilator days [96]. Moreover, prone positioning may have complementary benefits on high PEEP mitigating its deleterious effect such as overdistention [106]. Prone positioning and PEEP produce greater effects in indirect ARDS [11]. Furthermore, in experimental models prone positioning reduced cellular signaling pathways known to be present during VILI, and, in patients with ARDS this supportive method also decreased inflammatory mediators of serum and BAL [107]. In addition, in populations with severe ARDS, prone position further diminishes right ventricular dilatation and septal dysfunction.

Contraindications of prone positioning are severe facial or neck trauma, pelvic/spinal instability, elevated cranial pressure, hemoptysis, cardiac arrhythmias or requirement of cardiopulmonary resuscitation. Moreover, in obese patients with ARDS, prone position might worsen intraabdominal hypertension and produce renal and hepatic dysfunction [106].

Future studies need to find out the optimal strategy for prone positioning, considering its use until a clear improvement in gas exchange and mechanics is observed, and relating its employment to the appropriate PEEP and neuromuscular blockade use.

1.3.1.3 Neuromuscular blockers

The neuromuscular transmission is blocked by neuromuscular blocking agents at the neuromuscular junction, paralyzing the affected skeletal muscles [108].

In a multicenter randomized trial, the early administration of neuromuscular-blocking agent cisatracurium in patients with moderate to severe ARDS improved outcomes [97]. Another clinical trial is nowadays being performed in the United States in order to confirm the results found by Papazian *et al.* The mechanism underlying the beneficial effect of neuromuscular blocking agents in ARDS is not clear. Although neuromuscular blockers produce a brief paralysis in early ARDS that facilitates lung-protective mechanical ventilation improving patient synchrony with the ventilator and ameliorating an accurate adjustment of tidal volume and pressure, this agents also reduce lung and systemic inflammation [108]. Treatment with neuromuscular blocking agents is recommended for 3

days, as long-term administrations are associated with muscle weakness, although the risk varies depending on the agent. Cisatracurium appears to be a safer compound and it is the most used at the moment.

1.3.2 Future therapies

Despite advances in supportive care of ARDS, new therapies focused in ARDS pathophysiology development are needed [109]. Although further trials are required, herein a small overview of the ongoing alternative therapies is summarized.

Anti-platelet and anti-coagulant treatment might have an important contribution in ARDS. Aspirin acts on platelets, which have an important role in ARDS pathogenesis, being activated when inflammation is started. Another potential therapy might be heparin, which reduces alveolar fibrin deposition and presents anti-inflammatory effects.

Anti-inflammatory and immune-modulatory agents might either be a good candidate for ARDS patients. Statins might have a potential role in ARDS thanks to its anti-inflammatory and endothelial protective effects. Furthermore, anti-inflammatory drugs such as IFN-β promote protective effects in pre-clinical models of ARDS acting on endothelial cells. Moreover, tumor necrosis factor receptor 1 blockade results in decreased inflammation in the lung.

Vasoactive agents such as beta adrenergic agonists or inhaled nitric oxide may improve the oxygenation of ARDS patients. In addition, angiotensin converting enzyme 2 can be a potential therapy for ARDS, as it is a homolog of angiotensin converting enzyme that blocks its function resulting in protective pulmonary effects. Adrenomedulin is an amino acid peptide that prevents endothelial contraction and intercellular gap formation, reducing permeability.

Also, keratinocyte growth factor treatment increases ATII cell proliferation promoting reparative processes. Another emerging therapy is mesenchymal stem/stromal cells, which have been found to regulate innate and adaptive immune system reducing pro-inflammatory cytokines.

New therapeutic strategies using inhaled carbon monoxide (CO) or AP301 peptide are also ongoing. CO exerts anti-inflammatory effects and reduces oxidative stress, ischemia/reperfusion injury, ARDS and other pathological conditions, and might be especially useful in septic patients and in pneumonia-induced ARDS. AP301 is a synthetic peptide that activates apical epithelial sodium channels (ENaC), restoring the alveolar fluid clearance and the lung fluid homeostasis. AP301 has proved beneficial effects in mechanically ventilated patients reducing edema [110].

ARDS is a complex disease regarding its pathophysiology, so the therapy administrated should face different pathways and processes to ameliorate patient's outcomes. Another challenge for administrated therapy is to avoid off-target effects of drugs and concomitant MODS. Furthermore, identify subtypes in ARDS heterogeneous population might help to predict responsiveness to a specific treatment, although it is important to have in mind that the time to initiate a treatment is decisive. At last, we should not forget that data between pre-clinical and clinical models might be affected because animal models are a homogenous young population that does not reproduce the complexity of human ARDS, and that animal models also isolate all the other factors that interfere in humans such as comorbidities and side-diseases.

1.3.2.1. Anti-coagulants

Pharmacological targets for the coagulation cascade and fibrinolytic pathway might be promising candidates for ARDS treatment and prevention given the increased pro-coagulant activity found in the alveolar compartment. Higher levels of soluble TF, factor VIIa, thrombin-antithrombin complexes (TATC), thrombin, TM and PAI-1, and lower levels of the natural anti-coagulants, TFPI, protein C and antithrombin-III (ATIII) are found in BAL from ARDS patients, indicating increased coagulation activation and reduced fibrinolysis activity [73, 111, 112].

Previous studies point out that anti-coagulants such as TFPI, ATIII, TM, heparin and APC, together with fibrinolytics may help restore the coagulation cascade and treat ARDS (Figure 11, Table 3 and 4) [69].

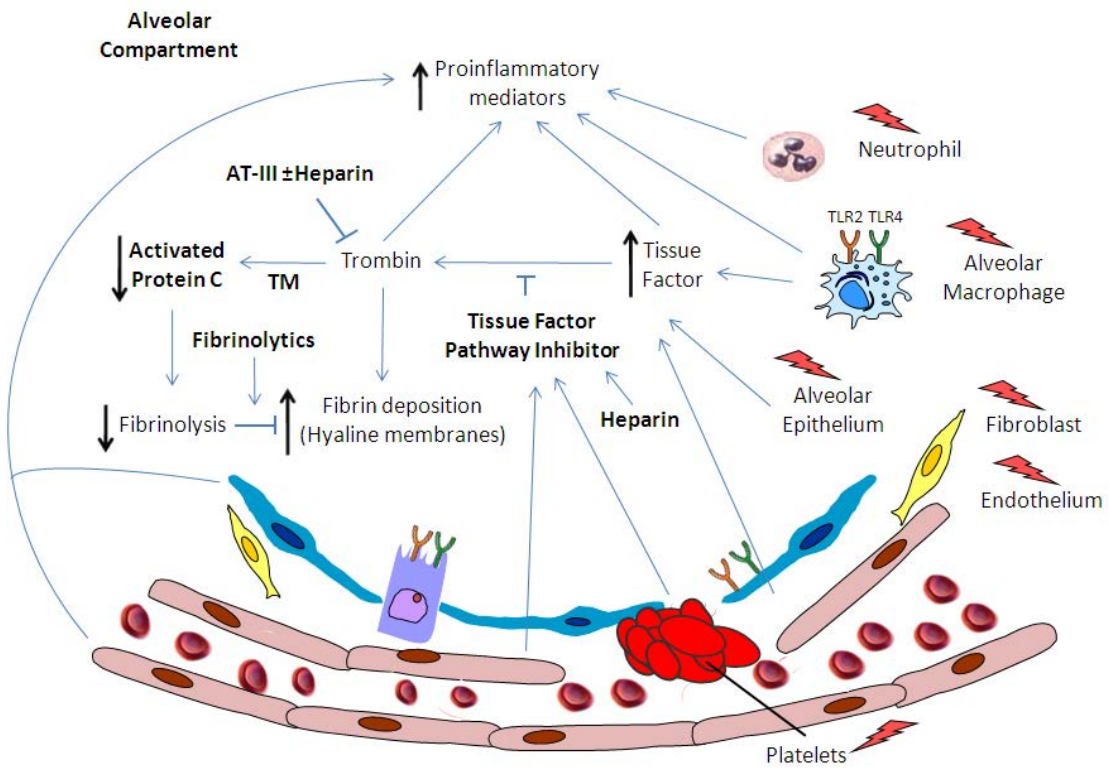

Figure 11. Anti-coagulants action on inflammation and coagulation

Given the essential role that coagulation and inflammation play in ARDS and the close relation between these pathways, anti-coagulants might act on ARDS pathophysiology not only because of its anti-coagulant activities but also because of its anti-inflammatory effect [113].

1.3.2.1.1 Tissue Factor Pathway Inhibitor

Due to its functions on the coagulant pathway, TFPI could be a good option for ARDS treatment. In a septic model in baboons, neutralized TF activation with TFPI administration decreases coagulation and cell injury [114] and reduces pulmonary injury and coagulation through inhibiting leukocytes activation in a model of LPS [115].

In addition, activated factor VIIa was developed as an anti-coagulant, and has protective effects in a septic model in baboons [116]. Nebulized recombinant human TFPI seem feasible and safe in a direct and indirect rat model of ALI (Table 3) [117]. It reduced

pulmonary and systemic coagulation in both models, although just in the intratracheal model of *Pseudomonas aeruginosa* the pulmonary inflammation was decreased [117].

Intravenous recombinant TFPI failed in reducing sepsis [118] and severe community-acquired pneumonia mortality in a phase III clinical trial [119]. Given the positive results obtained with nebulized recombinant human TFPI in pre-clinical models, further investigation should focus on this form of delivery.

1.3.2.1.2 Activated Protein C

In animal models of ALI nebulized APC administration reduces lung injury [120–122] and pulmonary coagulopathy [123–125], stimulates fibrinolysis [125], diminishes inflammation [120, 123, 125] and ameliorates oxygenation (table 3) [120, 124]. Systemic coagulation was only decreased in one of the studies with pulmonary infection [124].

In an ARDS patient that received inhaled APC (Drotrecogin alpha activated), the alveolar compartment resulted with anti-coagulant, pro-fibrinolytic and anti-inflammatory effects and reduced neutrophils recruitment into the alveolar space, without causing nor local nor systemic adverse effects [93, 126]. Unfortunately, the negative results obtained in the PROWESS-Shock trial, a phase III trial with 1967 patients with severe sepsis receiving intravenous recombinant human APC [127], and the APC removal from the market ended with the use of APC [128].

1.3.2.1.3 Thrombomodulin

ART-123 is a recombinant human soluble TM which ameliorates disseminated intravascular coagulation in animal models and clinical studies through anti-coagulant and anti-inflammatory effects [129–132]. TM modulates the generation of thrombin preventing the activation of coagulation and inflammation. Furthermore, ART-123 inhibited pro-inflammatory cytokines and ameliorated survival in a pre-clinical model of cecal ligation and puncture induced sepsis. Intravenous ART-123 reduced HMGB1 plasma levels and mortality in a LPS model of endotoxemia [133]. In addition, in a phase II study, intravenously administered ART-123 proved to be safe and effective in patients with

sepsis-associated disseminated intravascular coagulation, reducing pro-thrombin fragment and TATC concentrations [134]. In a retrospective study of intravenously combined therapy with sivelestat and recombinant human soluble TM, beneficial effects on survival of patients with ARDS and disseminated intravascular coagulation were suggested [135]. Nowadays there is an ongoing phase III study of intravenous ART-123 in septic shock patients with disseminated intravascular coagulation and multiorgan failure.

1.3.2.1.4 Heparin

Heparin is one of the most common anti-coagulants used in the clinics and could be also useful for the treatment of ARDS.

1.3.2.1.4.1 Mechanisms of action

Heparin is a potent natural anti-coagulant synthesized by mast cells in the intestine or lungs, basophils in the blood and endothelial cells [136]. It is a heteropolymer composed by repeating sequences of disaccharide formed by an uronic acid and a glucosamine residue [137]. A variable number of sulphate groups are also present. This glycosaminoglycan is extensively applied in the clinics for its anti-coagulant properties. The pharmaceutical drug is produced from porcine or bovine mucosa. Unfractioned heparin (UFH) weights 13,000-15,000 Dalton (Da) (approximately 50 monosaccharide chains) while low-molecular-weight heparin (LMWH) weights 3,000-5,000 Da [138]. LMWH is manufactured from UFH by chemical or enzymatic depolymerisation methods, which yield different end groups. Clinical prepared heparin has a negative charge due to sulphate groups of the disaccharides [137].

Both UFH and LMWH present anti-coagulant properties, potentiating ATIII inhibitory activity in the coagulation pathway and acting through other serine protease inhibitors such as TFPI [139]. All these effects reduce thrombin generation and consequently, coagulation.

The synergistic effect of UFH or LMWH on ATIII is different. On the one hand, a pentasaccharide sequence to bind ATIII with high affinity and enhance its anti-factor X

activity is need. The presence of this sequence is higher in UFH, as in LMWH some of these sequences have been destroyed during the polymerization process. On the other hand, a chain of 18 saccharides to bind ATIII with high affinity and potentiate its anti-thrombin properties is required. This chain is only present in UFH. Therefore, ATIII in the presence of LMWH exerts more specific anti-factor X activity than UFH, while in the presence of UFH, ATIII inhibits both thrombin and factor X.

A part of determining its anti-coagulant activity, heparin molecular size also influences the test used and its clearance. While the activity of UFH can be evaluated by activated partial thromboplastin time (aPTT), LMWH has to be monitored by anti-Xa activity measurement. International guidelines prescribe calibration of the aPTT to 0.3-0.7 IU/ml anti-Xa activity to establish UFH therapeutic range [140]. Regarding its metabolism, the clearance of UFH from the circulation is quicker, as it is metabolized by binding to endothelial cells and renal clearance, while LMWH only is metabolized by renal clearance, reason why LMWH is accumulated in the circulation for longer. So, the half-life of UFH is 1 h 30 min and of LMWH is 4 h [141].

Furthermore, heparin presents anti-inflammatory activities related to thrombin inhibition together with anti-inflammatory actions not related with its anti-coagulant properties [136, 142]. *In vitro*, heparin was found to inhibit the NF-κB pathway in monocytes treated with LPS [143, 144]. Moreover, heparin may interfere with the recruitment of neutrophils to the inflammatory sites [145, 146].

Biological action of heparin is wider, as it has been shown to reduce the adhesion of bacteria and viruses and the days of mechanical ventilation in ARDS patients [147]. Additionally, heparin inhibits fibroblast proliferation and collagen deposition and may thus affect tissue remodeling [148].

1.3.2.1.4.2 Pre-clinical and clinical studies

In direct and indirect ARDS, experimental models and patients studies pointed out that heparin diminish lung injury although it produces systemic bleedings. Local administration of heparin by nebulization might increase its effectiveness and prevent systemic adverse

effects (Table 3) [68, 113]. In a model of smoke inhalation and sepsis nebulized heparin ameliorated oxygenation [149]. Moreover, in pre-clinical and clinical studies inhaled anti-coagulants (heparin, heparinoids, ATIII, or fibrinolytics such as tissue plasminogen activator) favored survival [150].

Regarding inflammation, controversial results about the anti-inflammatory effect of heparin are observed in *in vivo* models of ALI. Intravenous heparin ameliorated lung injury through the inhibition of the nitric oxide synthase expression and the TGF-β/Smad pathway in a model of ALI induced by LPS [151] and nebulized heparin decreased inflammatory cells in the BAL of a model of chlorine exposure [152]. However, in an animal model of endotoxemia [153] and pneumonia [123, 124] the positive effect of nebulized heparin in coagulopathies was confirmed, although no changes on inflammation were found.

Controversial results have been found in patients with ARDS while administering local heparin (Table 4). In ARDS patients nebulized heparin did not present adverse effects, attenuated pulmonary coagulopathy and reduced the days of mechanical ventilation [147, 154, 155]. A recent multicenter trial, HEPBURN, focused in the safety and efficacy of burn patients receiving nebulized heparin, was stopped due to an elevated systemic clotting times and further detected adverse effects [113, 156]. No convincing benefit of heparin nebulization was found under mechanical ventilation [157] nor for prophylaxis for pneumonia patients receiving mechanical ventilation [158, 159]. In 16 patients with VILI heparin was nebulized proving safety and increasing the number of ventilator-free days [160].

1.3.2.1.5 Antithrombin (ATIII)

Another anti-coagulant which could have beneficial effects is ATIII, which actions are enhanced by the presence of heparin.

1.3.2.1.5.1 Mechanisms of action

Antithrombin (AT), also termed ATIII is a broad-spectrum serine protease inhibitor synthesized in the liver [161]. It is a single-chain glycoprotein that has a molecular weight of 58 kDa [162]. ATIII neutralizes several enzymes in the coagulation cascade, including thrombin and factor Xa, IXa, XIa and XIIa [139, 163].

As explained above at the heparin section, ATIII contains a heparin-binding domain on its active site. When the pentasaccharide sequence from the heparin molecule binds to ATIII, it induces local conformational changes that increase the attraction between protein and ligand [139]. Further conformational changes propagate on the structure of the protein, inducing the expulsion of the reactive center loop, which allows the interaction with the protease [139]. Heparin enhances the inhibitory activity of ATIII for the pro-coagulant proteins of the coagulation pathway in an approximate 1000-fold increase [164].

Anti-inflammatory properties of ATIII have also been observed. Given the close relation between inflammation and coagulation, ATIII modulates the inflammatory response through the inhibition of the molecules of the coagulant cascade. Moreover, ATIII has several coagulation independent anti-inflammatory effects mediated by its interaction with heparin-like glycosaminoglycans (HSPGs) receptor on the endothelial cells. Activation of HSPGs induces the prostacyclin release and regulates neutrophil activation, rolling and adhesion on the endothelium [163–165]. Syndecan-4, an HSPG member of the syndecan family is expressed by neutrophils, monocytes, lymphocytes, eosinophils and endothelial cells [164]. Heparin competes with syndecan-4 or other HSPGs to bind ATIII. Moreover, it has been described that ATIII blocks LPS-mediated TLR4 and NF-κB activation in monocytes and endothelial cells inhibiting IL-6, TNF-α and iNOS production [164, 166].

As inflammatory cytokines and thrombin are involved in the regulation of tight junctions and previous studies proved that APC reduces the permeability induced by thrombin [93], ATIII might regulate the tight junctions through its action on the coagulation and inflammatory pathways. Thus, ATIII might have a major role on avoiding damage of the alveolar-capillary barrier.

1.3.2.1.5.2 Pre-clinical and clinical studies

In order to reestablish the natural anti-coagulant cascades, therapeutic strategies with ATIII have been tested in pre-clinical models of ARDS (Table 3). In LPS-induced lung injury, intravenous ATIII reduced vascular injury, leukocyte accumulation, and vascular permeability [139, 163, 167]. Furthermore, in a model of intratracheally *Streptococcus pneumoniae* induced pneumonia, nebulized ATIII as a pre-treatment attenuated pulmonary coagulopathy and fibrinolysis, reduced bacterial outgrowth, decreased inflammation and did not produce systemic bleeding [123]. In models of *Pseudomonas aeruginosa* [124] and endotoxemia [153] nebulized ATIII reduced pulmonary coagulation and did not affect systemic coagulation.

Currently, nebulized ATIII has not been administered in any clinical trial. As explained above, pre-clinical studies with combined ATIII and heparin have proved positive results.

1.3.2.1.6 Combined heparin and ATIII

Studies where nebulized heparin and ATIII have been combined also proved benefit in ARDS. A pre-clinical model of combined aerosolized recombinant human AT and heparin in a sheep with burn and smoke inhalation improved pulmonary pathophysiology (Table 3) [168]. Moreover, intravenous recombinant human AT together with aerosolized heparin diminished the lung injury in a model of sheep with burn and smoke inhalation [169]. Intravenous AT together with nebulized heparin and tPA in a model of burn smoke inhalation sheep restored gas exchange but not inflammation [165].

Lung injury in ventilated smoke inhalation ARDS patients [170] and duration of mechanical ventilation in burn inhalation injury [171] was reduced by aerosolized heparin and N-acetylcystine (Table 4). No drug incompatibilities were found in a case of a patient with smoke inhalation injury receiving nebulized heparin with N-acetylcysteine and epoprostenol [172]. Nebulized heparin together with a beta-agonist and a mucolytic in burn patients reduced duration of mechanical ventilation, was safe and no bleeding events were recorded [173].

Table 3. Pre-clinical models of ARDS with nebulized anti-coagulants

Pathogenesis	Ref.	Nebulized agent (dose)	Species	Model details	Pulmonary coagulation	Pulmonary inflammation	Physiology and other outcomes	Side effects
Pulmonary infection	[174]	Hep (5 µg/mouse)	Mouse	*Legionella spp*	NR	↓	↑Bacterial clearance ↓mortality	NR
	[123]	Hep (1,000 IU/kg) Dan (250 IU/kg) APC (5000 µg/kg) AT (500 IU/kg)	Rat	*Streptococcus pneumoniae*	↓ Hep, ↓Dan, ↓APC ↓AT	↓AT	AT: ↑Bacterial clearance ↓lung injury	Dan: ↓systemic coagulation Hep Dan: ↑bacteremia
	[124]	Hep (1,000 IU/kg) Dan (250 IU/kg) APC (5000 µg/kg) AT (500 IU/kg)	Rat	*Pseudomonas aeruginosa*	↓Dan, ↓APC ↓AT	=	=bacterial clearance	APC: ↓systemic coagulation
	[117]	TFPI (10 mg/kg)	Rat	*Pseudomonas aeruginosa*	↓	↓	↑Bacterial clearance	↓systemic coagulation
Inhalation trauma	[149]	Hep (10,000 IU) or i.v. 5,300 IU/kg)	Sheep	Skin burn+smoke inhalation	NR	=	↓lung injury ↓airways obstruction ↑arterial oxygenation ↑hemodynamics	No side effects
	[175]	Hep (10,000 IU) ± lisofylline (10 mg/kg/h)	Sheep	Skin burn+smoke inhalation	NR	=	=lung injury ↓mechanical ventilation need ↓pulmonary shunt	No side effects
	[168]	Hep (10,000 IU) ± AT (290 U)	Sheep	Skin burn+smoke inhalation	NR	↓Hep+AT	↓airways obstruction ↓pulmonary shunt ↑arterial oxygenation	No side effects
	[169]	Hep (10,000 IU) + AT (0.34 mg/kg/h)	Sheep	Skin burn+smoke inhalation	NR	↓	↓lung edema ↓ airways obstruction ↑arterial oxygenation ↑hemodynamics	No side effects
	[165]	Hep (10,000 IU) + AT (6 IU/kg/h) + tPA (2 mg)	Sheep	Skin burn+smoke inhalation	NR	↓	↓lung injury+edema ↓ airways obstruction ↑arterial oxygenation ↑hemodynamics	No side effects

Lung injury	[176]	Hep (1,000 IU/kg)	Rat	Direct	NR	↓	NR	NR
	[152]	Hep (not reported)	Mouse	Direct	NR	↓	NR	No side effects
	[177]	Dan (50 IU/kg)	Rat	Indirect	NR	↓	↓mortality	NR
	[121]	APC (48 µg/kg/h)	Sheep	Indirect	NR	NR	↓atelectasis =lung edema ↑arterial oxygenation	No side effects
	[125]	APC (100 µg/mouse)	Mouse	Direct	↓	↓	NR	NR
	[120]	APC (4 mg/ 3 ml)	Mouse	Direct	NR	↓	NR	NR
	[153]	Hep (1,000 IU/kg) Dan (250 IU/kg) APC (5,000 µg/kg) AT (500 IU/kg)	Rat	Indirect	↓Hep ↓Dan ↓APC ↓AT	=	NR	Hep and Dan: ↓systemic coagulation
	[117]	TFPI 10 (mg/kg)	Rat	Indirect	↓	↓	NR	↓systemic coagulation

Abbreviations: ↓: decreased, ↑: increased, =: unchanged, APC: activated protein C, ARDS: acute respiratory distress syndrome, AT: antithrombin, direct/indirect model of ARDS, Dan: danaparoid, Hep: heparin, NR: not reported, ref: reference, TFPI: tissue factor pathway inhibitor, tPA: tissue plasminogen activator. Adapted from Juschten *et al.* [113]

Table 4. Nebulized anti-coagulants in patients with ARDS

Pathogenesis	Ref.	Nebulized agent (dose)	Patients	Study details	Physiological endpoints	Clinical endpoints	Side effects
Burn patients with inhalation trauma	[178]	Hep (60,000 IU/day)+NAC+ bronchodilator	90 children	Retrospective study using historical controls	↓atelectasis	↓reintubations ↓duration of mechanical ventilation ↓mortality	NR
	[179]	Hep (30,000 IU/day)+NAC+ bronchodilator	150 children or adults	Retrospective case-control study	=arterial oxygenation	=duration of mechanical ventilation =mortality	=pulmonary infections
	[170]	Hep (60,000 IU/day)+NAC+ bronchodilator	30 adults	Retrospective study using historical controls	↓lung injury ↑arterial oxygenation	↓mortality	NR
	[180]	Hep (30000 IU/day)+NAC+ bronchodilator	63 adults	Retrospective study using historical controls	=systemic coagulation	NR	=systemic bleeding
	[181]	Hep (30,000 IU/day)+NAC+ albuterol	40 adults	Retrospective study using historical controls	NR	=duration of mechanical ventilation =mortality	↑pulmonary infections
	[173]	Hep (60,000 IU/day)+NAC/$NaHCO_3$+ bronchodilator	72 adults	Retrospective case-control study	=lung injury	↓duration mechanical ventilation ↑ventilator-free days =mortality	=pulmonary infections =systemic bleeding
	[156]	Hep (100,000 IU/day)	-	RCT, stopped early (safety issues)			
ARDS patients	[155]	Hep (50,000 – 400,000 IU/day)	16 adults	Open-label phase I trial	≥100000 IU/day ↓systemic coagulation	=lung compliance =arterial oxygenation	=systemic bleeding
	[154]	Hep (50,000 – 400,000 IU/day)	16 adults	Open-label phase I trial	≥400000 IU/day ↓systemic coagulation	NR	NR
Patients at risk of ARDS	[147]	Hep (100,000 – 150,000 IU/day)	50 adults	RCT	=pulmonary coagulation ↓systemic coagulation =arterial oxygenation	↑ventilator-free days =mortality	=systemic bleeding
	[158]	Hep (20,000 IU/day)	214 adults	RCT	NR	=duration of mechanical ventilation	=systemic bleeding

	[182]	Hep (50,000 IU, single dose)	40 adults	RCT	↓atelectasis = arterial oxygenation =hemodynamics ↑CO_2 elimination	=duration of mechanical ventilation =mortality	=systemic bleeding

Abbreviations: ↓: decreased, ↑: increased, =: unchanged, Hep: heparin, NAC: N-acetylcysteine, NR: not reported, RCT: randomized controlled trial, ref: reference. Adapted from Juschten *et al.* [113]

1.3.2.1.7 Systemic versus local treatment

Even though previous findings indicate that anti-coagulants have shown beneficial effects in pre-clinical and clinical ARDS, systemic bleeding offset its positive effects. Local pulmonary administration of anti-coagulants might increase local efficacy and reduce the risk of systemic adverse effects [68, 113].

