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Optimization of the Therapeutic Potential of Umbilical Cord-Mesenchymal Stem Cells for Staphylococcus Aureus Induced Pneumonia

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Chapter 1. Introduction. Section A.

"Definition and epidemiology of acute respiratory distress syndrome"

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Abstract

Fifty years ago, Ashbaugh and colleagues defined for the first time the acute respiratory distress syndrome (ARDS), one among the most challenging clinical condition of the critical care medicine. The scientific community worked over the years to generate a unified definition of ARDS, which saw its revisited version in the Berlin definition, in 2014. Epidemiologic information about ARDS is limited in the era of the new Berlin definition, and wide differences are reported among countries all over the world. Despite decades of study in the field of lung injury, ARDS is still so far under-recognized, with 2 out of 5 cases missed by clinicians. Furthermore, although advances of ventilator strategies in the management of ARDS associated with outcome improvements—such as protective mechanical ventilation, lower driving pressure, higher PEEP levels and prone positioning—ARDS appears to be undertreated and mortality remains elevated up to 40%. In this review, we cover the history that led to the current worldwide accepted Berlin definition of ARDS and we summarize the recent data regarding ARDS epidemiology.

Keywords: Acute respiratory distress syndrome (ARDS); definition; epidemiology

Introduction

Acute respiratory distress syndrome (ARDS) is an acute inflammatory lung process, which leads to protein-rich non-hydrostatic pulmonary edema, causes refractory hypoxemia, increases lung “stiffness” and impairs the ability of the lung to eliminate carbon dioxide.

At a macroscopical level, computed-tomography (CT) studies gave

birth to the fascinating concept of "baby lung", suggesting a change of perspective from a "stiff" to a "small" lung (1). Furthermore, the CT study of the gravity effect on the lungs, using the prone position, helped to better understand how the "baby lung" was not an anatomical and static concept, but a functional one, visualized in a "sponge" model (2). High dead space fraction was discovered to be correlated to increased mortality in ARDS patients (3). In other words, the higher was the amount of lungs that did not participate to gas exchanges, the higher was the proportion of ARDS mortality. The continuous efforts aimed to improve the ARDS definition, together with the advanced diagnostic tools and therapeutic strategies, had an impact on the ability to recognize the onset of ARDS and to change the clinical history of ARDS. In this review, we will provide the readers the essential information to understand the