1.3.2.1.7.1 Nebulization systems

Aerosol medication has been used for thousands of years [183]. They are attractive because drugs can be delivered directly to the respiratory tree, to the alveolar epithelium, or both. Furthermore, the capacity of the lungs for absorption and diffusion ensures the quick entrance of drugs to the systemic circulation.

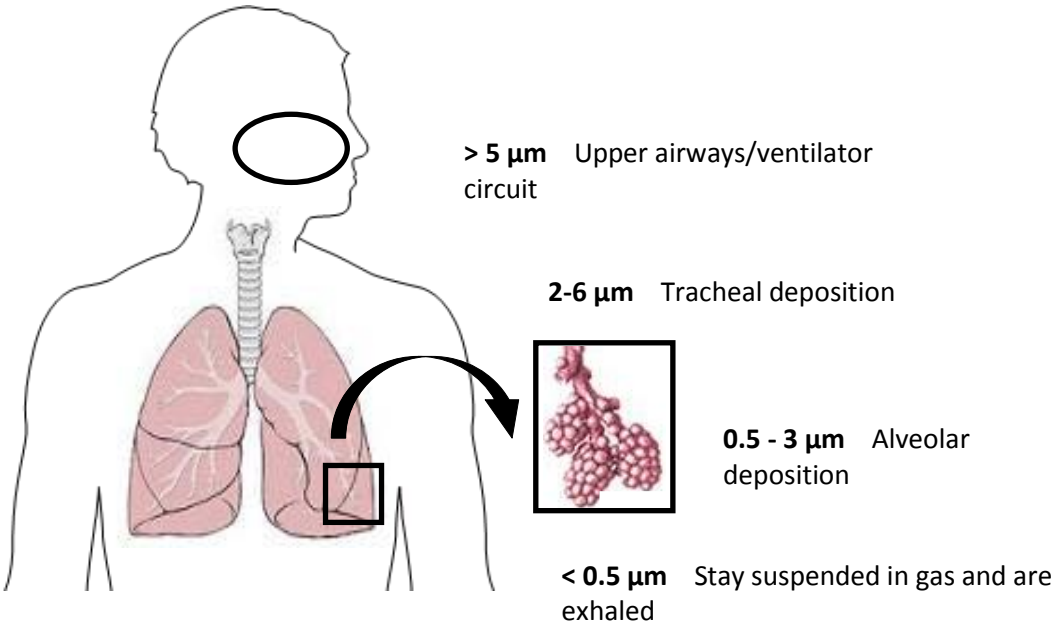

Figure 12. Probable site of aerosol deposition related to particle size. From Artigas *et al.* [128]

Aerosols are colloidal suspensions of particles in gas. Particles size, shape, and density together with gas density and viscosity define the extent to which particles can endure suspended. In order to characterize aerosols two variables are used: the mass median aerodynamic diameter indicates the size of the particles, whereas the geometric standard

deviation reflects the degree of variation in the size of the particles. These properties allow to predict where will be the particles deposited within the tracheobronchial tree/ventilator circuit (Figure 12). With a known rate of particle production and geometric standard deviation the efficiency of an aerosol generator can be determined. The ventilator circuit and settings have a bigger impact than the aerosol generator on time required for drugs to reach their targets and on the proportion of drugs that reach these targets.

In patients that receive mechanical ventilation, ensure that aerosolized particles delivered by the generator reach the distal airways and the alveoli is a challenge, as the proportion of the drug that arrives to the target site depends on various factors (Table 5). The delivery of the drug is determined by the ventilator settings and the physiological/pathophysiological factors of the patient's airflow. The ventilator should be set for the following characteristics to ensure peripheral drug deposition: 1) low bias flow; 2) higher tidal volume and/or recruitment maneuver to distribute the drug more extensively; 3) a long, slow, continuous inspiratory profile; 4) a long pause at end-inspiration to maximize the impaction/dropout of particles in peripheral regions; and 5) PEEP to prevent alveolar collapse during expiration. In modern ventilators, aerosol production and inspiration are synchronized to ameliorate drug delivery [184].

Table 5. Factors that affect the delivery of aerosol to the distal airways/alveoli in mechanically ventilated patients

Ventilator	• Bias flow • Tidal volume • Respiratory rate • Inspiratory profile-time, flow rate • End inspiratory pause • Positive end-expiratory pressure • Gas composition
Circuit	• Method, location and efficiency humidification • Presence of any restrictions distal to aerosol generator • Temperature • Geometry of entire circuit • Position of nebulizer within the circuit
Patient	• Proximal airway geometry • Degree and pattern of ventilator heterogeneity • Airway and/or parenchymal pathology • Ventilation-perfusion matching • Body position • Spontaneous respiratory efforts and ventilator synchrony

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

The hypothesis of this thesis is that nebulized heparin and/or ATIII exhibit an anti-inflammatory and anti-coagulant effect in the lung after ALI. The local co-administration via nebulization into the lung produces a synergistic effect enhancing the properties of both anti-coagulants without producing systemic bleeding, also promoting the restoration of the epithelial barrier and reducing ALI.

2.2 Objectives

Main objective: To evaluate the effect of local heparin and/or ATIII in the lung after ALI focusing on inflammation, coagulation and lung permeability.

Objective 1: To characterize the effect of UFH in inflammation, proliferation and permeability in primary lung cells such as human alveolar macrophages (hAM), human alveolar type II (hATII) cells and human fibroblasts (hF) *in vitro* after activation with LPS.

Objective 2: To evaluate the effect of nebulized heparin in a LPS-induced ALI in rats. To study the role that heparin might exert in alveolar macrophages focusing in the activation of the coagulation and the inflammatory pathways.

Objective 3: To determine the action of ATIII in inflammation and lung permeability in hATII cells *in vitro* after activation with LPS.

Objective 4: To study the effect of nebulized ATIII or the combination of both anti-coagulants, ATIII and heparin, in a rat model of ALI induced by Hydrochloric acid (HCl) and LPS, focusing on its anti-inflammatory and anti-coagulant activities, and the restoration of the epithelial barrier.

3. RESULTS

In this section the published articles concerning the first and the second objective are included. Subsequently, at the appendix section, as results presented in two congresses, the methods and results concerning the third and fourth objectives are described (Appendix 8.1).

3.1 Role of heparin in pulmonary cell populations in an *in-vitro* model of acute lung injury

Camprubí-Rimblas M, Guillamat-Prats R, Lebouvier T, Bringué J, Chimenti L, Iglesias M, Obiols C, Tijero J, Blanch L, Artigas A. Role of heparin in pulmonary cell populations in an in-vitro model of acute lung injury. Respir Res. 2017 May 10;18(1):89. doi: 10.1186/s12931-017-0572-3. PubMed PMID: 28486961; PubMed Central PMCID: PMC5424410. Q1. Impact factor: 3.841.

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Role of heparin in pulmonary cell populations in an in-vitro model of acute lung injury

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Abstract

Background: In the early stages of acute respiratory distress syndrome (ARDS), pro-inflammatory mediators inhibit natural anticoagulant factors and initiate an increase in procoagulant activity. Previous studies proved the beneficial effects of heparin in pulmonary coagulopathy, which derive from its anticoagulant and anti-inflammatory activities, although it is uncertain whether heparin works. Understanding the specific effect of unfractioned heparin on cell lung populations would be of interest to increase our knowledge about heparin pathways and to treat ARDS.

Methods: In the current study, the effect of heparin was assessed in primary human alveolar macrophages (hAM), alveolar type II cells (hATII), and fibroblasts (hF) that had been injured with LPS.

Results: Heparin did not produce any changes in the Smad/TGFß pathway, in any of the cell types evaluated. Heparin reduced the expression of pro-inflammatory markers (TNF-α and IL-6) in hAM and deactivated the NF-kß pathway in hATII, diminishing the expression of IRAK1 and MyD88 and their effectors, IL-6, MCP-1 and IL-8.

Conclusions: The current study demonstrated that heparin significantly ameliorated the cells lung injury induced by LPS through the inhibition of pro-inflammatory cytokine expression in macrophages and the NF-kß pathway in alveolar cells. Our results suggested that a local pulmonary administration of heparin through nebulization may be able to reduce inflammation in the lung; however, further studies are needed to confirm this hypothesis.

Keywords: Acute Respiratory Distress Syndrome (ARDS), Alveolar macrophages, Alveolar cells, Fibroblasts, Anticoagulants, Inflammation

Background

Acute respiratory distress syndrome (ARDS) is a common and devastating illness characterized by lung inflammation, endothelial and epithelial injury, increased vascular permeability and oedema; all of these factors lead to organ dysfunction. ARDS results from a direct injury such as a pneumonia or aspiration of gastric contents or from a systemic injury such as sepsis or severe trauma [1–4].

Current therapeutic strategy to decrease ARDS-associated mortality is to utilize protective mechanical ventilation in combination with low tidal volume. However, morbidity and mortality remain high, both exceeding 40% [5–7]. The need for new specific pharmacological therapies has carried to examine the role of altered coagulation and fibrinolysis in the pathogenesis of ARDS.

Pulmonary coagulopathy is intrinsic to ARDS and directly dependent on the severity of acute lung injury (ALI) and linked to the outcome of ARDS. This disease is characterized by the coagulation cascade activation and reduced fibrinolysis that leads to fibrin deposition in the airspaces and triggers inflammation. All of this occurs because of the alveolar type II cell damage and also the pro-inflammatory activation of macrophages.

In recent years, several preclinical studies have supported the use of nebulized or systemic anticoagulants to prevent and treat ALI in diverse animal models [8, 9]. Moreover, numerous clinical trials were performed with patients with ARDS or sepsis requiring unfractionated

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heparin (UFH), although results have been conflicting. High dose of nebulized heparin decreased pulmonary coagulation in the lung of patients with ARDS without causing systemic bleedings [10]. Furthermore, patients treated with nebulized heparin were ventilated with lower tidal volumes compared to control patients during the first 7 days of ventilation; determining an improvement in the lung function that was associated with the dosage and the days that the nebulized heparin was administered. However the administration of heparin was ineffective in the improvement of patient's outcomes such as the survival at day 28 [11–15]. Moreover, nebulized heparin combined with N-acetylcysteine for burn inhalation injury decreased lung injury scores and the duration of mechanical ventilation. Besides, heparin was tested as a treatment in other lung diseases such as cystic fibrosis with favourable results [16].

Cellular and molecular mechanisms that control intra-alveolar fibrin deposition are not completely understood. The modulation of fibrin deposition, coagulation and fibrinolysis could be important targets in ARDS treatment. The use of anticoagulants or/and antithrombotic agents such as heparin could ameliorate lung pathology. Inhaled heparin was administered in healthy patients with no side effects [17]. Elucidate the pathways involved in the injury improvement will allow us to look for new therapeutically strategies. As well, cell-cell interaction during ARDS worsens the damage and to design new target treatments for each specific cell are need.

It is known that heparin has anti-inflammatory as well as anticoagulant effects, though the mechanism is unclear. Determine the cross-talk between coagulation and inflammation pathways that are modified by heparin would be useful for ARDS treatment.

The objective of this study was to test the effect of UFH, in inflammation–proliferation–permeabilisation in various primary lung cells such as human alveolar macrophages (hAM), human alveolar type II cells (hATII), and human fibroblasts (hF) in vitro after lipopolysaccharide (LPS)-induced acute injury. Effects on the inflammatory response and potential mechanisms such as the NF-kß and Smad/TGFß pathways were investigated.

Methods

Ethics statement

Fourteen biopsies from human lungs for isolation of primary human alveolar macrophages (hAM), fibroblasts (hF) and alveolar type II cells (hATII) were obtained from patients that were submitted to a lobectomy and had previously provided informed consent. The biopsies were obtained from distal areas of the tumour and were histologically normal. Patients did not have interstitial diseases, they were not smokers in the last 2 years and they were between 55 and 75 years old. This study was reviewed and approved by the respective local Ethics Committee.

Isolation of alveolar macrophages

hAM were obtained by broncho-alveolar lavage (BAL), which was performed with 50 ml of sterile saline (0.15 M; 0.9% NaCl). BAL cells were centrifuged (800 x g, 10 min) and the cell pellet with hAM was resuspended in RPMI 1640 medium (Gibco, USA) supplemented with 10% inactivated foetal bovine serum (FBS) (Gibco, USA), 1 mM L-glutamine, penicillin-streptomycin (50 U/ml, 0.05 mg/ml, respectively), 0.025 mg/ml Vancomycin (Pfizer, Spain) and 0.1 mg/ml cefotaxime (Normon, Spain) and 1 mM HEPES. hAM were plated in 24-well plastic dishes ($2x10^5$ cells/well) with 1 ml of supplemented medium and precultured for 24 h at 37 °C and 5% CO2.

To estimate the purity of isolated hAM, cytospin preparations were stained using the Diff-Quick kit (Diagnostics Grifols, Spain), according to the manufacturer's protocol. To reinforce the purity of hAM, preparations were fixed in 4% paraformaldehyde and blocked with a solution of PBS, 3% FBS and 1% BSA for 2 h at room temperature. hAM were incubated overnight with mouse anti-rat CD68 (1:100) (Acris Antibodies, USA), washed with PBS and incubated for 1 h at 37 °C with goat anti-mouse IgG-FITC (1:500) (Santa Cruz Biotechnology, USA). After a PBS 1X lavage, cells were incubated 5 min with HOECHST (1:1000) (Invitrogen, USA) (Fig. 1).

Isolation of human alveolar epithelial type II cells

hATII cells were isolated from lung biopsies after digestion using 0.25% of trypsin type I (Sigma, Germany) in Hanks Balanced Salt solution (HBSS) (Gibco, USA) at 37 °C for 20 min. Once digested, the tissue was chopped in the presence of albumin (Grifols, Spain) and DNase (250 μg/ml) (Roche, Germany). The cell suspension was filtered through 100- and 40-μm nylon meshes, then centrifuged with a density gradient of 1.077 g/ml of Lypmphoprep solution (Sigma, Spain), at 600 x g for 25 min. The interface containing ATII cells and interstitial macrophages was collected, mixed with DNAse (100 μg/ml) and centrifuged at 600 x g for 15 min. The pellet was then resuspended in DNAse (100 μg/ml) and incubated on Petri dishes for 1 h at 37 °C to remove the remaining interstitial macrophages. Unattached cells were hATII cells, which were collected and seeded at a density of 2×10^5 on 24-well plastic dishes, in 1 ml of supplemented DCCM1 (10% FBS (Gibco, USA), 1 mM L-glutamine, penicillin-streptomycin (50 U/ml, 0.05 mg/ml, respectively), μg/ml Vancomycin and cefotaxime and 1 mM HEPES). The cells were precultured for 48 h at 37 °C and 5% CO2.

The purity of isolated hATII was measured by the presence of intracellular alkaline phosphatase (Sigma,.

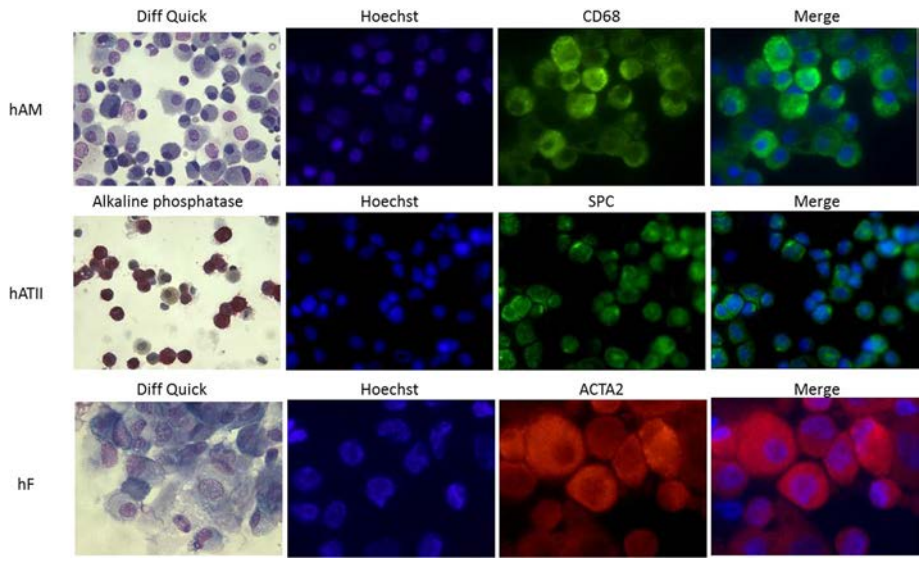

Fig. 1 Purity of isolated cells. hAM were stained with Diff quick to differentiate macrophages, neutrophils and lymphocytes and also CD68 immunofluorescence. hATII were stained with alkaline phosphatase and also with Surfactant C protein immunofluorescence. hF were stained with Diff Quick and ACTA2 immunofluorescence. Diff quick and alkaline phosphatase images had x400 magnification. Images of Hoechst, CD68, SPC and ACTA2 immunofluorescences had x600 magnification (hAM: human alveolar macrophages; hATII: human alveolar type II cells; hF: human fibroblasts, SPC: surfactant C protein and ACTA2: alpha smooth actin)

Spain). Further, hATII cells were fixed in paraformaldehyde, and the immunofluorescence protocol described for hAM was performed. Antibodies used were mouse anti-rat SPC (1:100) (Santa Cruz Biotechnology, USA) and goat anti-mouse IgG-FITC (1:500) (Santa Cruz Biotechnology, USA) (Fig. 1).

Isolation of fibroblasts

hF were obtained from lung explants. Lung slices (1 mm) were cut and placed in Petri dishes of 90 mm^2. The small pieces were separated by 2–3 cm and incubated with RPMI 1640 medium (Gibco, USA) supplemented with 10% inactivated foetal bovine serum (FBS) (Gibco, USA), 1 mM L-glutamine, penicillin-streptomycin (50 U/ml, 0.05 mg/ml, respectively), 0.025 mg/ml Vancomycin (Pfizer, Spain) and 0.1 mg/ml cefotaxime (Normon, Spain) and 1 mM HEPES. The explants were removed 21 days later, and fibroblasts grew until confluence. Fibroblasts were used between passages 2 and 7. The cells at 85% confluence were trypsinised (Sigma, Spain) and seeded at a density of 5×10^4 on 24-well plastic dishes.

HF cytospin preparations were stained with Diff-Quick kit following manufacturer's protocol. Moreover, hF cells were fixed in paraformaldehyde and the immunofluorescence protocol described above was performed. Antibodies used were mouse anti-rat ACTA2 (1:100) (Proteintech, USA) and goat anti-rabbit IgG-TR (1:500) (Santa Cruz Biotechnology, USA) (Fig. 1).

Injury induced by LPS and treatment with heparin

All cell types were exposed to Lipopolysaccharide from *Escherichia coli* 055:B5 (LPS) alone (hAM: 50 ng/ml, hATII: 50 μg/ml and hF: 50 μg/ml) (Sigma, Spain) in serum-free media or in combination with unfractionated sodium heparin (0.1 IU/ml) (Hospira Products Farmac, Spain), which was added 2 h after the LPS exposure. A control of untreated cells and a control with heparin alone were established. hAM were collected 7 h after heparin addition with 500 μl of TRIzol reagent (Ambion, USA) and frozen at −80 °C. hATII cells and hF were collected 24 h after heparin administration. Heparin optimal dose was established from literature [18] and previous studies performed in our laboratory. A time course study determined the maximal efficacy point, which was used to perform our analysis.

RNA isolation and real-time PCR analysis

Total RNA was extracted from isolated cells using chloroform, isopropanol and ethanol. The optical density at 260 nm and the ratio 260 nm/280 nm were measured with spectrophotometerND-1000 (Nanodrop, USA) to determine the RNA concentrations. Total RNA was reverse-transcribed into cDNA according to the Reverse Transcriptase Core kit (Eurogentec, Belgium), using Alpho-SC (Analytikjena, Germany) thermocycler. PCR amplification was performed in 7500 RealTime PCR System (Applied Biosystems, USA) using SYBR

green (Kapa Biosystems, Germany) and the corresponding human primers (Table 1). The PCR started at 95 °C for 10 min, followed by 40-cycle amplification (15 s at 95 °C, 60 s at 60 °C and 2 min at 72 °C). Data are shown as target gene expression relative to GAPDH and fold over Control group; ΔΔCt method was used to correct all the PCR data.

Multiplex analysis

Media of all the cells in all treated conditions was collected after the treatment, 7 h in the case of hAM and 24 h in the case of hF and hATII. A 4-plex for TNF-α, IL-6, IL-8 and MCP-1 and a single multiplex for TGF-β was performed with the samples of 4 biopsies. The multiplex were performed following the manufacturer's protocol (eBioscience, Germany). The results are expressed in pg/ml. The sensivity for TNF-α and IL-6 was 0,4 pg/ml, 1,2 pg/ml per IL-8, 0,6 pg/ml per MCP-1 and 0,96 pg/ml per TGF-β.

Immunofluorescence

Cells were seeded in cell chambers (Merck Millipore, Germany) and treated as explained before. After 7 h for hAM and 24 h for hATII and hF, cells were fixed with formalin (4%) during 5 min and permeabilized with PBS + 0,2% Triton during 10 min. After, we incubated cell chambers with blocking solution (PBS with 1% albumin and 3% fetal bovine serum) for 2 h at room temperature. NF-kß (1:200, Ref: CPA9199), IRAK-1 (1:100; Ref: TA305934), MyD88 (1:100; Ref: TA30599) and Smad2/3 (1:200; CPA-1707) antibodies (ACRIS, Spain) were used to determine the protein expression by immunofluorescence in hAM, hATII and hF after all the treatments. The cells were incubated 2 h at room temperature with the primary antibodies. After 3 washes with PBS, slides were incubated with the secondary antibodies for 1 h 30 min at room temperature in the dark. We used anti-mouse-FITC as a secondary per NF-kß, anti-rabbit-FITC as a secondary per IRAK-1 and anti-rabbit-Alexa647 as a secondary per MyD88 and Smad2/3. All the secondary antibodies were used a 1:800 dilution (Santa Cruz, USA). After nucleus were stained with Hoechst (Thermo Fisher, Germany) (1:10000 dillution) for 2 min. Slides were washed again in PBS and mounted with Fluoromount™ Aqueous Mounting Medium (Sigma; USA). Light and fluorescence microscopy were performed using a Nikon Eclipse Ti microscope.

Flow cytometry

A cell death assay was performed using the Annexin V-FITC and propidium iodide (PI) apoptosis and necrosis detection Kit (Clinisciences, France). All cell types (hAM, hATII and hF) were injured with LPS and treated with heparin, as previously described. Cells were trypsinised (Sigma, Spain), washed with PBS 1X and 2×10^7 cells were resuspended in 1 ml of binding buffer (Clinisciences, France). Ten microliters of Annexin V-FITC solution and 15 μl of PI were added to each treatment condition and incubated for 45 min at 4 °C in the dark. Three hundred microliters of PBS 1X were added, and the stained cells were analysed with flow cytometry (FACSCanto).

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and the $n = 8$ for all the study groups. All results were analysed by one-way ANOVA with multiple comparisons, and Newman-Keuls post-hoc test was applied. Statistical significance was considered at $p \leq 0.05$.

Results

Purity

Primary cells (hAM, hATII and hF) of human biopsies were obtained with purities of 98, 78 and 100%, respectively (Fig. 1). The main impurities in hATII are caused by macrophages and after the media change at 48 h the major part of the impurities are removed. The hATII purity is similar to the purities obtained in other published papers [19, 20].

Table 1 List of primers and their corresponding sequences used for PCR analysis

Gene	Forward primer	Reverse primer
GAPDH	5′ GAT CAT GAG CAA TGC CTC CT 3′	5-TGT GGT CAT GAG TCG TTC CA-3
TNF-α	5′TCC TTC AGA CAC CCT CAA CC 3′	5-AGG CCC CAG TTT GAA TTC TT-3
IL8	5′ ATTTCTGCAGCTCTGTGTGAAGGTGC 3′	5 -TTGTGGATCCTGGCTAGCAGA C-3
MCP1	5′ CAAACTGAAGCTCGCACTCTCGCC 3,	5 -ATTCTTGGGTTGTGGAGTGAGTGTTCA-3
IRAK1	5′ CCAGCCCCTTCTTCTACCAA 3′	5′ AGCATACACCGTGTTCCTCA 3′
TGF-β	5′ CGGATCAGCGTTTATCAGGT 3′	5′ CAACTTGGGGTTGATGCTCT3′
Myd88	5′ TCACCACACTTGATGACCCC 3′	5′ CGGCACCTCTTTTCGATGAG 3′
SMAD2	5′ TAAAGTGCCTGGGATTGAGG 3′	5′ GTGTGCCTGGGACTTGTTTT 3′
SMAD3	5′ ATAGGTGCTTTGGGCGTATG 3′	5′ CTGCTATCCAGTCACCAGCA 3′
IL6	5′ TACCCCCAGGAGAAGATTCC 3′	5′ TTTTCTGCCAGTGCCTCTTT 3′

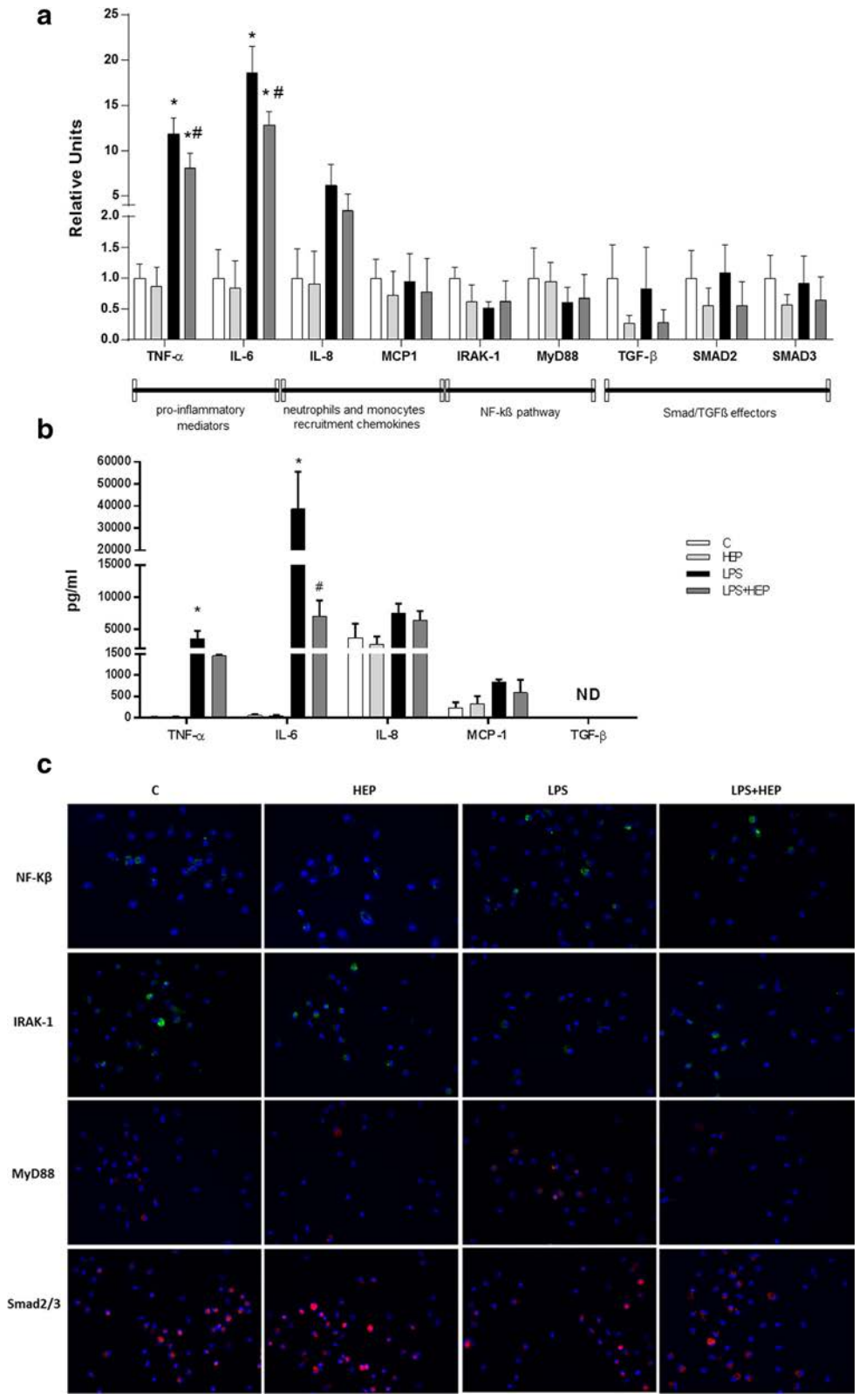

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Human alveolar macrophages gene expression. **a** Expression of TNF-α, IL-6, IL-8, MCP-1, IRAK-1, MyD88, TGF-β, Smad2 and Smad3 evaluated by q-PCR at 7 h after LPS treatment. Data are expressed mean ± SEM (ΔCt correction was applied using GAPDH as a housekeeping gene and units are relative to the expression of control group) ($n = 8$ samples per group). **b** Protein expression for TNF-α, IL-6, IL-8, MCP-1 and TGF-β ($n = 4$ samples per group). Data are expressed mean ± SEM. *$p \leq 0.05$ vs control groups; #$p \leq 0.05$ vs LPS group **c** Immunofluorescence for NF-kß, IRAK-1, MyD88 and Smad2/3 and all the treatments are shown. Magnification is 400x. (ND: non-detectable; LPS: Lipopolysaccharide from Escherichia coli 055:B5 and HEP: unfractionated heparin)

Heparin reduces classical pro-inflammatory cytokines on hAM

The effect of heparin on LPS-injured hAM was assessed after 7 and 24 h by q-PCR. The response of macrophages was observed at 7 h (changes not found at 24 h, data not shown). No changes were observed in the expression of IRAK-1 and MyD88 in different treated hAM. LPS increased significantly the expression of the most common pro-inflammatory mediators of ARDS, IL-6 and TNF-α and heparin reduced them significantly. Moreover, no changes in the expression of monocytes recruitment and neutrophils chemokines were measured. No changes were found in the expression of TGF-β or its effectors (Smad2 and Smad3) in the different hAM groups (Fig. 2a). TNF-α and IL-6 protein expression was also increased by the LPS and after the treatment with heparin was significantly reduced (Fig. 2b). In Fig. 2c we can observe different stainings for NF-kß, IRAK-1, MyD88 and Smad2/3; no differences with the different treatments were perceived.

Heparin inhibits NF-kß pathway on hATII cells

The gene expression produced by hATII cells cultured with LPS and heparin were measured 24 h after heparin administration (no changes were observed at 7 h, results not shown). IRAK-1 and MyD88 levels were increased when cells were injured with LPS, and the addition of heparin diminished their expression significantly. Furthermore, higher levels of IL-6, MCP-1 and IL-8 were observed in the LPS group and heparin reversed those increases significantly. TGF-β, Smad2 and Smad3 did not present any changes when hATII cells were injured by LPS or treated with heparin (Fig. 3a). These mRNA expression results were confirmed by the protein expression. hATII increased their expression of IL-6 and IL-8 after LPS treatment and heparin was able to reduced significantly the levels of this both cytokines (Fig. 3b). However, the effect in MCP-1 observed in mRNA could not be confirmed at protein level. In Fig. 3c, it could be noticed an increase in the labelling for NF-kß, IRAK-1, MyD88 in the LPS treated group compared to the other groups. In the group LPS + HEP we cannot observe any difference compared to control.

Heparin does not affect NF-kß nor TGF-β pathways on hF

The effect of heparin on LPS-injured hF was examined 24 h after heparin treatment (no changes were observed at 7 h, results not shown). No changes were observed in the expression of IRAK-1 and MyD88 in the different hF groups. An observable increase of IL-6, MCP-1 and IL-8 was observed in the LPS group and reduced by heparin in the case of IL-6 and MCP-1, although the decrease was not significant. No changes were found in the expression of TGF-β or its effectors in the different hF treated groups (Fig. 4a). In protein expression, TNF-α was increased after treatment with LPS, however no other changes were observed in the effectors evaluated (Fig. 4b). In Fig. 4c no labelling for any antibody was observed.

Apoptosis and Necrosis are not affected by heparin

Annexin V/PI assay was performed to detect apoptotic and necrotic cells after LPS injury and heparin treatment. There was no evident increase in apoptosis or necrosis in the LPS-treated group in any of the cell populations studied. Heparin did not produce any change in apoptosis or necrosis (Fig. 5).

Discussion

In the present study, we demonstrated the specific anti-inflammatory and immunomodulatory effect of heparin in various cell lung populations. We studied primary human macrophages (hAM), alveolar type II cells (hATII) and fibroblasts (hF). Few studies are now focused in the evaluation of infection and inflammation response in specific cell populations. The mechanisms underlying the effects of heparin on the inflammatory response are not fully understood, which is why we decided to study the individual response of human cell lung populations stimulated by LPS after heparin treatment in vitro. In this study we determined that heparin regulates inflammation through the NF-kß pathway. Moreover, all of the previous studies are performed in cell lines and not in primary cells, which is less useful as cell line response is less translational to in vivo results than primary cell response.

Heparin, a natural anticoagulant, is synthesized by various cells, including mast cells [18]. Heparin is broadly applied in the clinics as an anticoagulant drug [21, 22]. Several studies showed controversial results about the anti-inflammatory effects of heparin in in-vivo and in-vitro LPS-induced ALI models [10, 18, 23–26].

When infectious bacteria invade the lung, they activate inflammation that leads to cytokine release and endothelial

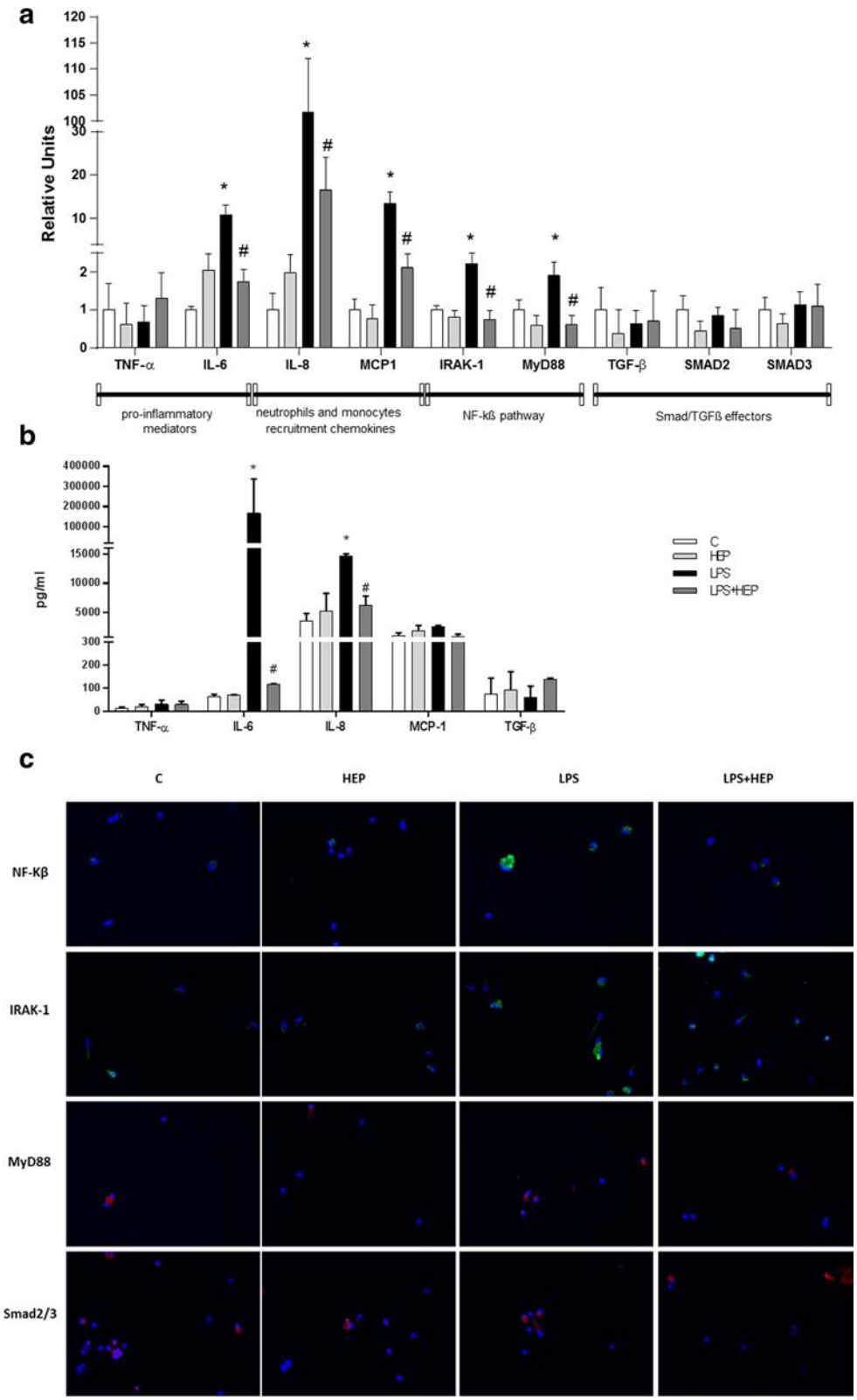

Fig. 3 (See legend on next page.)

(See figure on previous page.)
Fig. 3 Human alveolar type II cells gene expression. **a** Expression of TNF-α, IL-6, IL-8, MCP-1, IRAK-1, MyD88, TGF-β, Smad2 and Smad3 evaluated by q-PCR at 24 h after LPS treatment. Data are expressed mean ± SEM (ΔCt correction was applied using GAPDH as a housekeeping gene and units are relative to the expression of control group) ($n = 8$ samples per group). **b** Protein expression for TNF-α, IL-6, IL-8, MCP-1 and TGF-β ($n = 4$ samples per group). Data are expressed mean ± SEM. group **c** Immunofluorescence for NF-kß, IRAK-1, MyD88 and Smad2/3 and all the treatments are shown. Magnification is 400x.*$p \leq 0.05$ vs control groups; #$p \leq 0.05$ vs LPS group (LPS: Lipopolysaccharide from Escherichia coli 055:B5 and HEP: unfractionated heparin)

activation and dysfunction [27]. LPS is a potent inductor of pro-inflammatory factors such as TNF-α and IL-1β as well as IL-8 and CXCL family chemokines that reproduce the effect of an infection [28]. TNF-α is one of the first inflammatory factors released in the inflammatory process, and it plays a key role in the network of inflammatory mediators. There is a significant body of evidence that concentration of TNF-α and IL-6 is increased in BALF of patients with ARDS [29], and the persistent elevation of pro-inflammatory cytokines has been associated with a worse outcome in patients with ARDS or sepsis. However, the inflammatory response is a multi-step process involving different cytokines and chemokines released from numerous and different cells at different time-points.

It is remarkable that macrophages are the first activated cells when an infection happens [30]. Our results showed that heparin effectively inhibits slightly LPS-induced TNF-α, and IL-6 mRNA and also protein expression (Fig. 2) in macrophages suggesting that heparin inhibits the induction of this pro-inflammatory cytokines. These findings suggest that heparin exerts its anti-inflammatory effects by suppressing the production of cytokines and chemokines swiftly in macrophages. The production and release of cytokines occurs more rapidly with macrophages as compared to alveolar cells and fibroblasts, so, small changes in the production of proinflammatory cytokines by these cells could also induce a huge inhibition of inflammation in the lung. So, the quick de-activation of macrophages would be useful to control the classical pro-inflammatory cascade activation.

In case LPS is not rapidly eliminated, other cell types such as alveolar and fibroblasts cells will be activated, which increases inflammation. In addition to classical cytokines, further pathways will be either stimulated, amplifying the inflammatory response. Our findings demonstrate that heparin is able to interrupt the expression of IL-6, MCP-1 and IL-8 by alveolar epithelial cells and also reduce the protein expression in IL-6 and IL-8. MCP-1 is the most important chemokine that regulates migration and infiltration of monocytes and is used for the prediction of prognosis in some diseases as sepsis [31]. IL-8 is a neutrophil chemotactic factor and induces the migration of neutrophils to the site of infection. However the decrease of the expression of MCP-1 protein is not significantly reduced by heparin at this time point. It is known that MCP-1 is regulated and produced after IL-8 secretion, so it could be that at later points a reduction of MCP-1 protein expression might be observed.

It has previously been described that heparin treatment significantly reduces the expression of pro-inflammatory markers, such as TNF-α and IL-1β in lungs of rats with lung injury. This effect was found to be mediated by the inhibition of NF-κB nuclear translocation in the lung. In agreement with these previous observations, we propose that the diminution of the expression of both factors demonstrates an interrupted activation of NF-κB signalling pathway [18]; which is involved directly in the regulation of MCP-1, IL-6 and IL-8. Our results showed correspondingly that the expression of IRAK-1 and MyD88 by the alveolar Type II cells decrease after heparin treatment. MyD88 plays an active role in the phosphorylation and activation of IRAK-1. When IRAK-1's kinase activity is induced, autophosphorylation occurs and leads to a conformational change that strongly leads to the downstream activation of NF-kß pathway. Here, we show that heparin specifically blocks this pathway in alveolar type II cells, which produce high amounts of MCP-1 and IL-8. Heparin has no effect on IRAK-1 or MyD88 in fibroblasts or macrophages; consequently, NF-kß is not de-activated. The non-regulation of MCP-1 and IL-8 is driven through this pathway.

In our studies, fibroblasts presented higher levels of TNF-α protein at the LPS group, although heparin did not exert any change to this cellular type. Furthermore, any significant change due to the LPS or the heparin treatment was assessed in the other markers evaluated. Lung fibroblasts are usually not taking part in the response to an infection and the secretion of LPS and it seems that the effect of heparin in this cells is not important at this concentrations.

The main limitation of our study is that we checked mRNA levels and the determination of protein expression was difficult due to the lower amounts of cells that we obtained from the human biopsies. It would be interesting the effects on IRAK phosphorylation's and of some effectors of NF-kß pathway. However, we were not able to perform western blots because the quantity of protein was not enough to perform this technique. So, we quantified some secreted cytokines effectors of NF-kß and Smad/TGFß pathways in media, and the results fitted to the mRNA results. Also, we did several immunostainigs of some NF-kß and Smad/TGFß mediators

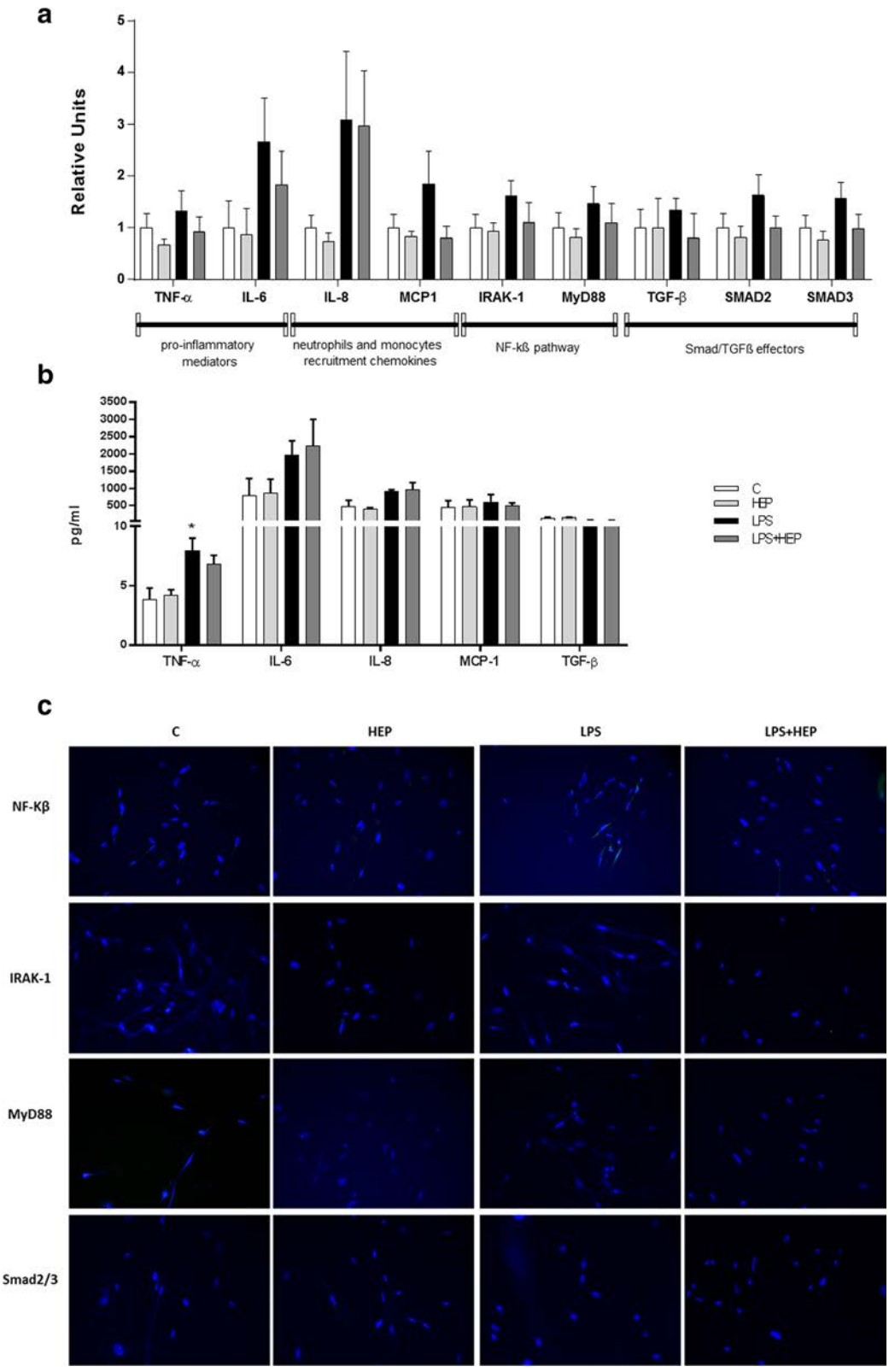

Fig. 4 (See legend on next page.)

(See figure on previous page.)
Fig. 4 Human fibroblasts gene expression. **a** Expression of TNF-α, IL-6, IL-8, MCP-1, IRAK-1, MyD88, TGF-β, Smad2 and Smad3 evaluated by q-PCR at 24 h after LPS treatment. Data are expressed mean ± SEM (ΔCt correction was applied using GAPDH as a housekeeping gene and units are relative to the expression of control group) ($n = 8$ samples per group). **b** Protein expression for TNF-α, IL-6, IL-8, MCP-1 and TGF-β ($n = 4$ samples per group). Data are expressed mean ± SEM group **c** Immunofluorescence for NF-kß, IRAK-1, MyD88 and Smad2/3 and all the treatments are shown. Magnification is 400x. *$p \leq 0.05$ vs control groups; #$p \leq 0.05$ vs LPS group (LPS: Lipopolysaccharide from Escherichia coli 055:B5 and HEP: unfractionated heparin)

that help to consider in the mRNa results. Previous studies have also reported that heparin ameliorated the lung injury induced by LPS in rats via inhibition of the TGF-β/Smad pathway [32]. We were unable to verify this claim. The TGF-β/Smad pathway is upregulated in fibroblasts after treatment with LPS and down-regulated after heparin administration, but these trends were not significant. The involvement of other cell types such as endothelial cells or myofibroblasts could explain the changes in TGF-β/Smad pathway in vivo. Further studies should be performed to confirm this.

We also investigated the apoptotic and necrotic processes that might be involved. LPS stimulation at the used concentrations and at 7 h or 24 h did not induce apoptosis in our human primary cells [33]. It is broadly demonstrated that LPS induces apoptosis in in-vivo lung injury models at late phases [33, 34], however we are just working in early stages of the damage and apoptosis or necrosis are not involved at the intervals we studied. Heparin had no effect in apoptosis nor on necrosis.

In recent years, the number of studies evaluating macrophage activation has increased. This phenomenon is often explored in acute lung injury models. We demonstrated that the role of macrophages is certainly important in the first steps of the inflammatory response; however, alveolar type II cells have more relevance in the maintenance

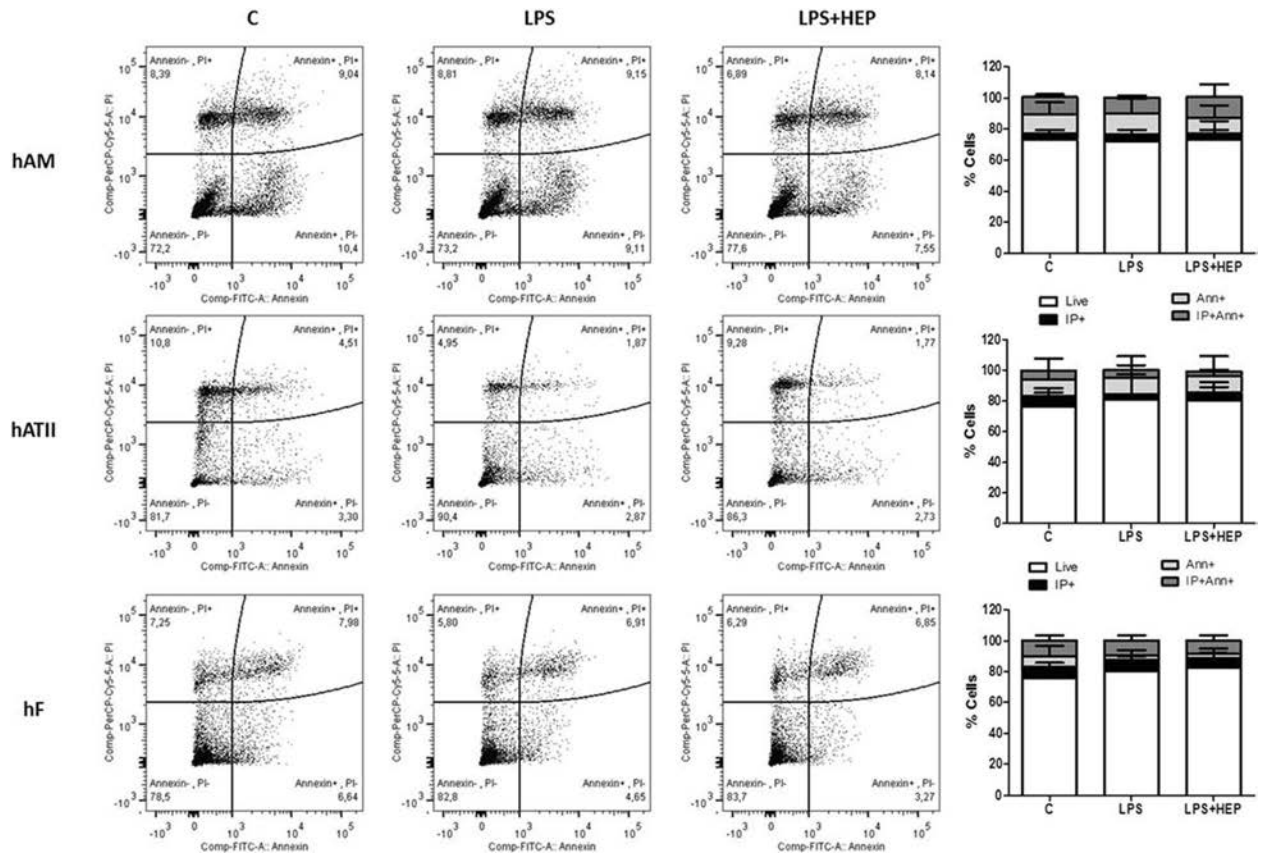

Fig. 5 hAM, hATII cells and hF apoptosis/necrosis measured by Annexin V and PI staining with flow cytometry. The proportion of live cells (Annexin V-FITC-/PI-), early apoptotic cells (Annexin V-FITC+/PI-), necrotic cells (Annexin V-FITC-/PI+), late apoptotic/necrotic cells (Annexin V-FITC+/PI+). No changes were observed in LPS treated samples or in LPS + Hep treated ones (hAM:human alveolar macrophages; hATII: human alveolar type II cells; hF: human fibroblasts; FITC: Fluorescein; PI: propidium iodide; LPS: Lipopolysaccharide from Escherichia coli 055:B5 and HEP: unfractionated heparin)

of this response because of NF-kß activation. Additionally, hATII cells might be the responsible in the recruitment of new pro-inflammatory cells, so therapies addressed to regulate these processes may target hATII cells. Nevertheless, in-vivo the crosstalk with different cells could modify the effects found in vitro. New studies with human primary cells and co-cultures evaluating the specific response to infection and inflammation and the crosstalk between cells are necessary to increase the knowledge and improve the targets for new therapies.

Conclusions

In conclusion, the current study demonstrated that heparin significantly ameliorated the cells lung damage induced by LPS through the inhibition of pro-inflammatory cytokine expression in macrophages and blocking NF-kß pathway in alveolar cells, consequently reducing the production of some of this pathway effectors such as IL-8 and IL-6. Our results suggested that a local pulmonary administration of heparin through nebulization may be able to reduce inflammation in the lung; however, further studies are needed to confirm this hypothesis.

Abbreviations

ALI: Acute lung injury; ARDS: Acute respiratory distress syndrome; BAL: Broncho-alveolar lavage; FBS: Fetal bovine solution; hAM: Human alveolar macrophages; hATII: Alveolar type II cells; HBSS: Hanks Balanced Salt solution; hF: Fibroblasts; LPS: Lipopolysaccharide from Escherichia coli 055:B5; UFH: Unfractionated heparin

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

MI and CO provided the human biopsies; MC-R and RG-P designed the experiments; MC-R, JB and JT contributed to cell cultures; MC-R, TL and RG-P carried out the experiments; MC-R, RG-P, LC and AA interpreted the data; MC-R, RG-P, JB and AA wrote the manuscript and LB and AA helped to revise the manuscript. All the authors approved the last version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All participants gave their informed consent to participate on this study. The study was reviewed and approved by our hospital Ethic Committee.

Publisher's Note

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3.2 Nebulized Heparin Attenuates Pulmonary Coagulopathy and Inflammation through Alveolar Macrophages in a Rat Model of Acute Lung Injury

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*these authors co-share the first author position and contribute equally to the work

Nebulized Heparin Attenuates Pulmonary Coagulopathy and Inflammation through Alveolar Macrophages in a Rat Model of Acute Lung Injury

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Abstract

Objective Alveolar macrophages play a key role in the development and resolution of acute respiratory distress syndrome (ARDS), modulating the inflammatory response and the coagulation cascade in lungs. Anti-coagulants may be helpful in the treatment of ARDS. This study investigated the effects of nebulized heparin on the role of alveolar macrophages in limiting lung coagulation and inflammatory response in an animal model of acute lung injury (ALI).

Methods Rats were randomized to four experimental groups. In three groups, ALI was induced by intratracheal instillation of lipopolysaccharide (LPS) and heparin was nebulized at constant oxygen flow: the LPS/Hep group received nebulized heparin 4 and 8 hours after injury; the Hep/LPS/Hep group received nebulized heparin 30 minutes before and 4 and 8 hours after LPS-induced injury; the LPS/Sal group received nebulized saline 4 and 8 hours after injury. The control group received only saline. Animals were exsanguinated 24 hours after LPS instillation. Lung tissue, bronchoalveolar lavage fluid (BALF) and alveolar macrophages isolated from BALF were analysed.

Results LPS increased protein concentration, oedema and neutrophils in BALF as well as procoagulant and proinflammatory mediators in lung tissue and alveolar macrophages. In lung tissue, nebulized heparin attenuated ALI through decreasing procoagulant (tissue factor, thrombin–anti-thrombin complexes, fibrin degradation products) and proinflammatory (interleukin 6, tumour necrosis factor alpha) pathways. In alveolar macrophages, nebulized heparin reduced expression of procoagulant genes and the effectors of transforming growth factor beta (Smad 2, Smad 3) and nuclear factor kappa B (p-selectin, CCL-2). Pre-treatment resulted in more pronounced attenuation.

Conclusion Nebulized heparin reduced pulmonary coagulopathy and inflammation without producing systemic bleeding, partly by modulating alveolar macrophages.

Keywords
- acute respiratory distress syndrome
- acute lung injury
- anti-coagulants
- heparin
- alveolar macrophages

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Introduction

Acute respiratory distress syndrome (ARDS) is a major cause of morbidity and mortality (30–40%) in critically ill patients.[1,2] Defined by non–heart-failure-related acute respiratory failure and inflammation, ARDS can arise from various local and systemic insults.[3] It is characterized by bilateral pulmonary infiltrates, increased endothelial permeability and oedema.[4] Although lung protective ventilation strategy and prone position have produced a major breakthrough in supportive care of ARDS patients, an effective pharmacological therapy for ARDS is not available yet.

Even though neutrophil influx and activation within the lungs contribute to the induction of ARDS, increasing evidence suggests that alveolar macrophages are critical to the initiation and maintenance of the inflammatory response and to the resolution phase.[5,6] More specifically, lung inflammation during ARDS is deeply correlated to the alveolar macrophages phenotype, function and cell–cell interactions.[6,7] Due to their plasticity, macrophages can be proinflammatory (M1) or anti-inflammatory activated (M2) depending on environmental signals. At the initial acute phase of ARDS, classically activated macrophages (M1) release early response cytokines such as tumour necrosis factor alpha (TNF-α) or inducible nitric oxide synthase (iNOS), stimulating the cells of the alveoli and recruiting neutrophils to the alveolar space, amplifying the inflammatory response and promoting the elimination of pathogens. At the resolution phase, alternative macrophages (M2) are activated releasing anti-inflammatory cytokines such as interleukin 10 (IL-10) or arginase 1 and promoting tissue remodeling.[5,7]

ARDS is also associated with pulmonary activation of coagulation mediated by the tissue factor (TF) pathway. Exposure to proinflammatory cytokines causes alveolar macrophages and alveolar epithelial cells to produce TF, the key mediator of coagulation in severe infections.[8] Pulmonary coagulation is evident in increased markers of thrombin generation, soluble TF and factor VIIa activity found in bronchoalveolar lavage fluid (BALF) from ARDS patients, together with increased release of plasminogen activator inhibitor-1 (PAI-1) resulting in decreased fibrinolytic activity.[9–11]

Previous findings indicate that anti-coagulants may help restore the coagulation cascade and treat ARDS; however, in experimental models of acute lung injury (ALI) and in ARDS patients, the beneficial effects of systemic anti-coagulants were outweighed by systemic bleeding.[12–18] Local administration of nebulized anti-coagulants to the lungs might reduce the risk of systemic bleeding and might be more effective than intravenous administration.[19] Preclinical studies with animal models of direct and indirect ALI have found that local administration of nebulized heparin improved pulmonary coagulopathy.[20] Intravenous anti-thrombin combined with nebulized heparin and tissue plasminogen activator restored gas exchange but not inflammation in a model of burn and smoke inhalation injured sheep.[21] In a phase I trial of nebulized heparin to ARDS patients, the activation of pulmonary coagulation was reduced without producing systemic bleeding.[22,23] In addition, nebulized heparin decreased the duration of mechanical ventilation in burn inhalation injured patients.[24]

Besides its anti-coagulant effects, intravenously administered heparin showed anti-inflammatory effects, ameliorating lipopolysaccharide (LPS)-induced lung injury in rats via the inhibition of the nitric oxide synthase expression and transforming growth factor beta (TGF-β)/Smad pathway.[25] Heparin was also found to inhibit the nuclear factor kappa B (NF-κB) pathway in monocytes treated with LPS.[26,27] Furthermore, data recently obtained by our group showed that after LPS injury in human alveolar macrophages heparin limited the expression of TNF-α and IL-6, while in human alveolar type II cells heparin inhibited the NF-κB pathway and their effectors IL-6, MCP-1 and IL-8.[28]

Since alveolar macrophages play an important role in the development and resolution of ARDS, modulating the inflammatory response and the coagulation cascade in lungs, and heparin exhibits both anti-inflammatory and anti-coagulant properties, the hypothesis of this work was that nebulized heparin could attenuate ARDS through the involvement of alveolar macrophages. Accordingly, the current study aimed to assess the effects of nebulized heparin in a rat model of ALI induced by intratracheal instillation of LPS with special regard to the role that alveolar macrophages might have in limiting the coagulation and the inflammatory response. More in details, we postulated that: (1) in lungs, nebulized heparin decreases procoagulant markers and inflammation in terms of lung neutrophil influx, oedema, proinflammatory cytokines and histopathology; (2) in alveolar macrophages, nebulized heparin reduces the effectors of TGF-β and NF-κB pathways and the expression of procoagulant genes.

Materials and Methods

Animals

We studied 64 pathogen-free male Sprague-Dawley rats (8 weeks old; 250–300 g; Charles River, Chatillon-sur-Chalaronne, France) housed in 12-hour light–dark-cycle, air-conditioned (23°C and 60% relative humidity) quarters with free access to standard food pellets (A04; Panlab, Barcelona, Spain) and tap water. The Animal Research Ethics Committee of the Autonomous University of Barcelona (UAB) approved the study.

Experimental Design

Rats were sedated with sevoflurane and randomized to four experimental groups (16 animals/group). ►**Fig. 1** illustrates the experimental design. ALI was induced by intratracheal instillation of LPS (*Escherichia coli* 055: B5, 10 μg/g body weight) (Sigma Chemical, St. Louis, MO).[29] Heparin (Vister, Parke-Davis, Linate, Milan, Italy) was nebulized through Aeroneb system (Philips Healthcare) at constant oxygen flow (2 L/min). Rats in the LPS/Hep group received two doses of 1,000 IU/kg nebulized heparin, administered 4 and 8 hours after LPS instillation. Rats in the Hep/LPS/Hep group received three doses of nebulized heparin: one dose 30 minutes before LPS instillation and one dose 4 and 8 hours after LPS instillation. Rats in the LPS/saline group received nebulized saline solution (0.9% NaCl) 4 and 8 hours after LPS instillation. Rats in the control group received only saline solution, by tracheal instillation at the time of ALI induction in the other animals and by nebulizer 4 and 8 hours

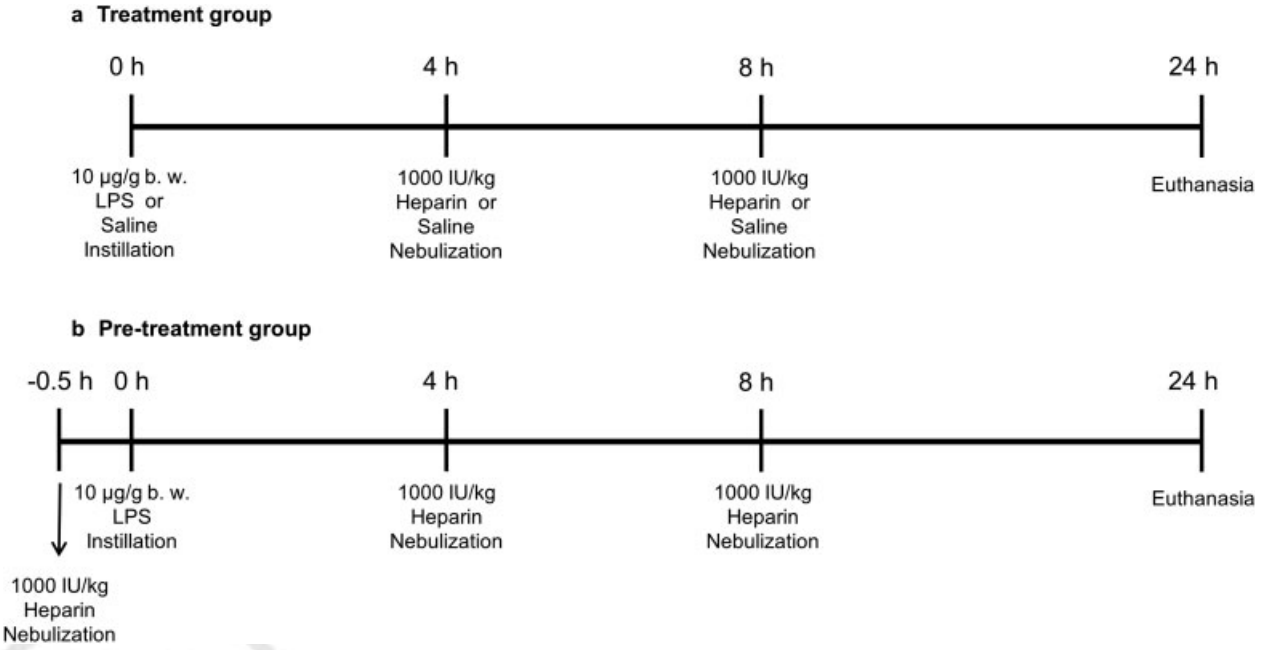

Fig.1 **Experimental design.** (**a**) Treatment group and (**b**) pre-treatment group.

afterwards. Animals were anesthetized (90 mg/kg ketamine and 10 mg/kg xylazine) and exsanguinated 24 hours after LPS instillation. BALF, lung tissue and blood were collected for further analyses; BALF and lung homogenate were obtained from eight animals in each group, and the lungs for histological examination and lung wet/dry weight ratio were obtained from the remaining eight animals in each group.

Obtaining and Processing Bronchoalveolar Lavage Fluid

The left main bronchus was tied with a string at the hilum. BALF from all animal groups was obtained from the right lung by connecting a syringe to the cannula placed in the trachea and then gently flushing through it 5 mL sterile 0.9% NaCl solution with 1-mM Ethylenediaminetetraacetic acid (EDTA) five times. BALF volume recovery was always greater than 85%. BALF was spun at 800 × g for 10 minutes and the supernatant was stored at –80°C for subsequent analysis. Cells were counted using a haemocytometer (Neubauer, Marienfeld, Lauda-Königshofen, Germany), and slides were prepared by cytocentrifugation (Shandon Cytospin 4, Thermo Electron Corporation, Marietta, OH) and Diff-Quick staining (Panreac Quimica SAU; Castellar del Vallès, Spain). For each rat, approximately 500 cells were counted.

Lung Wet/Dry Weight Ratio

To assess oedema, the left lung was dissected immediately after exsanguination and the wet weight was recorded. The lung was then placed in an incubator at 80°C for 48 hours, the dry weight recorded and the lung wet-to-dry weight ratio was measured.

Histological Examination

The right lungs were removed and fixed in 4% paraformaldehyde. Two independent experts blinded to treatment analysed 4-µm sections excised of lung stained with haematoxylin and eosin (H&E). The entire surface of the lung was analysed for inflammation and damage, and was scored as follows: normal lung (0), haemorrhage (on a 0–1 scale), peribronchial infiltration (on a 0–1 scale), interstitial oedema (on a 0–2 scale), pneumocyte hyperplasia (on a 0–3 scale) and intra-alveolar infiltration (on a 0–3 scale).

Cytokine and Protein Measurements

Total protein concentration in BALF was quantified using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). IL-6, GRO-κC, TNF-α and IL-10 in lung homogenate were determined by multiplex assay following the manufacturer's protocol (Luminex, Merck Millipore, Darmstadt, Germany). PAI-1 in lung homogenate was determined by uniplex assay (Luminex, Merck Millipore, Darmstadt, Germany). Levels of TF, fibrin degradation products (FDPs), and thrombin-antithrombin complexes (TATc) in lung homogenate were measured by enzyme-linked immunosorbent assay (USCN Life Sciences, Hubei, China).

Activated Partial Thromboplastin Time

Blood (0.3 mL) was collected from the abdominal aorta in tubes containing 11-mM sodium citrate 24 hours after LPS instillation. Activated partial thromboplastin time (aPTT) was measured according to standard protocols (Echevarne Laboratories, Spain).

Isolation of Alveolar Macrophages

BALF pellet from all animal groups was seeded in Petri dishes with RPMI 1640 Medium supplemented with 10% foetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco, Langley, OK), for 1 hour at 37°C. The supernatant was discarded and purified attached alveolar macrophages were cryopreserved in 500 µL of TRIzol reagent (Ambion, Thermo Fisher Scientific, Madrid, Spain).

Alveolar macrophages' purity was assessed by Diff-Quick staining and immunofluorescence. To perform immunofluorescence, cells were fixed in 4% paraformaldehyde and incubated for 2 hours in a blocking solution (3% FBS and 1% bovine serum albumin in phosphate buffered saline [PBS]). Cells were then incubated overnight with a mouse anti-rat CD68 antibody (1:100) (Acris Antibodies, Rockville, MD), washed with PBS1X and incubated at 37°C for 1 hour with a goat anti-mouse IgG-FITC antibody (1:500) (Santa Cruz Biotechnology, Dallas, TX). Cells were finally washed with PBS 1X and incubated 5 minutes with HOECHST (1:1,000) (Invitrogen, Waltham, MA).

RNA Extraction and Real-Time PCR

Total RNA was extracted from isolated alveolar macrophages using chloroform, isopropanol and ethanol. The optical density at 260 nm and the ratio 260 nm/280 nm were measured to determine the RNA purity (spectrophotometer ND-1000, Nanodrop, Thermo Fisher Scientific, Wilmington, DE). Total RNA was reverse-transcribed into cDNA with a reverse transcriptase core kit (Eurogentec, Seraing, Belgium), using an Alpho-SC (Analytik Jena AG, Jena, Germany) thermocycler. DNA was amplified in a real-time polymerase chain reaction (PCR) system (7500 Real-Time PCR System, Applied Biosystems, Thermo Fisher Scientific, Madrid, Spain) using SYBR green (Kapa Biosystems, Cultek, Mataró, Spain) and the corresponding rat primers (►**Table 1**). Data were corrected by ΔΔCt method and shown as target gene expression relative to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fold over saline group.

Statistical Analysis

Before starting the experiments, a power analysis was performed using the Gpower 3 program. The analysis indicated that 44 animals were needed to detect large effects (0.5) with 85% power using an analysis of variance (ANOVA) test between factors with α at 0.05. We used 20 extra animals to perform further investigational assessments. One-way ANOVA with Fisher's protected least-squares differences (PLSD) test as post hoc analysis was used for multigroup comparisons (StatView 5.0.1; Abacus Concept, Berkeley, CA). Results are reported as mean ± SEM. Statistical significance was set at $p < 0.05$.

Results

Cellular and Histological Response

Compared with control rats, animals in the LPS-instilled groups had higher total neutrophil counts in BALF; nebulized heparin limited the increase in neutrophils (►**Fig. 2a**). There were no major differences in total macrophage counts among groups (►**Fig. 2b**). Compared with the LPS/saline group, only heparin pre-treatment led to a decrease in the total number of cells in BALF (Sal/Sal: 16.8 ± 7.7, LPS/Sal: 45.7 ± 5.7, LPS/Hep: 40.8 ± 6.8, Hep/LPS/Hep: 31.1 ± 3.3; $p < 0.05$ vs. LPS/Sal group). The total concentrations of BALF proteins (►**Fig. 2d**) and the wet-to-dry ratio (►**Fig. 2c**) were significantly greater in LPS-instilled animals than in control animals, suggesting increased permeability of the alveolocapillary barrier. Nebulized heparin markedly reduced total BALF proteins and the wet-to-dry ratio. Histological analysis of lung tissue detected evidence of lung injury (haemorrhage, interstitial oedema, peribronchial and intra-alveolar infiltration, and alveolar pneumocyte hyperplasia) in LPS-instilled rats; lung injury was considerably reduced only in the Hep/LPS/Hep rats (►**Fig. 3**).

Coagulation Effects

LPS instillation increased lung-tissue levels of TF, TATc, FDP and PAI-1 compared with control animals (►**Fig. 4a–d** respectively). Nebulized heparin reduced lung TF, but TATc and FDP levels decreased only in the Hep/LPS/Hep group. PAI-1 levels were not altered after heparin nebulization (►**Fig. 4d**). Heparin had no effects on systemic coagulation, as no changes were observed in the aPTT (data not shown).

Table 1 Rat primers

Gene	Forward primer	Reverse primer
GAPDH	5′ CTGTGCTTTCCGCTGTTTTC 3′	5′ TGTGCTGTGCTTATGGTCTCA 3′
TNF-α	5′ AACTCCCAGAAAAGCAAGCA 3′	5′ CGAGCAGGAATGAGAAGAGG 3′
iNOS	5′ CTTGGAGCGAGTTGTGGATT 3′	5′ GGTGGGAGGGGTAGTGATG 3′
IL-10	5′ CATCCGGGGTGACAATAA 3′	5′ TGTCCAGCTGGTCCTTCT 3′
Arginase-1	5′ GGGAAGACACCAGAGGAGGT 3′	5′ TGATGCCCCAGATGACTTTT 3′
TGF-β	5′ TGCTTCAGCTCCACAGAGAA 3′	5′ TGGTTGTAGAGGGCAAGGAC 3′
Smad 2	5′ ACTCGTGGGGGAAGAAAAGT 3′	5′ CATGCTGCACTGCTTTGAAT 3′
Smad 3	5′ GACCAGGCATTTTGAGGAAA 3′	5′ AGACCACAGCACCCCATAAG 3′
IRAK1	5′ TACAAAGTGATGGACGCCCT 3′	5′ GGTGCCAGGCTGTAATGATG 3′
P-Selectin	5′ AGGTTGGCAATGGTTCACTC 3′	5′ ACCATTGGGAGCTACACCTG 3′
CCL-2	5′ GCTGCTACTCATTCACTGGC 3′	5′ GGTGCTGAAGTCCTTAGGGT 3′
TF	5′ ACAATCTTGGAGTGGCAACC 3′	5′ TGGGACAGATAGGACCCTTG 3′
PAI-1	5′ AGGGGCAGCAGATAGACAGA 3′	5′ CACAGGGAGACCCAGGATAA 3′
Plasminogen	5′ AAACGAAAGGGACTCCAGGT 3′	5′ TCTCGAAGCAAACCAGAGGT 3′

Note: Table of the primers used for the real-time polymerase chain reaction.

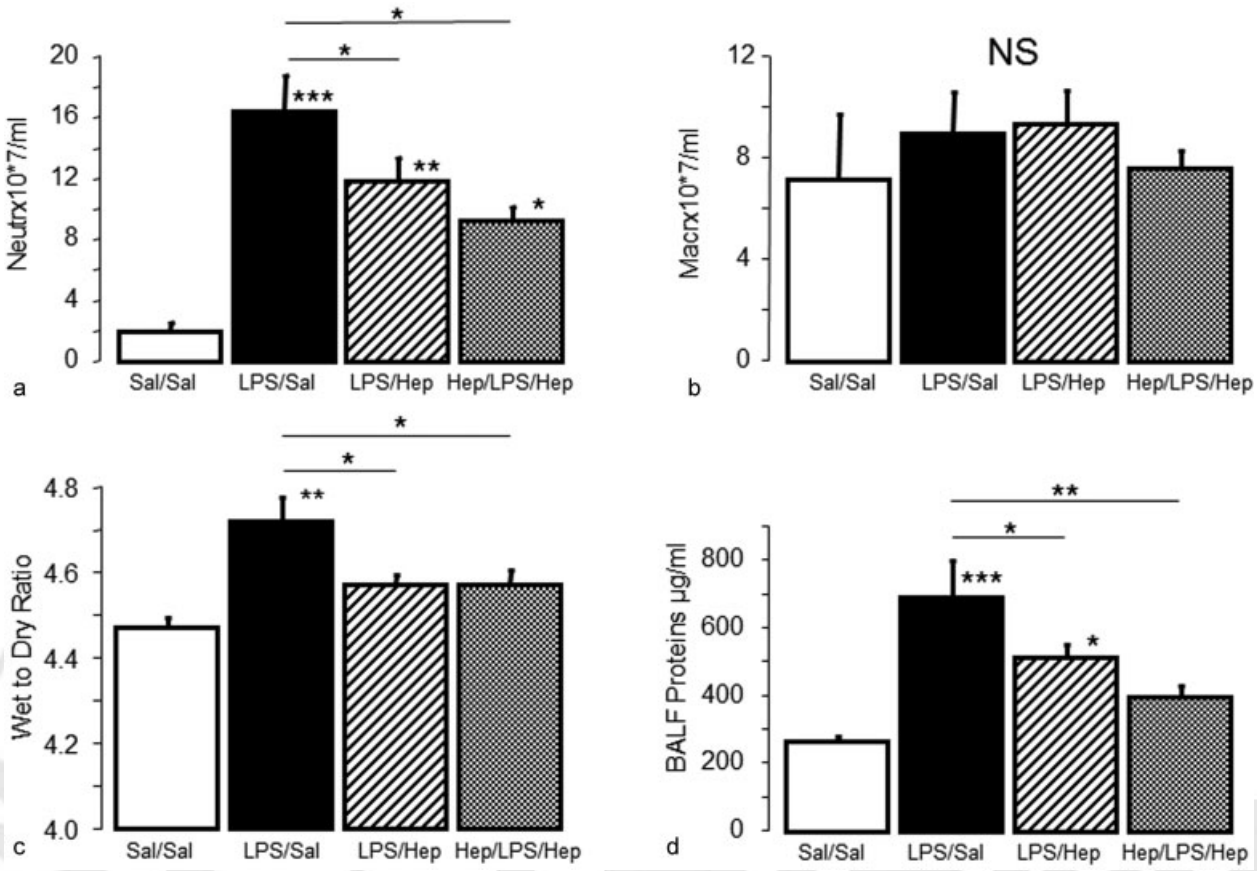

Fig. 2 Bronchoalveolar lavage analysis and wet/dry weight ratio. Absolute (**a**) neutrophil (PMN) and (**b**) alveolar macrophage (AM) cell counts in the bronchoalveolar lavage fluid of rats 24 hours after the induction of the injury. (**c**) Wet/dry weight ratio and (**d**) protein concentration in the bronchoalveolar lavage fluid. Data are presented as mean ± SEM. ANOVA followed by the post hoc Fisher's PLSD test were used. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

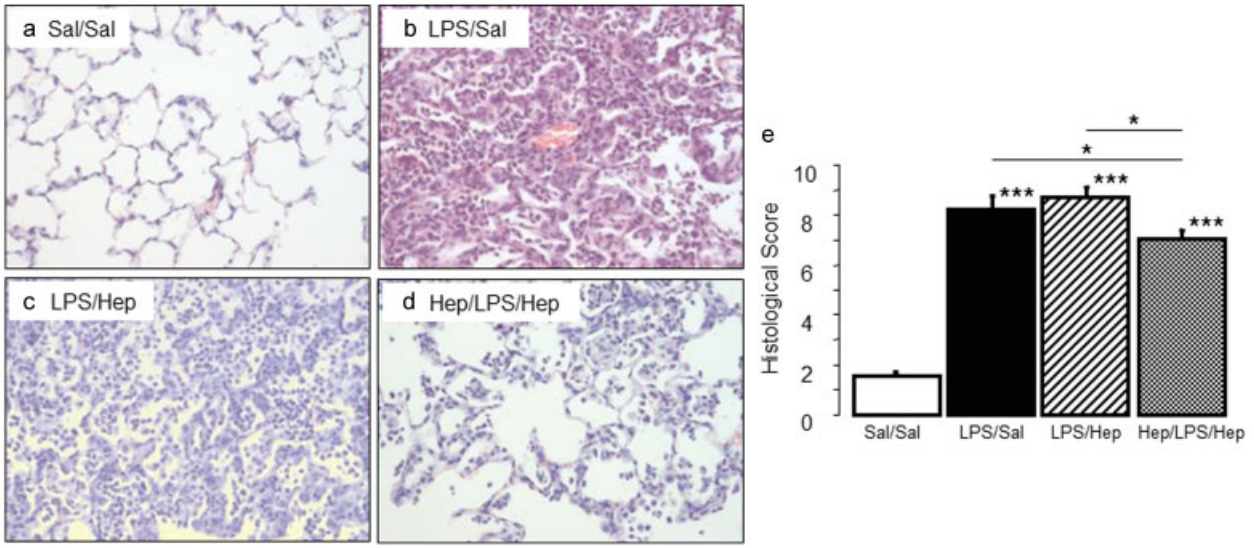

Fig. 3 Lung tissue analysis. (**a–d**) Representative images of haematoxylin and eosin staining lung tissue sections and (**e**) histological score in animals 24 hours after induction of the injury. Original magnification ×200. Data are presented as mean ± SEM. ANOVA followed by the post hoc Fisher's PLSD test were used. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

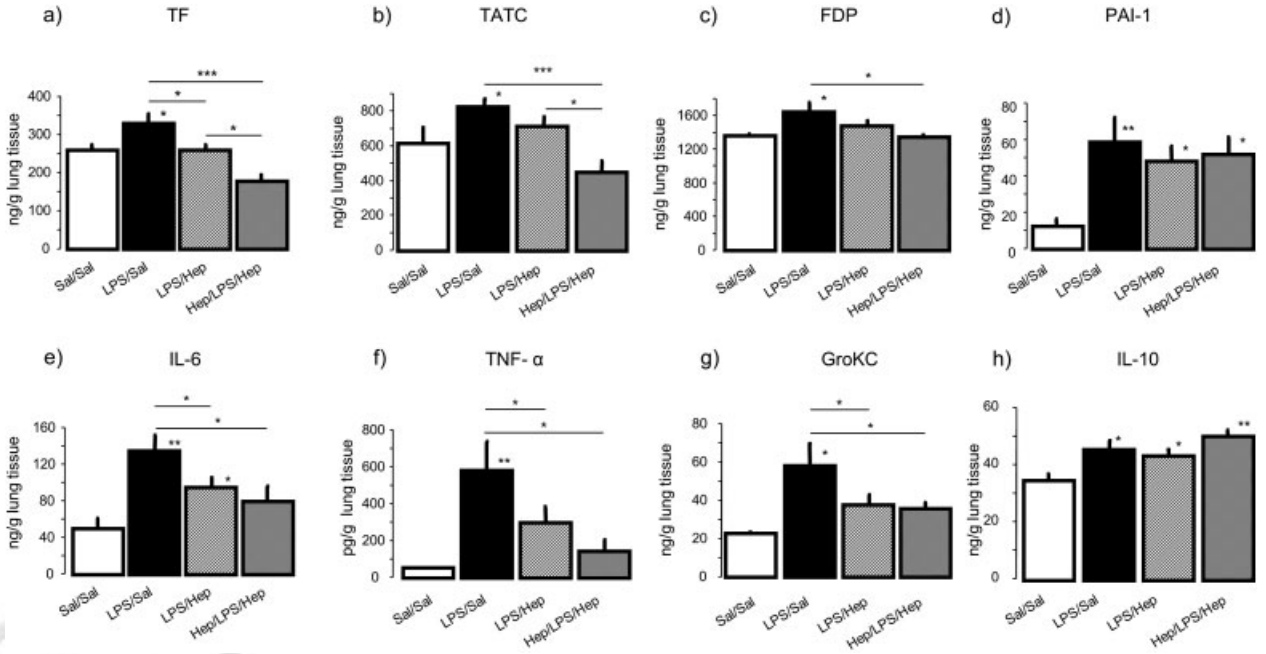

Fig. 4 Coagulant effectors and cytokines. (**a**) Tissue factor (TF), (**b**) thrombin–anti-thrombin complexes (TATc), (**c**) fibrin degradation products (FDPs), (**c**) plasminogen activator inhibitor type-1 activity (PAI-1), (**e**) IL-6, (**f**) TNF-α, (**g**) GRO-κC and (**h**) IL-10 concentrations were measured in lung homogenate of animals 24 hours after induction of the injury. Data are presented as mean ± SEM. ANOVA followed by the post hoc Fisher's PLSD test were used. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

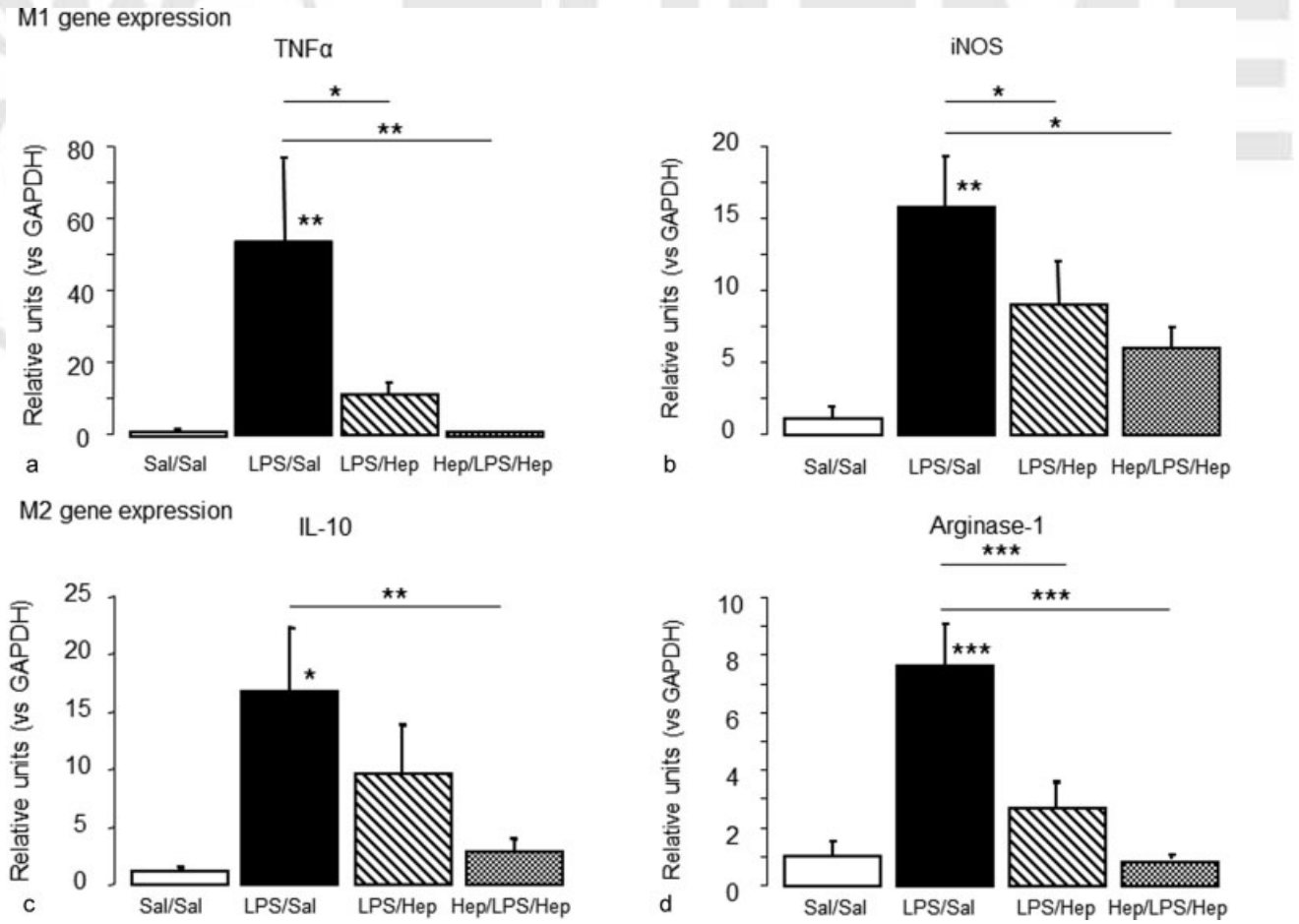

Fig. 5 Activation of alveolar macrophages. Gene expression of proinflammatory (M1): (**a**) TNF-α, (**b**) iNOS and alternative (M2), (**c**) IL-10, (**d**) arginase-1 mediators in alveolar macrophages isolated from BALF of animals 24 hours after induction of the injury. Data are presented as mean ± SEM. ANOVA followed by the post hoc Fisher's PLSD test were used. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

Inflammatory Response

In lung homogenates, concentrations of the proinflammatory cytokines IL-6, TNF-α and GRO-κC diminished in both groups of animals nebulized with heparin compared with the LPS group (►**Fig. 4e–g** respectively). Levels of IL-10 were higher in all the rat groups compared with control (►**Fig. 4h**).

Macrophage pathway

Alveolar macrophages were isolated from BALF (98% purity; **Supplementary Material 1**). ►**Fig. 5** reports the gene expression of M1 proinflammatory cytokines (TNF-α [►**Fig. 5a**] and iNOS [►**Fig. 5b**]) and M2 regulatory and reparative markers (IL-10 [►**Fig. 5c**] and arginase-1 [►**Fig. 5d**]) from alveolar macrophages. Nebulized heparin–deactivated alveolar macrophages stimulated with LPS; TNF-α, iNOS and arginase-1 expression significantly decreased in both LPS/Hep and Hep/LPS/Hep rats, but IL-10 expression significantly decreased only in the Hep/LPS/Hep group.

►**Fig. 6** reports the expression of genes involved in proinflammatory and coagulation pathways analysed in alveolar macrophages. LPS had no effect on TGF-β expression (►**Fig. 6a**) but increased the expression of Smad 3 (►**Fig. 6b**) and Smad 2 (►**Fig. 6c**). Nebulized heparin diminished the increase in Smad 3 expression in both groups, but diminished the increase in Smad 2 expression only in the Hep/LPS/Hep group. LPS increased the expression of IRAK1 (►**Fig. 6d**), p-selectin (►**Fig. 6e**) and CCL-2 (►**Fig. 6f**) in alveolar macrophages; CCL-2 and p-selectin expression were lower in the Hep/LPS/Hep group. LPS increased expression in alveolar macrophages of TF (►**Fig. 6g**), PAI-1 (►**Fig. 6h**) and plasminogen (►**Fig. 6i**), but only pre-treatment with nebulized heparin mitigated the increase in plasminogen expression.

Discussion

The pathogenesis of ARDS involves both proinflammatory and procoagulant mediators, and the breakdown of the epithelial and endothelial barrier results in pulmonary oedema and infiltration of neutrophils in the alveolar space. In this rat model of LPS-induced ALI, administrating nebulized heparin diminished recruitment of neutrophils into the lung and attenuated pulmonary coagulopathy and inflammation without producing systemic bleeding. Part of this positive effect of heparin might be ascribed to the alveolar macrophages, in which the expression of markers of TGF-β effectors, NF-κB and coagulation pathways was decreased after heparin nebulization.

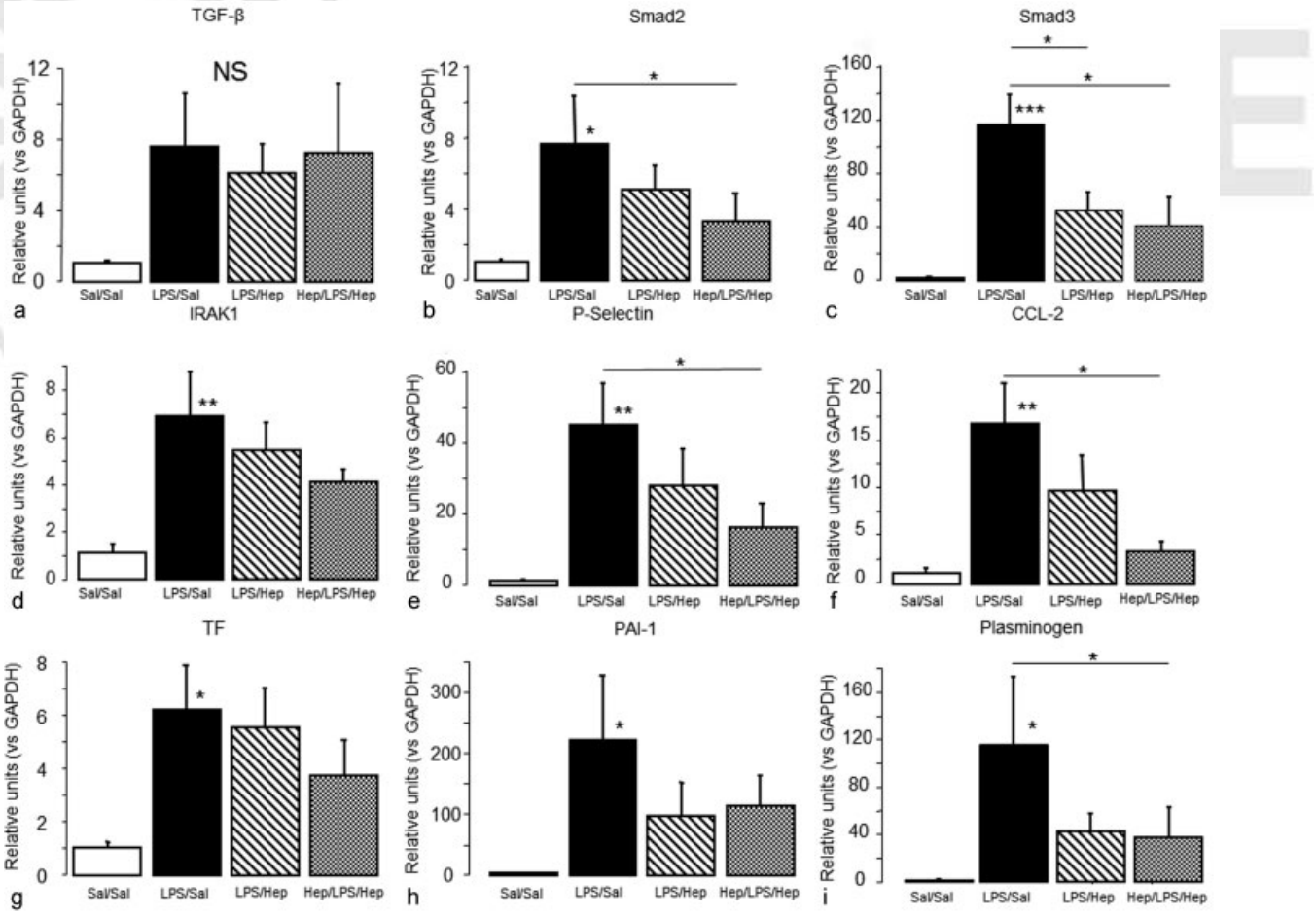

Fig. 6 Inflammatory and coagulation pathways of alveolar macrophages. Gene expression of TGF-β pathway: (**a**) TGF-β, (**b**) Smad 2 and (**c**) Smad 3, NF-κB pathway, (**d**) IRAK-1, (**e**) p-selectin and (**f**) CCL-2 and coagulation pathway, (**g**) TF, (**h**) PAI-1 and (**i**) plasminogen in alveolar macrophages isolated from BALF of animals 24 hours after induction of the injury. Data are presented as mean ± SEM. ANOVA followed by the post hoc Fisher's PLSD test were used. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

Administering anti-coagulants directly to the lungs allows higher dosages and increases local efficacy without producing systemic bleeding.[19] We tested the effects of two nebulized heparin treatment regimens. Rats in the LPS/Hep group received two doses after ALI induction, while rats in the Hep/LPS/Hep group received an additional dose before LPS. Although the prophylactic pre-treatment plus treatment (Hep/LPS/Hep) model can help elucidate mechanisms, the treatment (LPS/Hep) model better reflects the clinical situation. Heparin reduced the number of neutrophils and their recruitment in both groups. Furthermore, heparin diminished lung permeability, reducing the concentration of BALF proteins and oedema in both groups. However, only in the pre-treatment group, heparin decreased the number of total BALF cells and lung injury.

In the first stages of ALI/ARDS, proinflammatory mediators inhibit natural anti-coagulant factors and induce procoagulant activity.[18] In our ALI model, heparin nebulization decreased pulmonary coagulation and inflammation. Previous studies showed that nebulized heparin could reduce pulmonary coagulopathy in an animal model of endotoxemia[20] or pneumonia,[30] although heparin did not produce any changes on inflammation. This result could be attributed to the different timing and dosage of heparin. In Hofstra et al's experimental model,[20] heparin was given every 6 hours, while in our model the time interval between each heparin nebulization was 4 hours (heparin biological lifetime is ~1.5 hours), although we administrated fewer doses (three to the pre-treatment group and two to the treatment group).

Alveolar macrophages play important roles in both the development and resolution of ALI/ARDS.[5–7] In our ALI model, the expression of both proinflammatory (M1) and anti-inflammatory (M2) markers in alveolar macrophages increased after LPS administration, with a predominant activation of the proinflammatory M1 phenotype. Heparin attenuated the response of alveolar macrophages during ALI, reducing M1 (TNF-α, iNOS) and M2 (IL-10, arginase-1) markers to basal levels.

It has been shown that heparin anti-inflammatory effect could be produced by the inhibition of NF-κB nuclear translocation into the lung,[18,26] reducing the expression of TNF-α and IL-6.[25,28,31] This is consistent with our results. Nebulized heparin decreased proinflammatory cytokines in lung tissue and the expression of NF-κB effectors in alveolar macrophages. Moreover, some studies described that heparin ameliorated lung injury induced by LPS in rats via the inhibition of nitric oxide synthase expression and the TGF-β/Smad pathway.[25] In our model, heparin was also able to reduce the expression of TGF-β effectors in alveolar macrophages.

The expression of TF by inflammatory cells such as macrophages acts as one of the primary initiators of thrombosis.[32] Also, the release of TNF-α and IL-1β results in an increased TF expression. During ARDS, it is known that alveolar macrophages increase their PAI-1 activity, inhibiting fibrin degradation and promoting clots formation.[10] In our LPS model, higher levels of TF, TATc, PAI-1 and plasminogen were found, mimicking clinical ARDS.[10,33] Nebulized heparin decreased TF and TATc in lung tissue. It is known that TF expression on the monocytes surface induces their interaction with platelets and endothelial cells through the union of p-selectin.[34] Nebulized heparin significantly reduced p-selectin in alveolar macrophages. Furthermore, heparin was able to reduce plasminogen in alveolar macrophages, indicating that heparin may increase fibrinolysis through these cells. Accordingly, we ascertained that alveolar macrophages promote the deposition of thrombus and formation of clots, confirming that they are one of the main actors in the link between inflammation and coagulation.

This study has some limitations. First, LPS administration is a common ALI model that mimics human ARDS only in part, because this model cannot reflect the heterogeneous aetiology and management of ARDS. Second, the dose of heparin was chosen from previous studies based on the efficacy of the nose exposure system, the evaporative water loss during nebulization and the biological lifetime, and we cannot know whether lower doses would have similar effects. Third, our Hep/LPS/Hep group received an additional dose of heparin compared with the LPS/Hep group; since the early administration of heparin before LPS instillation might have reduced the development of the injury, it is not possible to know whether the differences between these two groups were due to the timing of administration or to the total dose administered; moreover, 24 hours may not have been long enough to detect some important histological changes in the LPS/Hep group.

Our experimental model focused on the acute phase of lung injury. A prolonged treatment of nebulized heparin and its effect in a late phase of ARDS need additional studies to determine its long-term effects.

What Is Known on This Topic

- Acute respiratory distress syndrome (ARDS) is associated with pulmonary activation of coagulation and inflammation. Previous studies suggest that anti-coagulants may help restore the coagulation cascade and treat ARDS.
- It has been shown that nebulized heparin was able to improve pulmonary coagulopathy in animal models of acute lung injury (ALI) and in ARDS patients without producing systemic bleeding.
- In vitro findings recently indicated that heparin may exert its effects through alveolar macrophages.

What This Paper Adds

- Our data confirmed that heparin nebulized directly into the lungs reduced pulmonary coagulopathy in a rat model of LPS-induced ALI without producing systemic bleeding. We also demonstrated that nebulized heparin significantly ameliorated lung injury, decreasing inflammation, permeability and neutrophils infiltration.
- We ascertained that heparin may act on alveolar macrophages limiting the inflammatory response through the reduction of TGF-β effectors, NF-κB and coagulation pathways.
- In our rat model of ALI, nebulized heparin was effective in attenuating pulmonary coagulopathy and inflammation. Some of the effects of heparin seem to be related with alveolar macrophages. Preclinical data need to be transferred to clinical studies to confirm the potential benefit of nebulized heparin in ARDS patients.

Conclusion

In our rat model of ALI, nebulized heparin was effective in attenuating pulmonary coagulopathy and inflammation. Some of the effects of heparin seem to be related with alveolar macrophages. Preclinical data need to be transferred to clinical studies to confirm the potential benefit of nebulized heparin in ARDS patients. A better understanding of the mechanisms involved in the pathogenesis of ARDS might open new fields for the treatment of this disease.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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Author Contribution

L. C., M. C.-R., R. G.-P. and A. A. designed the experiments; L. C., M. C.-R., R. G.-P., M. N. G. and J. T. performed the experiments; L. C., M. C.-R. and R. G.-P. analysed the data and, together with A. A., interpreted the data; M. C.-R., L. C., R. G.P. and A. A. wrote the manuscript and L. B. and A. A. helped to revise the manuscript. All the authors approved the last version of the manuscript.

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4. DISCUSSION

Substantial progress has been made in management of patients with ARDS; however, this syndrome still remains relatively common, being lethal or producing persistent sequelae on survivors. Effective therapies focused on the pathophysiology of ARDS are needed.

Pulmonary inflammation and coagulation are intrinsic to ARDS, together with the alveolar-capillary barrier breakdown, proteinaceous edema and neutrophils infiltration into the alveolar compartment. Considering that inflammation and coagulation play a critical role in ARDS development and that their cross talk may reciprocally stimulate and amplify both pathways, anti-coagulants might be a promising therapy because of their anti-inflammatory activities in addition to their anti-coagulant effects. Nebulized anti-coagulants might allow higher dosages, increase their local efficacy and overcome the risk of systemic bleeding produced by intravenous administration.

Heparin is a natural glycosaminoglycan widely used in the clinics because of its anti-coagulant properties. Previous pre-clinical and clinical studies have been performed using heparin as a treatment for ALI/ARDS, however the involved pathways are not well described and results have been conflicting [113, 185]. In order to elucidate the therapeutic role of heparin and its mechanism in lung injury we studied the action of heparin in specific primary human injured cell lung populations (ATII cells and alveolar macrophages) and its direct administration into the lungs by nebulization in a LPS-induced ALI rat model. In our results, animals treated with heparin presented attenuated pulmonary inflammation and coagulation and no systemic bleeding complications. Moreover, nebulized heparin modulated alveolar macrophages through reducing TGF-β and NF-κB effectors and the coagulation pathway and diminished the recruitment of neutrophils into the alveolar space.

Our experiments proved that nebulized heparin administration is safer than systemic administration cancelling the risk of systemic bleeding observed in other studies where heparin had been administered intravenously in lung injury models [68]. This fact was in line with what has previously been found in other pre-clinical and clinical studies in which heparin has been nebulized [113, 185]. However, in Hofstra *et al.* study nebulized heparin administration in a model of endotoxemia induced by intravenously administered LPS the

systemic coagulation was affected due to the administered constant doses over the time after the injury [153]. Additionally, alterations on systemic coagulation in the study of Hofstra *et al.* might be ascribed to the fact that it was a systemic damage model, as other models of direct lung injury such as pulmonary infection models [123, 124], chlorine-exposure models [152] or our model of LPS administration directly into the lungs did not present systemic coagulation alterations.

Furthermore, controversial results have been observed in clinical studies where heparin has been nebulized. Systemic coagulation was not affected in patients with ARDS that received different doses of nebulized heparin (50,000 IU/day, 100,000 IU/day, 200,000 IU/day, 400,000 IU/day), although a trend to increase aPTT levels was present with the highest dose used (400,000 IU/day) and, when nebulized heparin was stopped, aPTT returned to basal levels [147, 154, 155]. After a single nebulization of 90,000 IU in humans, the estimated UFH dose that is deposited in the low respiratory tract is of 7,000 IU and the peak value is found at 1 h 30 min after the nebulization [186]. The deposition of 32,000 IU of UFH to the low respiratory tract, which requires a nebulizer charge of 400,000 IU of UFH, has been proved to be a safe dose [186]. Furthermore, in the recent multicenter trial HEPBURN administration of nebulized heparin in burn patients with inhalation trauma produced adverse effects [113, 156]. In HEPBURN study, 25,000 IU of UFH were administered every 4 h until extubation or for a maximum of 14 days, administering 150,000 IU of UFH every day. The dosing, frequency and duration of local heparin administration in HEPBURN was based on the previous trials of patients with ARDS [147, 154, 155]. HEPBURN was prematurely stopped due to safety reasons: increased blood sputum and high levels of aPTT, some errors associated with the timing of nebulization and to the slow recruitment of patients, which made the study no viable [oral communication at the 23rd International Symposium on Infection in the Critically Ill Patient in February 2018 in Dublin, 113]. Differences on the effect of nebulized heparin between studies might indicate the need of a reduced dose of heparin for burn patients with inhalation trauma. Moreover, increased aPTT after repeated doses of this anti-coagulant might suggest that the processes involved in the storage of heparin in endothelial cells

and its metabolism by heparinases might become saturated [154]. Nevertheless, our pre-clinical model received two nebulizations of 1,000 IU/kg of heparin in the case of the treatment group and three nebulizations in the case of the pre-treatment group being less than the 70% of the dose used in the clinical study of patients with ARDS (150,000 IU of UFH/day) where aPTT levels tended to increase [147, 155] and taking into account that small animals metabolize drugs faster.

We studied the effect of nebulized heparin as both treatment and pre-treatment. In a retrospective cohort study, early administration of intravenous UFH decreased the mortality of patients with septic shock [187]. Given that patients under anti-coagulants treatments proved to develop less severe ARDS we planned to study the effects of heparin not only as a treatment for ALI but also as a pre-treatment. In our study, after the ALI induction, the treatment group received two doses of nebulized heparin, while an additional dose of nebulized heparin was given to the pre-treatment group before the LPS administration. A prophylactic pre-treatment model will prevent coagulation in the first stages of ALI, and a treatment model better mimics what is found in the clinics. Although close beneficial effects were observed in our results while comparing treatment and pre-treatment groups with the ALI group, the pre-treatment group presented a pronounced attenuation of lung injury. Concentration of BAL proteins and edema were decreased in both groups, but total cells in BAL and histological score were only declined in the pre-treatment group.

Increased effect of heparin in the pre-treatment group might be due to two reasons. The first explanation is that heparin administration before inducing the damage might have a protective repercussion avoiding the development of the injury. This protective effect can be ascribed to two mechanisms; heparin might block some receptors such as TLR and then LPS might not be able to bind and to induce a severe damage compared to the non-pre-treated groups. Also, prophylactic heparin administration might induce TFPI expression and restrain thrombin activation at the start, blocking the coagulation pathway and producing a compensatory effect to the activation of the coagulation pathway by LPS. The second explanation of why the pre-treatment group presents an increased heparin effect

while reducing injury might be due to the fact that this group is receiving an extra heparin dose compared to the treatment group. Differences between dosages is one of the limitations of our study; nevertheless, given that the biological half-life of heparin is 1.5 h and that dosages used in our experiment were much lower that dosages used in the clinical studies of heparin explained above, the extra-dose given to the pre-treatment group might be metabolized before the second dose is administered and the metabolism of heparin should not be saturated. In this line, the additional dose of heparin in the pre-treatment group compared to the treatment group might not be the reason of the beneficial results of the first group, giving us the rationale to administer two doses of heparin after LPS damage in both pre-treatment and treatment groups.

In line with previous studies of endotoxemia [153] and pneumonia [123], the anti-coagulant effect of heparin was clearly found in our studies in the treatment and pre-treatment groups. TF is the main initiator of extrinsic coagulation pathway and high levels of TF in patients with ARDS correlate with poor clinical outcomes [69]. Furthermore, increased levels of TATC, an inactive thrombin-antithrombin reaction, are elevated in these patients and point out enhanced thrombin levels and a hypercoagulant state. In our studies, both heparin as a treatment and a pre-treatment decreased TF in BAL, although only the effect of nebulized heparin as a pre-treatment reduced TATC. Regarding fibrinolysis, increased levels of FDP are observed when this system is altered. Heparin pre-treatment was able to reduce FDP levels in the LPS model. Reduced levels of TATC and FDP reflect a repercussion of heparin in all the coagulant processes involved in ARDS pathophysiology reducing the activation of the extrinsic coagulation through TF, and activating protein C pathway, which is translated in reduced coagulation. Furthermore, increased levels of FDP prove that heparin also promotes the fibrinolytic pathway.

Effects of nebulized heparin on inflammation have been controversial in previous pre-clinical and clinical studies of ALI/ARDS. In a model of chlorine exposure nebulized heparin reduced the presence of inflammatory cells in BAL [152], but in a model of endotoxemia [153] and pneumonia [123, 124] nebulized heparin did not produce any effects on inflammation. In our model, pro-inflammatory mediators (IL-6 and TNF-α) and the

mediator of neutrophils recruitment (Gro-kC) were dropped off in both the treatment and pre-treatment groups. Variations on inflammation between the different published studies might be attributed to the different models used and to variances in heparin timing and dosage administration. Models of LPS-induced ALI are simplified models of patients with ARDS. LPS is an endotoxin found in the cell wall of Gram-negative bacteria that can be a common cause of both direct (i.e. pneumonia) or indirect (i.e. sepsis) lung injury, and, depending on if it has been administrated intratracheally or intravenously, it can mimic direct or indirect ARDS, although LPS can also be used to reproduce other clinical disorders. Even though LPS is a strong activator of the host inflammatory response through TLR4 activation, it only reproduces part of the effects of live bacteria in the lungs, as together with LPS, bacteria further release other PAMPs and DAMPs that promote the activation of other pathways. Therefore, LPS models are really useful to study punctual damage after a specific PAMP; however, a part of including other activated processes, pneumonia models are more constant on time because of the continuous production of LPS and other molecules. Hence, our results prove that heparin is able to reduce inflammation through the specific LPS activated pathways.

Moreover, differences on inflammation between experiments might be due to the time interval of heparin administration. In our model two doses of heparin in the treatment group and three doses of heparin in the pre-treatment group were administered every 4 h, while in the studies of Zarogiannis SG *et al.* [152], Hofstra *et a*l. [123] and Cornet *et al.* [124] they nebulized heparin every 6 h. In the model of chlorine exposure that received nebulized heparin Zarogiannis SG *et al.* found reduced number of inflammatory cells in the BAL at 6 h post-exposure, but no differences on inflammation while nebulizing heparin were observed in the pneumonia models at 16 h or 40 h after the induction of the injury [123, 124]. Since heparin half-life is 1.5 h, counting that its effects last for 3 h, in our model we have a gap of 1 h between each heparin dose administration, while Hofstra *et al.* and Cornet *et al.*, although nebulizing more doses, might not find any effects on inflammation because there is a gap of 3 h between each heparin administration. In the model of Hofstra *et al.* [123] and Cornet *et al.* [124] the time interval might promote gaps

were heparin is not active and inflammation is enhanced, while in our model, although nebulizing less doses, the time interval might stop inflammation in the precise time. Also, anti-inflammatory effects found in the model of chlorine exposure [152] at 6 h after injury might indicate that differences found in inflammation between studies might be ascribed to the models, as explained above, and that the time interval of heparin nebulization in the pneumonia models should be reduced.

After proving that local administered heparin not only acts on coagulation but also has effects on inflammation, we deeply studied heparin action specifically in injured cell lung populations; ATII cells and alveolar macrophages. As our group had access to human fresh lung biopsies samples, we decided to study the mechanism of heparin in isolated human primary cells to demonstrate, also, the translational action into humans of this anti-coagulant. We found that heparin was able to hinder the response of ATII cells after LPS administration, decreasing the protein expression of IL-6 and IL-8 and the gene expression of MCP-1. As the expression of MCP-1 is regulated and released after TNF-α, IL-1β, IL-6 and IL-8 secretion, a variation in MCP-1 protein expression might be delayed on time after the first burst of cytokines, reason why its release might be observed at later stages. Given the role that NF-κB pathway plays in ALI/ARDS we also analyzed the effect of heparin on ATII cells through this pathway. Our group described for the first time a reduced expression of the mediators of the NF-κB pathway (IRAK-1 and MyD88) in ATII cells after heparin administration compared to the injured group. Hence, heparin proved effective blocking this pathway in ATII cells, since both mediators and effectors were decreased.

Thanks to their plasticity alveolar macrophages have a major role in both ALI/ARDS development and resolution. At the early stages of ARDS alveolar macrophages become pro-inflammatory activated presenting an M1 phenotype, while in the resolution phase of ARDS they are polarized towards an M2 phenotype exhibiting anti-inflammatory properties [37, 46, 47]. To further assess the role of this cellular type in lung injury and heparin treatment, alveolar macrophages were analyzed *in vitro* and *in vivo*. Heparin reduced the expression of TNF-α and IL-6 in *in vitro* treated alveolar macrophages after LPS injury. In line with these results, the expression of both M1 (TNF-α and iNOS) and M2

(IL-10 and Arg-1) mediators was decreased to basal levels in alveolar macrophages obtained from the BAL of our *in vivo* model. Altogether these findings indicate a direct effect of heparin in this cell population. In addition, nebulized heparin diminished the expression of NF-κB effectors in lung homogenates (IL-6, TNF-α and Gro-κC) and in alveolar macrophages from BAL (TNF-α, iNOS, P-Selectin and CCL-2), which together with the effects of heparin in *in vitro* isolated and injured alveolar macrophages point out the specific effect of heparin in this pathway and in this cell population, proving, also, the translational role of the studied anti-coagulant.

In addition, we demonstrated that the anti-inflammatory effect of heparin is maintained during the multi-step process of the inflammatory response. Alveolar macrophages are the first line of defense when there is an infection and, in case the bacterial agent is not rapidly eliminated, ATII cells become activated further enhancing and maintaining the inflammatory response. Given that heparin was able to immediately de-activate alveolar macrophages at the initial stages of the inflammatory response and suppress the expression of pro-inflammatory molecules by ATII cells at later stages, we not only demonstrated the specific effect of this anti-coagulant in ATII cells and alveolar macrophages, but also the anti-inflammatory effect of heparin at different time-points.

Consistent with previous studies that proved beneficial effects of intravenous heparin through the TGF-β/Smad pathway in a rat model of LPS [151]; in our results nebulized heparin also reduced the expression of TGF-β effectors in alveolar macrophages in the LPS-induced ALI model. As when we studied the specific effect of heparin in primary human cell lung populations no variations in this pathway were found in any of the cell types evaluated, these results further reinforce the theory that other cell types (endothelial cells or myofibroblasts) and interactions might influence in the regulation of TGF-β/Smad pathway when administering heparin *in vivo*.

Heparin exerts its major anti-coagulant properties by binding to ATIII. Several enzymes in the coagulation cascade are inhibited by ATIII, but in the presence of heparin, the rate at which ATIII inactivates thrombin and factor X is potentially increased. ATIII is an endogenous anti-coagulant that also presents anti-inflammatory activities dependent and

independent of its action on the coagulant cascade. Moreover, because of its effects on thrombin and inflammatory mediators, ATIII action might also restore the tight junctions and reinforce the alveolar-capillary barrier.

In order to study the response of local administered ATIII alone or combined with heparin in a whole organism, both treatments were nebulized in an HCl/LPS induced ALI model (Appendix 8.1). To our knowledge, this is the first time that ATIII and heparin are both administered nebulized together in a 48 h model of HCl/LPS induced ALI. ATIII alone and together with heparin reduced protein concentration in BAL and decreased coagulation and inflammation without producing systemic bleeding. Administration of ATIII alone increased beneficial effects in coagulation, while combined ATIII and heparin potentiated the reduction of permeability and macrophages infiltration into the alveolar compartment. Also, to further elucidate ATIII mechanisms in lung injury we evaluated the specific action of ATIII in primary human ATII cells.

We assessed the effects of the anti-coagulants in the HCl/LPS induced ALI rat model because we wanted to observe if their activity was beneficial in prolonged ALI and because two-hit model, a part from being more stable, is closer to what is found in the clinics and better mimics ARDS pathophysiology. Also, we have extensive experience with this model induced by HCl and LPS as it was previously developed and monitored in our laboratory [188]. Although we know that this model presents persistent damage until 72 h, we preferred to study the effects of anti-coagulants at 48 h in order to administer less anti-coagulants doses. Gastric aspiration is a cause of direct ARDS; in some cases it can progress to pneumonia when there is an aspiration of bacteria from the oral cavity or nasopharynx to the lower airway. Aspiration of HCl or gastric contents also impairs pulmonary bacterial clearance, further enhancing pneumonia development and pulmonary inflammation induced by LPS or bacteria.

In consonance with other pre-clinical models where ATIII has been nebulized, no effects on systemic coagulation were detected in our HCl/LPS induced ALI model [113, 185]. Furthermore, in a sheep model of burn and smoke inhalation [168] and in our studies combined ATIII and heparin nebulization did not alter systemic coagulation.

For the first time combined ATIII and heparin administered directly into the lungs demonstrated a beneficial effect on pulmonary coagulation, although effects of nebulized ATIII alone were more pronounced than effects of combined ATIII and heparin. Mitigated pulmonary coagulopathy while administering nebulized ATIII alone has also been reported in models of pulmonary infection [123, 124] and endotoxemia [154]. In our studies, ATIII alone decreased protein levels of TF in the BAL, blocking this pathway and reducing coagulation. Regarding the fibrinolytic pathway, diminished levels of PAI-1 while administering ATIII alone or combined with heparin were found at gene expression but not at protein level and only ATIII alone could reduce plasminogen and FDP. It is known that heparin alters ATIII conformation potentiating its inhibitory activity for thrombin 1000-fold more strongly and also enhancing the inhibition of factor Xa and IXa. In the present model, increased anti-coagulant effect of ATIII alone above combined ATIII and heparin might be due to the fact that although used dosage and timing of ATIII and heparin were based on their biological lifetime and previous studies of both anti-coagulants separately, a dose-response study for the combination of both anti-coagulants should have been performed in the used model. Nevertheless both ATIII alone or combined with heparin reduced coagulation in our model of HCl/LPS induced ALI. Discrepancies between RNA and protein might be explained by the post-transcriptional modifications of RNA, as there is not a perfect correlation between RNA expression and protein concentration. Moreover, given the interconnection between proteins, small alterations in the proteins of the coagulation cascade can have a strong impact on the final product.

In our results the combination of both treatments did not have a higher impact on inflammation compared to nebulized ATIII alone. Obtained results on inflammation correlate with the anti-inflammatory effect of nebulized ATIII alone in a model of *Streptococcus pneumoniae* induced pneumonia [123] and combined ATIII and heparin in a sheep model of burn and smoke inhalation [168, 169]. Nevertheless, nebulized ATIII in a pre-clinical model of *Pseudomonas aeruginosa* induced pneumonia [124] or endotoxemia [153] no anti-inflammatory effects were found 16 h after injury.

Differences of ATIII on inflammation between experiments might be linked to the number of administered ATIII doses and to the timing of the model used. Non-observed differences on inflammation in the models of *Pseudomonas aeruginosa* or endotoxemia might be due to the facts that they only received one dose of ATIII and that its effect was evaluated 16 h after injury, a too short time to find ATIII repercussion on inflammation. Positive anti-inflammatory effects of nebulized ATIII in HCl/LPS induced ALI model and in the model of *Streptococcus pneumoniae* might be ascribed to the two nebulisations of ATIII received and to the evaluation of its effect at later stages; 48 h and 40 h respectively.

Several anti-inflammatory effects of ATIII have been elucidated, both dependent and independent of its action on the coagulation pathway. In our studies, ATIII alone or combined with heparin reduced inflammation (TNF-α and IL-1β) gene expression but not its protein levels in the HCl/LPS model. Coagulation independent anti-inflammatory effects of ATIII are mainly mediated through its binding to syndecan-4, a HSPG receptor expressed by neutrophils, monocytes, lymphocytes, eosinophils and endothelial cells [164]. Given that syndecan-4 binds ATIII through its heparin-binding site, previous studies have proved that heparin reduces ATIII anti-inflammatory effect because of its binding to ATIII instead of syndecan-4 [164]. The non-observed synergistic effect of ATIII and heparin in inflammation could be due to syndecan-4 fact, although in our results a reduction of inflammatory mediators was found by both ATIII alone or together with heparin. Anti-inflammatory actions of combined anti-coagulants might be explained because of coagulation dependent anti-inflammatory effects of ATIII, produced through thrombin and factor X inactivation, together with independent heparin anti-inflammatory actions. So, although heparin does not potentiate the effects of ATIII on inflammation because it blocks the binding of syndecan-4 to ATIII, the same effects on inflammation are detected while administering ATIII alone or combined with heparin because coagulation dependent anti-inflammatory actions of ATIII and independent activities of heparin on inflammation are carried out.

This study differs from previous studies where heparin potentiates the anti-coagulant effect of ATIII and reduces its anti-inflammatory effect. This fact might be due to different

timing and dosage of ATIII and heparin between studies. We should not forget that time to initiate a treatment is decisive.

Disruption of the alveolar-capillary barrier is a critical characteristic of ALI/ARDS. Increased pro-inflammatory mediators activate NF-κB pathway promoting a negative regulation in tight junctions and reducing ZO-1 and occludin levels [52]. Given that ATIII has a direct action on thrombin and inflammation, which have a direct effect in endothelial and epithelial cells, ATIII might also produce beneficial effects in the alveolar-capillary barrier. As we had access to lung biopsies, we evaluated the effect of ATIII treatment directly in injured ATII cells *in vitro* and we found reduced levels of pro-inflammatory mediators and increased levels of tight junctions in this cellular type after ATIII treatment. These properties of ATIII were reinforced by the results obtained *in vivo* in our HCl/LPS-induced ALI model where anti-coagulants were administered. Reduced protein concentrations in the BAL of injured animals after anti-coagulant nebulization together with a decrease on the expression of neutrophils and monocytes recruitment in lung tissue prove ameliorations on permeability. Altogether is further supported by a non-significant reduction of lung injury evaluated by histology, although given that a beneficial action of nebulized anti-coagulants has been detected at different levels, 48 h might not have been long enough to detect histopathology scores changes or ameliorations in partial oxygen pressure in the anti-coagulants treated groups. In addition, a synergistic effect of heparin on ATIII in permeability is observed while analyzing the levels of tight junctions in lung tissue, as combined ATIII and heparin improved occludin levels but ATIII alone did not produce a significant increase. So, combined heparin and ATIII reparative effects in the alveolar-capillary barrier could explain the reduction of macrophages and total cells counts in BAL only in the HCl/LPS-induced ALI model treated with both anti-coagulants. Accordingly, *in vitro* and *in vivo* results from our experiments confirm that together with its repercussion in the coagulation and inflammatory pathways, ATIII also promotes the restoration of the epithelial barrier.

Given the importance of alveolar macrophages in ARDS induction and resolution, we further studied the effect of ATIII alone or combined with heparin in this cellular type

isolated from the BAL of the treated animals. ATIII alone inhibited NF-κB effectors, as reduced levels of iNOS were found. Although heparin alone was able to reduce iNOS expression in alveolar macrophages, the competition of heparin with syndecan-4 might be the reason why combined ATIII and heparin are not able to significantly decrease iNOS levels. Nevertheless, combined ATIII and heparin decreased the expression of the macrophage chemoattractant chemokine CCL2, while ATIII alone was not able to reduce CCL2 levels. These results further correlate with less macrophages cell numbers in the BAL. Regarding the alternative pathway of activated macrophages ATIII alone or combined with heparin reduced levels of Arg-1 to basal levels.

These studies present some limitations. First, animal models of both LPS and HCl/LPS induced-ALI cannot completely reproduce human ARDS or its heterogeneous pathophysiology. Second, ATIII and heparin doses were based on previous experiments but it was the first time that they were nebulized together; future studies should monitor the levels of these anti-coagulants throughout the experiment, in order to better elucidate ATIII and heparin interaction. Third, protein concentrations, partial oxygen pressure and histopathology scores were determined 48 h after inducing ALI; no significant differences could be due to the timeline. Further experiments increasing anti-coagulants dosage and timing should be performed, in order to assess if its effects are maintained or increased in sustained ALI or if more doses of anti-coagulants would enhance its actions.

These results confirm that nebulized anti-coagulants are able to face different pathways and processes involved in ARDS pathophysiology without causing systemic bleeding. Administer this treatment to a specific subtype of ARDS with increased coagulation and inflammation pulmonary levels might enhance patient response.

5. CONCLUSIONS

- Local heparin administration in primary cell lung populations injured with LPS was able to reduce inflammation in primary human alveolar macrophages and human ATII cells. At the first steps of the inflammatory response heparin administration inhibited the production of pro-inflammatory cytokines by alveolar macrophages. At later stages heparin deactivated the maintenance of the inflammatory response by ATII cells blocking NF-κB pathway.
- Local heparin administration in a LPS-induced ALI rat model decreased the recruitment of neutrophils into the lung, reduced pulmonary coagulation and inflammation and did not produce systemic bleeding. Part of this beneficial effect of heparin was exerted through alveolar macrophages, decreasing the expression of TGF-β and NF-κB effectors and reducing the expression of mediators of the coagulation pathway.
- Local ATIII administration after LPS injury in primary human ATII cells decreased inflammation and enhanced tight junctions reinforcing the alveolar epithelium restoration.
- Nebulized ATIII alone or combined with heparin attenuated lung injury in an HCl/LPS-induced ALI rat model. Both treatments reduced pulmonary coagulation and inflammation without producing systemic bleeding complications.
- Higher beneficial effects of ATIII alone were identified in coagulation, while increased actions of combined ATIII+Heparin were detected on permeability and cellular recruitment into the alveolar compartment.
- Nebulized anti-coagulants are able to face different pathways and processes involved in ARDS pathophysiology without causing systemic bleeding, being a potential treatment for this disease.

6. FUTURE DIRECTIONS

In this thesis we observed beneficial activities of local anti-coagulants effecting different pathways and processes involved in ARDS pathophysiology.

In our studies, local heparin administration in primary human cell lung populations injured with LPS was found to inhibit the production of pro-inflammatory cytokines by alveolar macrophages and the NF-κB pathway in ATII cells. Also, local ATIII reduced inflammation and reinforced the alveolar epithelium restoration. Further studies evaluating the effect of local heparin or ATIII administration in injured neutrophils, interstitial macrophages or endothelial cells might be helpful to increase our knowledge about the mechanisms and pathways of anti-coagulants.

In our results, nebulized heparin and/or ATIII reduced pulmonary coagulation and inflammation without producing systemic bleeding. Combined ATIII and heparin also reduced permeability and the cellular recruitment into the alveolar compartment. To find out other involved mechanisms such as the effect of anti-coagulants in apoptosis pathways or in limiting bacterial outgrowth are also of interest.

Anti-coagulant activities in our animal model of HCl/LPS at 48 h were greatly successful, and additional experiments increasing its dosage and timing should also be performed, in order to assess if its effects are sustained or increased in longer periods of lung injury or if higher doses or reducing the time between administration of anti-coagulants would enhance its actions without causing side effects. We would like to assess the effects of heparin and/or ATIII increasing its dosage or time in our animal model of HCl/LPS with persistent lung damage at 72 h [188]. Also, given that models of pneumonia present constant damage on time and a continuous production of LPS and other activating molecules for the coagulation it would also be of interest to observe the effects of the combination of nebulized heparin and ATIII after pneumonia.

Moreover, pre-clinical results need to be transferred to the clinics, in order to determine the benefit of anti-coagulants nebulization in ARDS patients. The obtained results and our group previous experience might allow the consideration of a Phase 1 trial in ARDS

patients to study heparin and/or ATIII safety, feasibility and determine the appropriated dose, and a Phase 2 trial to explore anti-coagulants efficacy.

In addition, our group has wide experience in the field of cellular therapies for the treatment of ARDS. Although we observed positive results while administering cellular therapies for the treatment of ALI/ARDS, we also found that ATII cells or MSC transplantation in animals with ALI increase the expression of coagulant factors such as TF and plasminogen in lung tissue. There are studies that indicate that both MSCs pretreatment with heparin or the co-administration of MSC and heparin prevent the pro-coagulant effects of MSC in a model of acute myocardial infarction and in a model of inflammatory colitis [189, 190]. Due to the key role of anti-coagulants on coagulation and inflammation and considering that cell therapies and anti-coagulants act via different mechanisms, our hypothesis is that the combination of both treatments; anticoagulants and cell therapy, target different pathways altered in ARDS and potentiate their therapeutic effect.

Further research on the field of anti-coagulants and ARDS is necessary because the scientific community still needs to elucidate some molecular and cellular pathways involved in their therapeutically effect.

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8. APPENDIX

8.1 Effects of antithrombin and heparin for the treatment of acute lung injury in rats

Abstracts of oral and poster presentations in international conferences and extended methods and results concerning these abstracts (8.1.1):

American Thoracic Society

Neus Tantinyà, Marta Camprubí-Rimblas, Raquel Guillamat-Prats, Josep Bringué, Mª Nieves Gómez, Lluís Blanch, Antonio Artigas. Effects of nebulized antithrombin and heparin for the treatment of acute lung injury in rats. *American Journal of Respiratory and Critical Care Medicine* 2018;197:A7532

Effects of Antithrombin and Heparin for the Treatment of Acute Lung Injury in Rats

N. Tantinyà[1], M. Camprubí-Rimblas[2], R. Guillamat-Prats[1], J. Bringue[1], M. Gómez[3], L. Blanch[4], A. Artigas[4]; [1]CIBER de Enfermedades Respiratorias, Sabadell, Spain, [2]Institut d'Investigació i Innovació Parc Taulí (I3PT), Sabadell, Spain, [3]Fundació Parc Taulí, Sabadell, Spain, [4]Sabadell Hospital, 08208 Sabadell, Spain.

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Rationale: Acute Lung Injury (ALI) is an acute respiratory failure that develops from a variety of clinical disorders and is associated with a high mortality rate. In the first stages of ALI, proinflammatory mediators inhibit natural anticoagulant factors, which alter the normal balance between coagulation and fibrinolysis leading to a procoagulant state. Thus, patients with ALI may benefit from anticoagulant therapy. Previous studies revealed that local administration of anticoagulants could not only re-establish coagulant activity in the lung but also prevent systemic bleedings. AIM: Evaluate the potential therapeutic effects of nebulized antithrombin (ATIII) and heparin (Hep) in an ALI model. Methods: Sprague-Dawley rats (~300g) underwent intratracheal administration of HCL (1µl/g of HCL 0.1M, pH=1.2). 2h later subjects underwent intratracheal administration of lipopolysaccharide (LPS 30µg/g body weight). Control groups received saline (0.9%) instead. ATIII (500IU/Kg body weight) alone or together with Hep (1000IU/Kg body weight) were nebulized through Aeroneb Pro Nebulizer system (Aerogen) 4h and 28h after HCL. An extra dose of nebulized Hep was administered 12h after HCL or saline administration in ATIII+Hep groups. Animals were sacrificed 48h after injury. Pro- and anti-inflammatory mediators as well as neutrophil and macrophages chemoattractant activity were evaluated in lung tissue using qRT-PCR. The permeability and the cellular infiltration were quantified in bronchoalveolar lavage (BAL) and the integrity of the alveolar epithelium was evaluated by histology. Statistics: One-way ANOVA and Newman Keuls post-hoc test ($p<0.05$). Results: Figure 1a showed a significantly higher concentration of protein in the BAL in ALI group and when treated, protein concentration drops to baseline levels. Neutrophil levels in BAL are significantly increased in ALI group in relation to control levels. However, a non-significant decrease has been found between ALI and ALI+treated groups. Figure 1b showed a significant increase of all the markers in ALI group. When treated with AT-III and the combination of AT-III and Hep, there is a significant reduction in the expression of all of them. Regarding figure 1c no significant difference can be observed between groups in tissue factor levels. However, HCL/LPS group showed a significant reduction of ATIII levels compared to the control group although there are no significant differences between this group and the injured/treated groups. Conclusions: These results indicate that nebulized anticoagulants are able to attenuate the lung injury through reducing inflammation, neutrophils and monocytes recruitment and permeability.

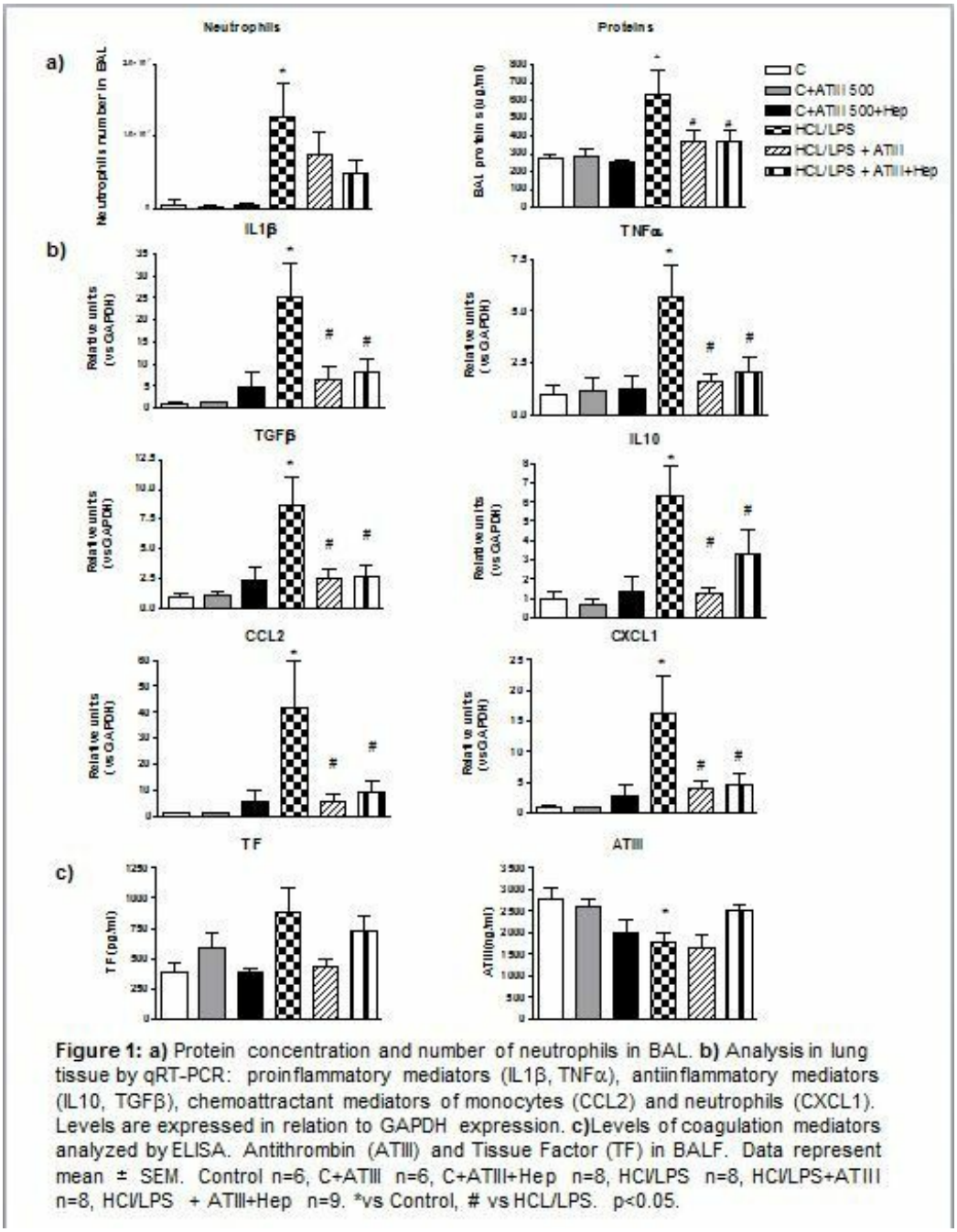

Figure 1: a) Protein concentration and number of neutrophils in BAL. **b)** Analysis in lung tissue by qRT-PCR: proinflammatory mediators (IL1β, TNFα), antiinflammatory mediators (IL10, TGFβ), chemoattractant mediators of monocytes (CCL2) and neutrophils (CXCL1). Levels are expressed in relation to GAPDH expression. **c)**Levels of coagulation mediators analyzed by ELISA. Antithrombin (ATIII) and Tissue Factor (TF) in BALF. Data represent mean ± SEM. Control n=6, C+ATIII n=6, C+ATIII+Hep n=8, HCl/LPS n=8, HCl/LPS+ATIII n=8, HCl/LPS + ATIII+Hep n=9. *vs Control, # vs HCL/LPS. $p<0.05$.

This abstract is funded by: GrÃfols, S.A

European Society of Intensive Care Medicine

Neus Tantinyà, Marta Camprubí-Rimblas, Raquel Guillamat-Prats, Josep Bringué, Mª Nieves Gómez, Lluís Blanch, Antonio Artigas. Effects of nebulized antithrombin and heparin for the treatment of acute lung injury in rats. *Intensive Care Medicine Experimental* 2017, 5 (Suppl2):0111

RESULTS. Tidal R/D decreased from 5.7 ± 7.4% to 2.3 ± 1.5% as PEEP was gradually increased from 0 cmH_2O to 15 cmH_2O ($p < 0,01$), and was increased to 5.5 ± 3.9% as PEEP was gradually decreased to 0 cmH_2O ($p < 0,01$). Mean tidal volume increased from 83 ± 39 ml at PEEP 0 cmH_2O to 386 ± 126 ml at PEEP 15 cmH_2O, while the respiratory rate decreased from 77 ± 20 bpm (PEEP 0) to 4.3 ± 1.9 (PEEP 15).
CONCLUSIONS. In this experimental early mild model of ARDS, tidal R/D occurred during spontaneous breathing using NAVA. However, the amount of tidal R/D was reduced by increasing PEEP. This indicates that PEEP higher than conventionally used clinically, in spontaneously breathing patients, might be applied in early ARDS in order to reduce R/D and, possibly, the risk of VILI.
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0111

Effects of antithrombin and heparin for the treatment of acute lung injury in rats

N. Tantinyà[1], M. Camprubí-Rimblas[2,3], R. Guillamat-Prats[1], J. Bringué[1,2,3], M.N. Gómez[2], L. Blanch[1,2,4], A. Artigas[1,2,4]
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Intensive Care Medicine Experimental 2017, **5(Suppl 2):**0111

INTRODUCTION. Acute Lung Injury (ALI) is the result of an acute respiratory failure that develops from a variety of clinical disorders and is associated with a high mortality rate. In the first stages of ALI, proinflammatory mediators inhibit natural anticoagulant factors, which alter the normal balance between coagulation and fibrinolysis leading to a procoagulant state (1). Thus, patients with ALI may benefit from anticoagulant therapy. Recent studies revealed that local administration of anticoagulants could not only re-establish coagulant activity in the lung but also prevent systemic bleedings (2).
OBJECTIVES. Evaluate the potential therapeutic effects of nebulized antithrombin (ATIII) and heparin in an ALI model.
METHODS. Sprague–Dawley rats (~300g) underwent intratracheal administration of HCL (1ml/g of HCL 0.1M, pH = 1.2). 2h later subjects underwent intratracheal administration of lipopolysaccharide (LPS 30mg/g body weight). Control groups received saline (0.9%) instead. ATIII (500IU/Kg body weight) alone or together with heparin (1000IU/Kg body weight) were nebulized through Aeroneb system (Philips Healthcare) 4h and 28h after HCL. An extra dose of nebulized heparin was administered 12h after HCL in heparin groups. Animals were sacrificed 48h after injury. Pro- and anti-inflammatory mediators as well as neutrophil and macrophages chemoattractant activity were evaluated in lung tissue using qRT-PCR. The permeability and the cellular infiltration were quantified in bronchoalveolar fluid and the integrity of the alveolar epithelium was evaluated by histology. Statistics: One-way ANOVA and Newman Keuls post-hoc test ($p < 0.05$).
RESULTS. A significant increase was observed in IL1beta, IL10, CXCL1, CCL2 and Caspase-3 in the lung tissue of the ALI group. AT-III and the combination of AT-III and heparin reduced the expression of all of them. A tendency was observed for plasminogen (Fig. 49).
CONCLUSIONS. These results indicate that nebulized anticoagulants are able to attenuate the lung injury through the decrease of neutrophils and monocytes recruitment and the reduction of inflammation.
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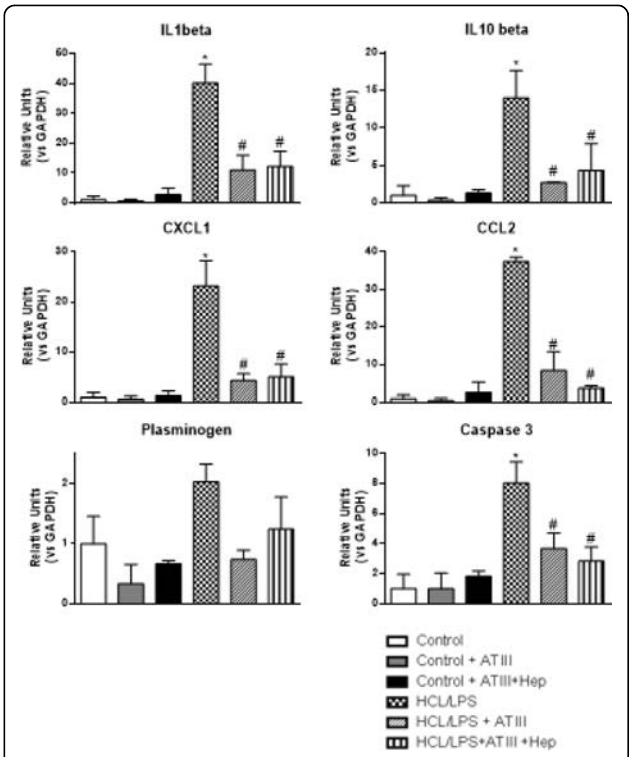

Fig. 49 (abstract 0111). Gene expression of pro- and anti-inflammatory markers, neutrophils and monocytes recruitment, coagulation and apoptosis. Groups: Control (n=8), control + ATIII (n=8), control+ATIII+Hep (n=8), HCL/LPS (n=8), HCL/LPS+ +ATIII (n=8), HCL/LPS+ +ATIII+HEP (n=8). *p≤0.05 vs Control: #p≤0.05 vs <HCL/LPS. Gene expression of inflammatory markers, coagulati

0112

The homing and protective effects of mesenchymal stem cells overexpressing CXCR7 in LPS-induced acute respiratory distress syndrome mice

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Intensive Care Medicine Experimental 2017, **5(Suppl 2):**0112

INTRODUCTION. Mesenchymal stem cells (MSCs) have potential for re-epithelization and recovery in acute respiratory distress syndrome (ARDS). In a previous in vitro study, the results showed that the CXCL12/CXCR7 axis promoted the homing of MSCs, suggesting that the CXCL12/CXCR7 axis might be one of the key mechanisms underling the therapeutic effect of mouse MSCs in ARDS.
METHODS. mMSCs stable transfected with CXCR7 were transplanted intratracheally into the ARDS mice induced by lipopolysaccharide. Lung tissue injury and repair assessment were examined using haematoxylin and eosin staining, lung injury scoring. Homing of mMSCs were assayed by labelling and tracing MSCs using NIR815 dye and immunofluorescent staining.
RESULTS. mMSCs overexpressing CXCR7 engraftment led to more significant effects than the ZsGreen controls, including the homing of the mMSCs in the lung, improvement in endothelial permeability, and the pathologic impairment of the lung tissue.
CONCLUSIONS. These results suggest that overexpressing CXCR7 in mMSCs could further promote their homing to injured lung and improve the therapeutic effects of mouse MSCs in ARDS mice.

8.1.1 Methods and results

8.1.1.1 Methods

Isolation and purification of hATII cells

Human lung biopsies were used for the isolation of primary hATII cells. Biopsies were obtained from the distal areas of the tumor part and the tissue was histologically normal. Patients did not have interstitial diseases, they were no smokers in the last 2 years and they were between 55-75 years old. The local Ethics Committee reviewed and approved the study and patients had previously provided informed consent.

In order to isolate hATII cells, biopsies were washed by perfusing sterile saline (0.9% sodium chloride (NaCl)) throughout the tissue with 20 ml for 1 cm^2 of tissue. Lung was digested with 30 ml of trypsin (0.25%) (Sigma, Darmstadt, Germany) prepared in Hanks Balanced Salt solution (HBSS) at 37ºC. The tissue was chopped in the presence of human albumin (Grifols, Barcelona, Spain) to stop the digestion and DNase (250 µg/ml) (Roche, Mannheim, Germany) to prevent the cell aggregates. The cell suspension was filtered through 100 µm and 40 µm nylon meshes and a density gradient with 1.077 g/ml with Lymphoprep solution (Axis-Shield, Oslo, Norway) was performed and centrifuged at 600 x g for 25 min to purify the cells. The interface band with the hATII cells and interstitial macrophages was collected, resuspended with DNase (100 µg/ml) prepared in HBSS and centrifuged at 600 x g for 15 min. The pellet was resuspended with DNase (100 µg/ml) and seeded in Petri dishes, in order to remove the interstitial macrophages. After 1 h at 37ºC, non-adherent cells were hATII cells, which were collected, counted, checked for viability and seeded at a density of 2 x 10^5 cells/wells on 24-well plastic dishes in 1 ml of supplemented DCCM1 (10% FBS (Gibco, USA), 1 mM L-glutamine, penicillin-streptomycin (50 U/ml, 0.05 mg/ml, respectively), 0.025 mg/ml vancomycin (Pfizer, Hospitalet de Llobregat, Spain) and 0.1 mg/ml cefotaxime (Normon, Barcelona, Spain) and 1 mM HEPES). Cells were precultured for 48 h at 37ºC and 5% CO_2.

Purity of isolated hATII cells was assessed by the presence of intracellular alkaline phosphatase (Sigma, Darmstadt, Germany) and by immunofluorescence. HATII cells were

fixed in 4% paraformaldehyde, blocked with a solution of PBS, 3% FBS and 1% BSA for 2 h at room temperature and incubated overnight with rabbit anti-rat SPC (1:100) (Santa Cruz Biotechnology, Santa Cruz, USA). After washing with PBS and incubate for 1 h at 37ºC with goat anti-rabbit IgG-FITC (1:500) (Santa Cruz Biotechnology, Santa Cruz, USA), cells were washed with PBS 1X and incubated 5 min with HOECHST (1:1000) (Invitrogen, Carlsbad, USA).

Treatment and analysis of hATII cells

HATII cells were injured with 100 ng/ml of LPS from *Escherichia coli* 055:B5 (Sigma Darmstadt, Chemical, St. Louis, USA, 30 µg/g body weight) and 2 h later they were treated with 5 IU/ml of ATIII (Grifols S.A, Barcelona, Spain). After 24 h, cells were collected with DireCtQuant 100T (DireCtQuant, Lleida, Spain) and stored at -80ºC for further analysis.

Table 6. Human primers

Gene	Forward Primer	Reverse Primer
GAPDH	5’ GAT CAT GAG CAA TGC CTC CT 3’	5’ TGT GGT CAT GAG TCG TTC CA 3’
iNOS	5’ CAC CAT CCT GGT GGA ACT CT 3’	5’ TCC AGG ATA CCT TGG ACC AG 3’
CD206	5’ CAGATGCCCGGAGTCAGATC 3’	5’ TTTATCCACAGCCACGTCCC 3’
ZO-1	5’ GTTCCAAAGCTGCTTCCACA 3’	5’ CCTTCCGCCACGATTGTAAG 3’
Occludin	5’ GGGCATTGCTCATCCTGAAG 3’	5’ GAGTAGGCTGGCTGAGAGAG 3’

Total RNA was reverse-transcribed into cDNA, which was amplified in a real time polymerase chain reaction (qRT-PCR) system (7500 Real-Time PCR System, Applied Biosystems, Thermo Fisher Scientific, Madrid, Spain) using SYBR green One-Step (Kapa Biosystems, Merck, Darmstadt, Germany). ΔΔCt method was used for correction and glyceraldehyde-3-phosphatase (GAPDH) was used as housekeeping to analyse the following markers (Table 6).

Animals

Male Sprague-Dawley rats (8 weeks old; 275-300 g) (Charles River, Chatillon-sur-Chalaronne, France) housed at 23ºC and 60% relative humidity in 12 h light-dark cycle were used. Food pellets (A04 Scientific Animal Food & Engineering, Panlab, Barcelona,

Spain) and tap water were available *ad libitum*. The Animal Research Ethics Committee of Autonomous University of Barcelona (UAB) and the Animal Experimentation Committee of Generalitat de Catalunya approved the study. Animals were supervised throughout all the experiment.

Acute lung injury model

A double-hit model was used to develop ALI, as previously described [188]. Rats were sedated with sevoflurane and received an intratracheal (IT) instillation of 300 µl of HCl (0.1 M, pH=1.3) followed 2 h later by IT instillation of 500 µl of LPS. Control groups received saline solution (0.9% NaCl) instead.

Nebulization

The nebulization of ATIII and heparin (Hospira Products Farmac, Madrid, Spain) was performed with the AeronebPro nebulizer system (Aerogen Limited, Galway, Ireland) at constant oxygen flow (2 L/min). This system was connected through restraint tubes to the bottle necks where animals were restricted, allowing direct exposure of the nebulized agents to the rats (Figure 13). Based on previous studies, the dose of ATIII was 500 IU/kg body weight and the dose for heparin was 1,000 IU/kg body weight [153]. The timing of administration was determined by the longer elimination half-life of ATIII (19-72 h) and the elimination half-life of heparin (1.5 h), considering that the second one pretended to produce a synergistic effect on the first one. No-treated groups received nebulized saline instead. In all cases, the total volume of each nebulization was 700 µl.

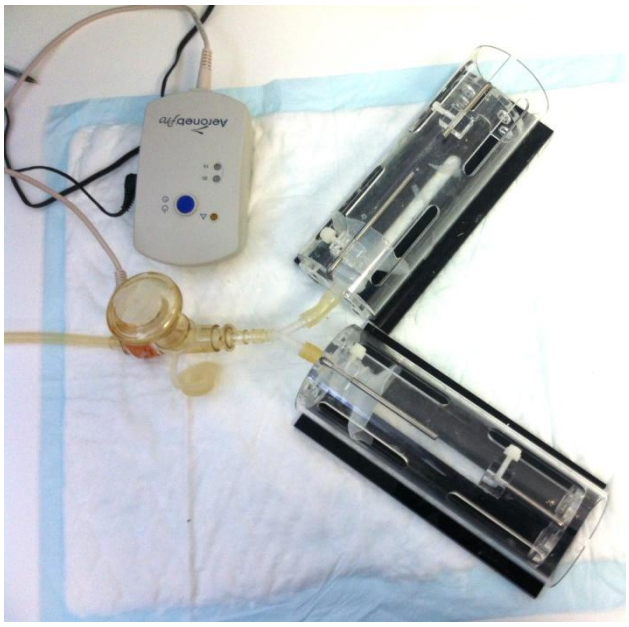


Figure 13. AeronebPro nebulizer system

Experimental groups

The animals were randomly assigned into 6 experimental groups (Figure 14):

- Control (n=8): Saline IT instillation at 0 h and 2 h followed by saline nebulization at 4 h, 12 h and 28 h.
- Control + ATIII (n=9): Saline IT instillation as in control group followed by ATIII nebulization at 4 h and 28 h and a saline nebulization at 12 h.
- Control + ATIII+Heparin (n=10): Saline IT instillation as in control group followed by a nebulization of ATIII combined with heparin at 4 h and 28 h and an extra dose of nebulized heparin at 12 h.
- HCl/LPS (n=12): HCl and LPS IT instillation at 0 h and 2 h, respectively. Saline nebulization 4 h, 12 h and 28 h after the first instillation.
- HCl/LPS + ATIII (n=12): HCl and LPS administration as in HCl/LPS group followed by ATIII nebulization at 4 h and 28 h and a saline nebulization at 12 h.
- HCl/LPS + ATIII+Heparin (n=10): HCl and LPS administration as in HCl/LPS group and a nebulization of ATIII combined with heparin at 4 h and 28 h and an extra dose of nebulized heparin at 12 h.

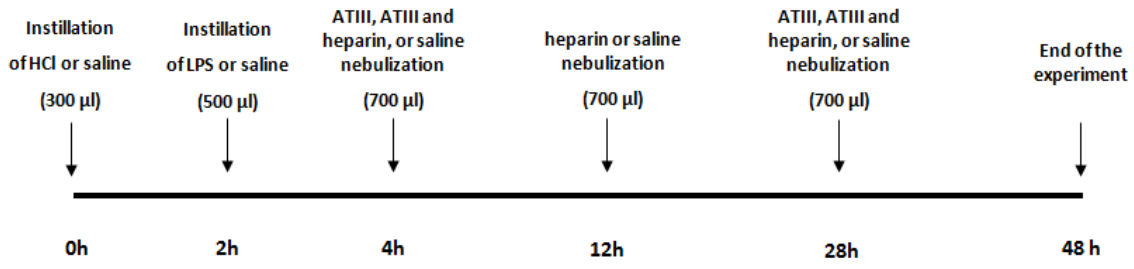


Figure 14. Experimental design

Collection of samples

Animal body weight was recorded at 24 h and 48 h. Rats were sacrificed at 48 h; they were anesthetized with an intraperitoneal administration of ketamine (90 mg/kg) and xylazine (10 mg/kg) and exsanguinated via the abdominal aorta artery. At this moment, blood was used for gasometry (epoc Blood Analysis System, Alere Healthcare, Ottawa, Canada) and further collected in citrate tubes (9NC Coagulation Sodium citrat 3.2% tube, Vacuette,

Greiner-Bio One, Monroe USA). Lungs were removed and weighted. The unilobular lung was used for both BAL or histology and the multilobular lung for lung tissue analysis.

Obtaining and processing BAL

The left bronchus was tied and removed. In order to obtain BAL, the unilobular lung was connected by a syringe to a cannula placed into the trachea and 5 ml of saline (0.9% NaCl) were gently flushed through the lung five times. Cells were counted in the BAL using a haemocytometer (Neubauer, Marienfeld, Lauda-Königshofen, Germany) and slides were prepared by cytocentrifugation (Shandon Cytospin 4, Thermo Electron Corporation, Marietta, USA) to determine each cellular type by Diff-quick staining (Panreac Quimica SAU, Castellar del Vallès, Spain).

BAL was spun at 800 x g for 10 min and the supernatant was stored at -80ºC for subsequent analysis. The pellet from BAL was seeded in Petri dishes with RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Big Cabin, USA) at 37ºC for 1 h. Purified attached alveolar macrophages were collected with TRIzol reagent (Thermo Fisher Scientific, Madrid, Spain) and stored at -80ºC for further analysis. The purity of alveolar macrophages was determined by Diff-Quick staining.

Gene expression, cytokine and protein measurements

Total RNA was extracted from both lung tissue homogenate and alveolar macrophages using chloroform, isopropanol and ethanol. RNA purity was assessed by a spectrophotometer ND-1000 (Nanodrop, Thermo Fisher Scientific, Wilmington, USA). Total RNA was reverse-transcribed into cDNA, which was amplified in a qRT-PCR system (7500 Real-Time PCR System, Applied Biosystems, Thermo Fisher Scientific, Madrid, Spain) using SYBR green One-Step (Kapa Biosystems, Merck, Darmstadt, Germany) and the corresponding rat primers (Table 7). The expression of the following markers was analysed using ΔΔCt method and with GAPDH as housekeeping in lung tissue and macrophages:

Table 7. Rat primers

Gene	Forward Primer	Reverse Primer
GAPDH	5’ CTGTGTCTTTCCGCTGTT TTC 3’	5’ TGTGCTGTGCTTATGGTCTCA 3’
IL-1β	5’ AAAAATGCCTCGTGCTGTCT 3’	5’ TCGTTGCTTGTCTCTCCTTG 3’
TNF-α	5’ AACTCCCAGAAAAGCAAG CA 3’	5’ CGAGCAGGAATGAGAAGAGG 3’
iNOS	5’ CTTGGAGCGAGTTGTGGATT 3’	5’ GGTGGGAGGGGTAGTGATG 3’
Arg-I	5’ GGGAAGACACCAGAGGAGGT 3’	5’ TGATGCCCCAGATGACTTTT 3’
CCL2	5’ GCTGCTACTCATTCACTGGC 3’	5’ GGTGCTGAAGTCCTTAGGGT 3’
CXCL1	5’ CCACACTCAAGAATGGTCGC 3’	5’ GTTGTCAGAAGCCAGCGTTC 3’
TF	5’ ACAATCTTGGAGTGGCAACC 3’	5’ TGGGACAGATAGGACCCTTG 3’
Plasminogen	5’ AAACGAAAGGGACTCCAGGT 3’	5’ TCTCGAAGCAAACCAGAGGT 3’
PAI-1	5’ AGGGGCAGCAGATAGACAGA 3’	5’ CACAGGGAGACCCAGGATAA 3’
ZO-1	5’ GCCTCGAACCTCTACTCTCC 3’	5’ TGGTGGTGGTACTTGCTCAT 3’
Occludin	5’ TCCAACGGCAAAGTGAATGG 3’	5’ ACCTGTCGTGTAGTCGGTTT 3’

Total protein concentration in BAL and lung homogenate was quantified using a BCA protein assay kit (Pierce BCA protein assay kit, ThermoFisher, Rockford, IL, USA). Protein from the lung tissue homogenate was extracted using a protease inhibitor cocktail (Roche, Merck Millipore, Darmstadt, Germany) together with orthovanadate sodic and lysis buffer solution (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and multiplex assays were performed to assess IL-1β and TNF-α following the manufacturer’s protocol (Luminex, Merck Millipore, Darmstadt, Germany). Levels of TF (Cloud-Clone, Birmingham, United Kingdom), PAI-1 (LifSpan BioScience, Seattle, WA, USA) and FDP (Cloud-Clone, Birmingham, United Kingdom) in the BAL were determined by ELISA.

Histopathology

The right lungs were removed and perfused with a fixative solution of 4% paraformaldehyde and embedded in paraffin. Sections of 4 µm were stained with haematoxylin and eosin and were evaluated by three independent investigators blinded to treatment, using a Nikon Eclipse Ti microscope and ImageJ software (ImageJ 1.40g; W. Rasband, NIH, USA). Inflammation and damage were scored as follows: normal lung (0),

hemorrhage (0-1), peribronchial infiltration (0-1), interstitial edema (0-2), alveolar hyperplasia (0-3) and intralveolar infiltration (0-3). All the variables were summed, leading to the total histopathology score.

Activated partial thromboplastin time

Blood (1.3 ml) was collected from the abdominal aorta in tubes containing 11mM sodium citrate, and aPTT measured according to standard protocols (Echevarne Laboratories, Spain).

Statistical analysis

Before starting the experiment, a power analysis with the Gpower computer program (Faul & Erdfelder, 1998) indicated that a total sample of 48 animals (8 animals/group) would be required to detect large effects (0.5) with 80% power using an ANOVA between factors with alpha at 0.05 at the end of the experiment. We used 61 animals in total to achieve 8-12 animals/group at 48 h, as we had to take in consideration the mortality of some groups and further investigational assessments performed. In the legend of each figure the number of animals of each analysis is specified.

Gaussian distribution was tested applying by D'Agostino Pearson omnibus or Shapiro Wilk normality test. Tukey's method (Q level of 5%) was used to detect possible outliers. One-way analysis of variance (ANOVA) was performed to compare between the experimental groups and Bonferroni selected pair comparisons post-hoc test was used. Results were reported as mean ± standard error of the mean (SEM). Statistical analysis were performed with Prism 4.0 (GraphPad Software Inc, US) and statistical significance was set at $p<0.05$.

8.1.1.2 Results

Protective effect of ATIII on hATII cells

The administration of ATIII on hATII cells produced a significant reduction of pro-inflammatory markers iNOS and TNF-α when compared to the LPS group (Figure 15A and 15B). Moreover, when looking at the tight-junctions, gene expression of ZO-1 was significantly increased in the LPS+ATIII group compared to the LPS group (Figure 15C).

Although no significant differences were found, ATIII tended to increase the levels of occludin compared to the injured group (Figure 15D).

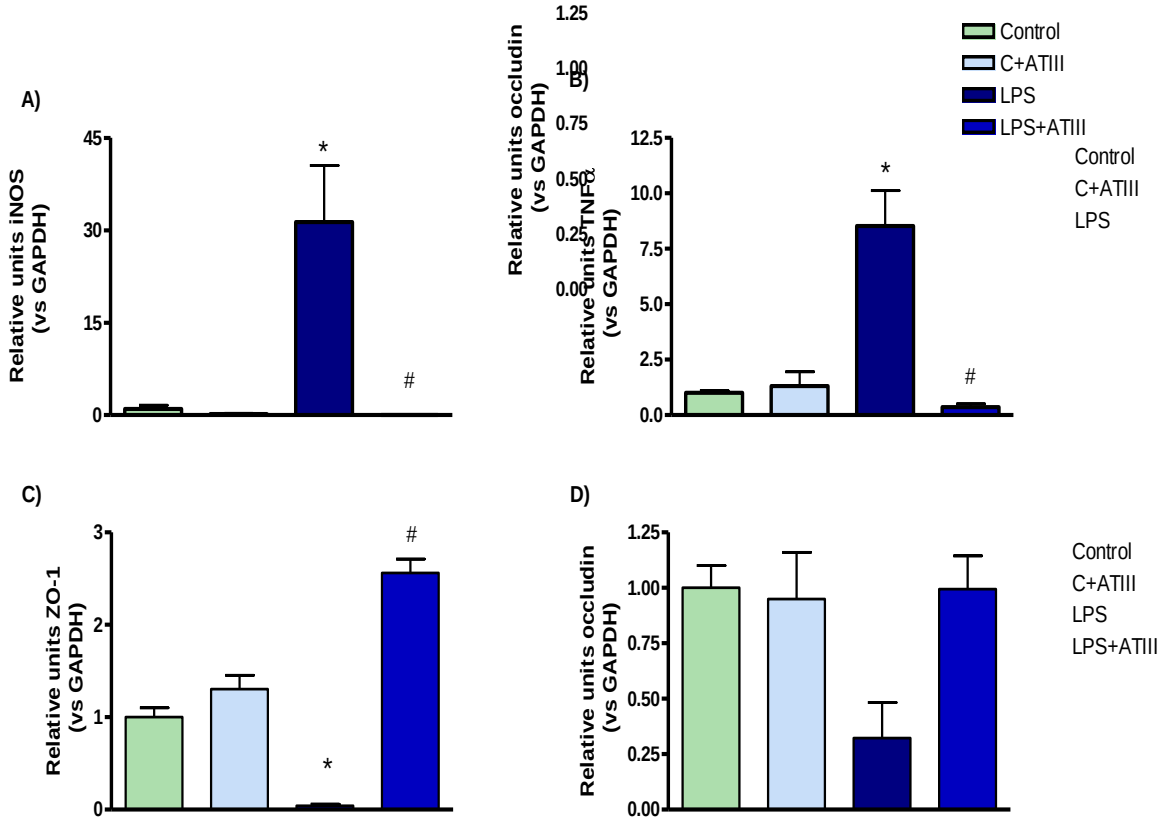


Figure 15. Effect of ATIII on hATII cells. Gene expression of A) iNOS and B) TNF-α C) ZO-1 and D) Occludin (n=3-5) in hATII cells from lung biopsies. RNA levels shown in the graphs are expressed in relation to

Body weight analysis and alteration of the alveolar-capillary barrier

In the first 24 h, all the studied groups lost weight, but HCl/LPS injured animals presented a stronger reduction. At 48 h HCl/LPS groups treated with both ATIII or ATIII+Heparin gradually increased weight compared to the HCl/LPS group, which continued losing weight, nevertheless no statistical differences were found (Figure 16A).

Regarding the lung weight, ATIII alone but not with heparin significantly decreased the lung/body weight ratio to control levels (Figure 16B). The increased protein concentration in the BAL of HCl/LPS group was reduced by both treatments to basal levels (Figure 16C).

In line with results obtained in lung weight and proteins the hypoxic state presented the same pattern, but differences were not statistically significant (Figure 16D).

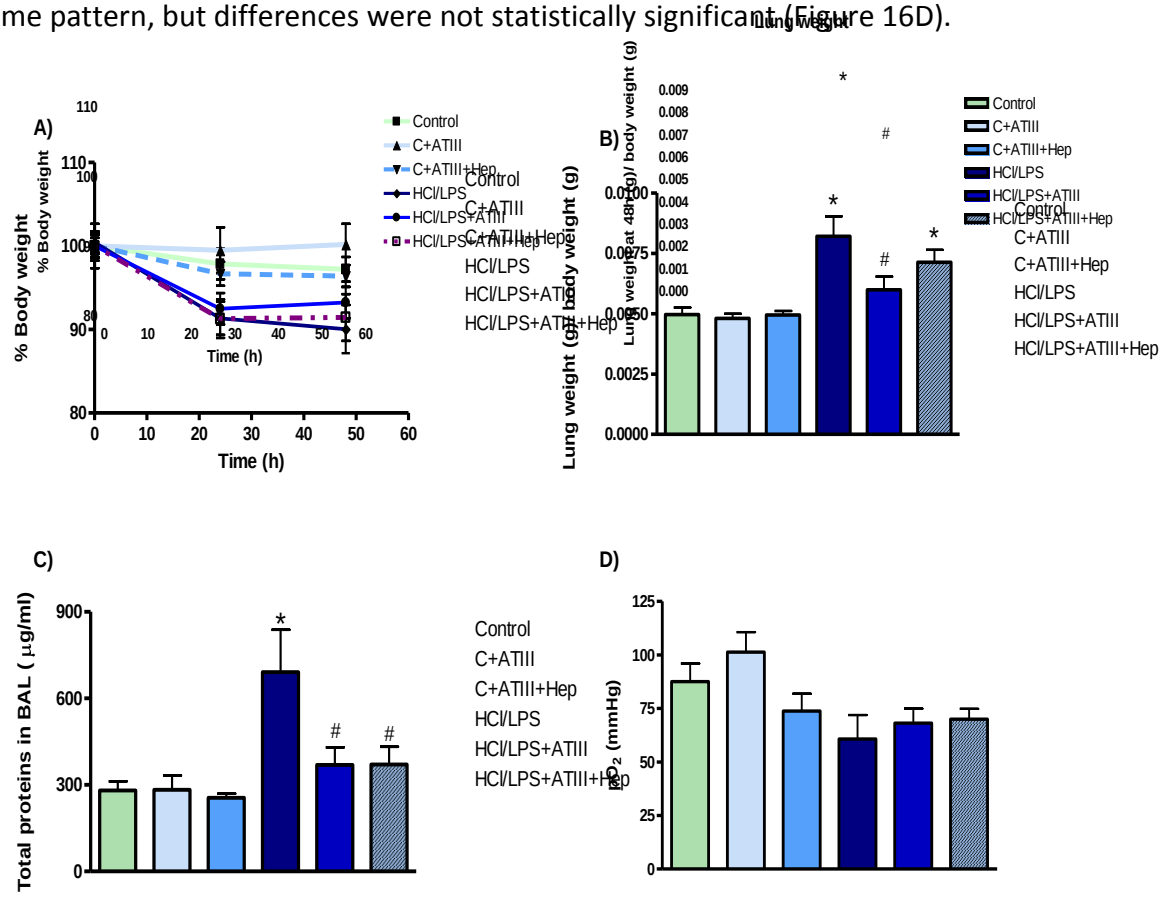


Figure 16: Body weight analysis and alteration of the alveolar-capillary barrier: A) Change in rat body weight at 24 h and 48 h after the induction of the injury. Percentage calculated in relation to baseline levels. (n=8-12). B) Lung/body weight ratio 48 h after injury (n=8-11) C) Protein concentration in bronchoalveolar lavage (BAL) at 48 h (n=6-9). D) Partial oxygen pressure (pO_2) 48 h after the injury (n=3-7). Data are represented as mean ± SEM.*p≤0.05 vs Control group, # vs HCl/LPS.

Coagulation

Expression of genes involved in the coagulation pathway was assessed in lung tissue 48 h after the injury. Compared to HCl/LPS group, treatment with ATIII alone significantly reduced TF, plasminogen and PAI-1 expression, while treatment with ATIII+Heparin only decreased PAI-1 expression (Figure 17A, 17B and 17C).

Coagulation protein expression were measured in BAL at 48 h. Analyses of coagulation proteins in BAL followed the same line than obtained results in lung tissue. TF and FDP were significantly decreased in the HCl/LPS+ATIII group compared to the HCl/LPS group, and a non-significant reduction was observed in the HCl/LPS+ATIII+Heparin group (Figure 17D and 17E). PAI-1 was reduced, unfortunately not significantly, by ATIII and ATIII+Heparin treatments (Figure 17F).

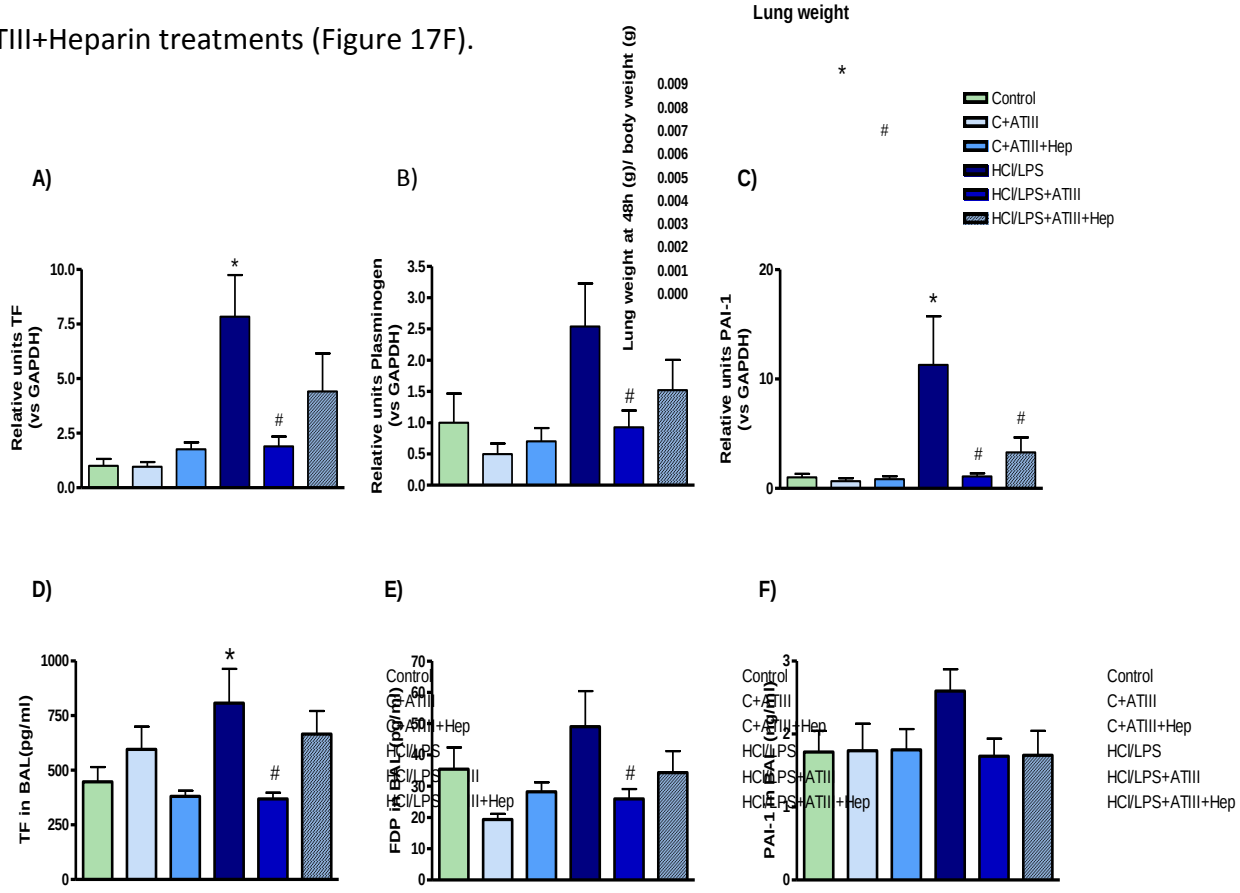
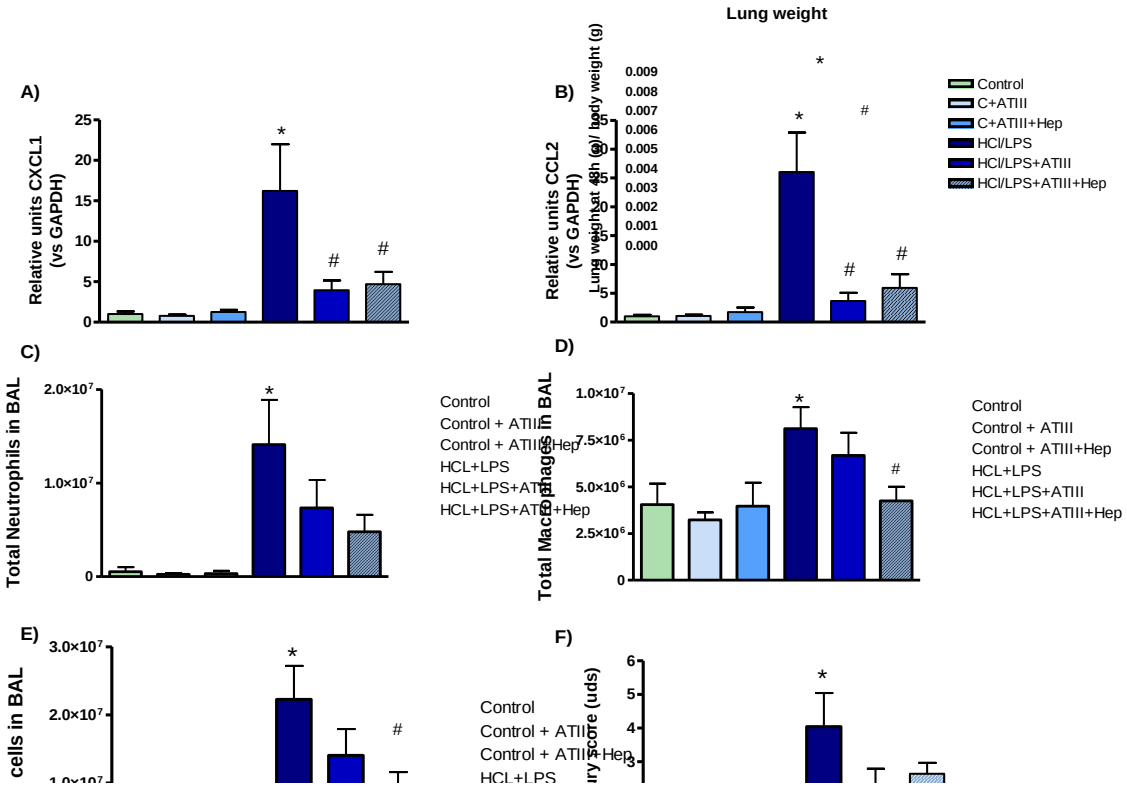


Figure 17: Coagulation factors levels of the experimental groups: Gene expression of coagulation markers: A) Tissue factor (TF), B) Plasminogen and C) Plasminogen Activator Inhibitor-1 (PAI-1) in lung tissue homogenate at 48 h after the induction of the injury. RNA levels shown in the graphs are expressed in relation to GAPDH expression (n=6-9). Protein levels in bronchoalveolar lavage (BAL) of D) TF, E) fibrin degradation products (FDP) and F) PAI-1. Protein levels are corrected for the total protein concentration in BAL (n=5-6). Data are represented as mean ± SEM.*p≤0.05 vs Control group, # vs HCl/LPS.

APTT analysis did not show systemic coagulation alterations in anti-coagulant treated groups (data not shown).

Cell infiltration and histology

Gene expression of CXCL1 and CCL2, neutrophil and macrophage chemoattractant chemokines, respectively, were significantly decreased by both, ATIII as well as ATIII+Heparin treatments compared to the HCl/LPS group (Figure 18A and 18B). In line with these results, total cells and total number of neutrophils and macrophages in the BAL were reduced in the treated groups compared to the HCl/LPS group; however this reduction just was significant in the total macrophage numbers and total cells of the ATIII+Heparin group (Figure 18C, 18D and 18E). Histological analysis of lung tissue after ATIII and ATIII+Heparin treatment did not differ to control group. Lung injury score was highly increased in HCl/LPS group compared to control indicating a reduced damage by ATIII and heparin in the treated groups (Figure 18F and 18G).



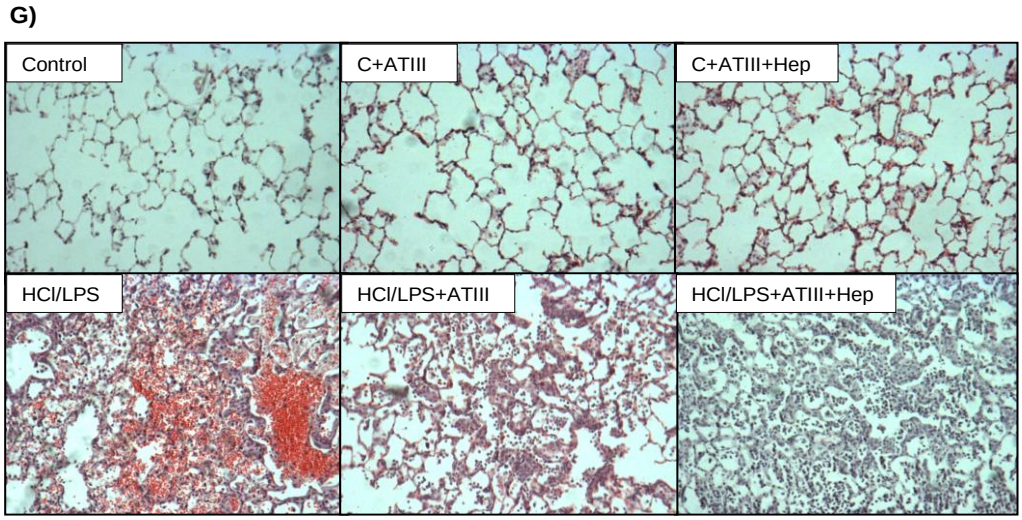


Figure 18: Analyses of bronchoalveolar lavage (BAL) infiltration and lung specimens: Gene expression of A) CXCL1 and B) CCL2 chemoattractant chemokines in lung tissue 48 h after damage (n=6-9). Absolute C) neutrophil, D) macrophages and E) total cell counts in the bronchoalveolar lavage (BAL) of rats 48 h after the induction of the injury (n=6-9). F) Lung injury score in animals 48 h after induction of the injury (n=4-7). G) Representative images of hematoxilin-eosin staining lung tissue sections 200x amplification. Data are represented as mean ± SEM.*p≤0.05 vs Control group, # vs HCl/LPS.

Inflammation response and tight junctions

At 48 h gene expression of TNF-α and IL-1β in lung homogenates were significantly reduced by nebulized treatments compared to the injured group (Figure 19A and 19B). At protein level, a tendency to decrease IL-1β was observed when administering ATIII or ATIII+Heparin to the injured groups but no differences were found while analysing TNF-α (Figure 19C and 19D).

Gene expression of tight junctions: ZO-1 and occludin were also analysed. Both treatments seemed to increase ZO-1 expression compared to the injured group, no significant differences were found (Figure 19E). Regarding occludin, ATIII+Heparin significantly increased its expression compared to the HCl/LPS group (Figure 19F).

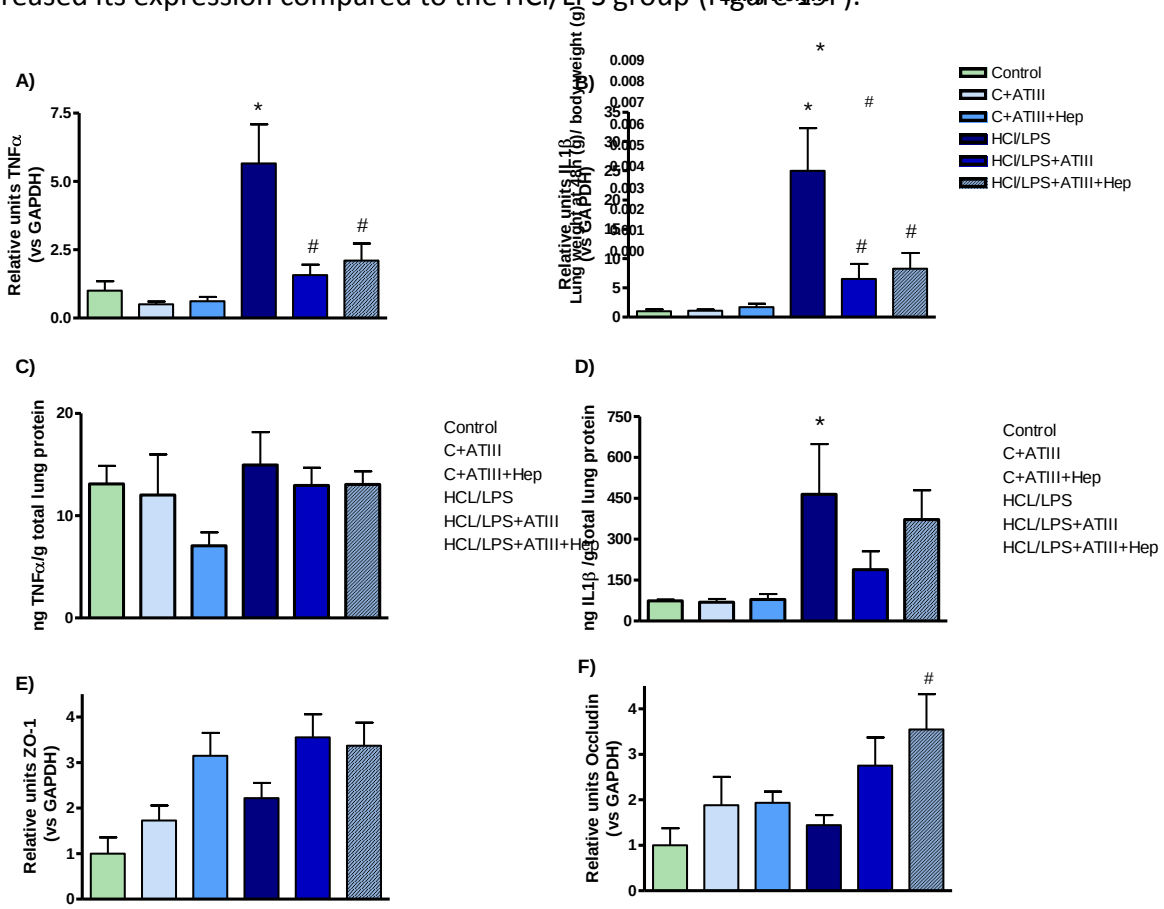


Figure 19: **Inflammatory response and tight junctions:** gene expression of A) TNF-α and B) IL-1β in lung tissue homogenate at 48 h after the injury (n=5-9). Protein levels in lung tissue of C) TNF-α and D) IL-1β (n=5-9). Gene expression of E) ZO-1 and F) Occludin. RNA levels shown in the graphs are expressed in relation to GAPDH expression and protein levels are corrected for the total protein concentration in lung tissue. Data are represented as mean ± SEM.*p≤0.05 vs Control group, # vs HCl/LPS.

Macrophage response to anti-coagulant therapy

Alveolar macrophages were isolated from the BAL of the animals 48 h after the induction of ALI. Mediators of both classical (iNOS) and alternative (Arg-1) pathway were decreased by ATIII treatment compared to the HCl/LPS group, although just Arg-1 was reduced by ATIII+Heparin (Figure 20A and 20B). Also, anti-coagulants declined the chemoattractant cytokines of neutrophils (CXCL1) and monocytes (CCL2) in the HCl/LPS group; however only the reduction of CCL2 in the ATIII+Heparin group was statistically significant (Figure 20C and 20D).

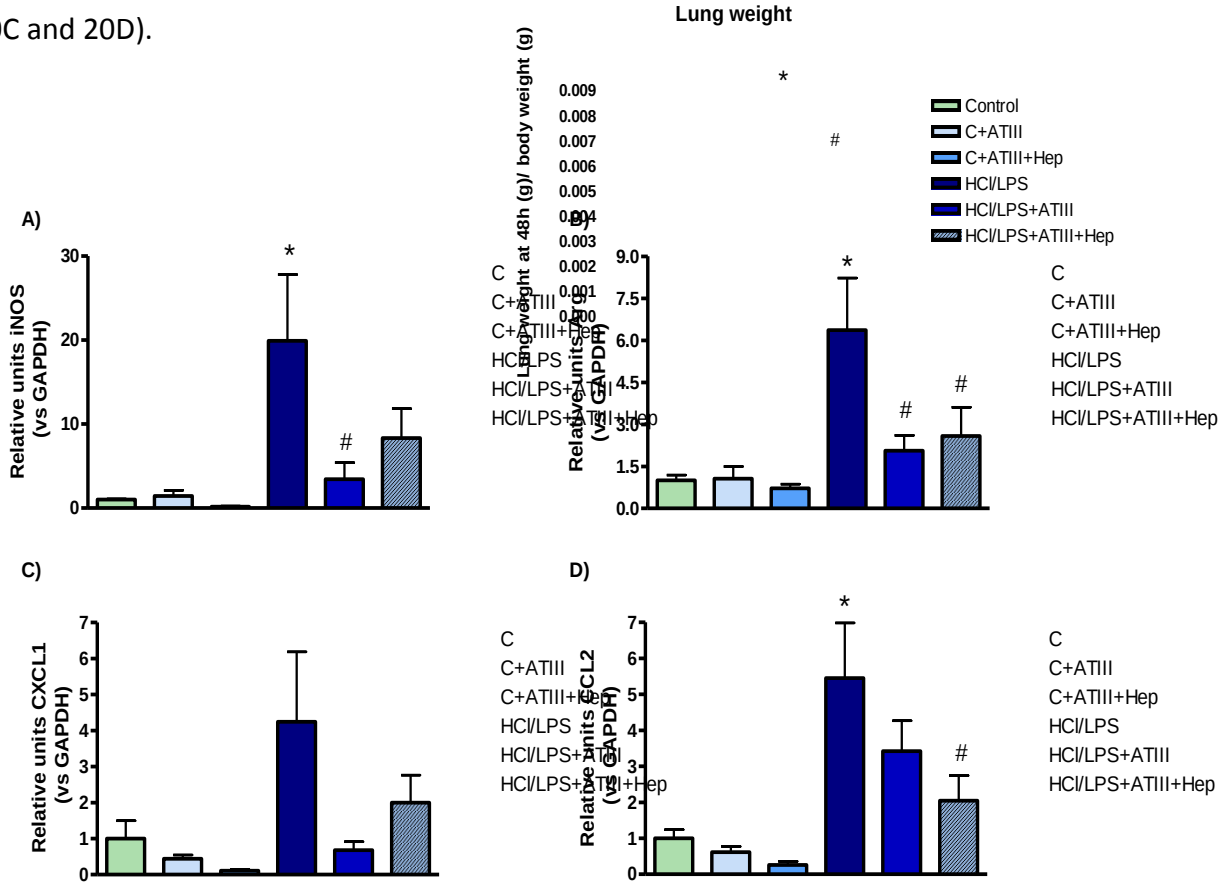


Figure 20: **Alveolar macrophages:** gene expression of A) iNOS, B) Arg-1, C) CXCL1 and D) CCL2 (n=3-8) in alveolar macrophages in BAL after the induction of the injury. RNA levels shown in the graphs are expressed in relation to GAPDH expression. Data are represented as mean ± SEM.*p≤0.05 vs Control group, # vs HCl/LPS.

8.2 Funding and scholarships

1. EFFECT OF ANTITHROMBIN FOR THE TREATMENT OF ACUTE LUNG INJURY

   Financial Entity: GATRA Award (GRIFOLS)

   Period: 2016 - 2017

   Colaborator
2. STUDY OF ALVEOLAR TYPE II CELLS AND MESENCHYMAL STEM CELLS TRANSPLANTATION TO TREAT ACUTE LUNG INJURY

   Financial Entity: Societat Catalana de Pneumologia (SOCAP)

   Period: 2016 - 2017

   Fellowship as a PhD student
3. IN VIVO AND EX VIVO TRANSLATIONAL MODELS OF INFECTIOUS LUNG INJURY

   Financial Entity: European Respiratory Society (ERS)

   Period: 2017

   Fellowship as a PhD student
4. MODELOS TRANSLACIONALES IN VIVO Y EX VIVO DE LA LESIÓN PULMONAR AGUDA

   Financial Entity: Sociedad Española de Neumología y Cirugía Torácica (SEPAR)

   Period: 2017

   Fellowship as a PhD student
5. EFFECT OF ANTITHROMBIN IN AN IN VITRO MODEL OF ACUTE LUNG INJURY

   Financial Entity: LABORATORIOS GRIFOLS, S.A.

   Period: 2014 - 2016

   Colaborator

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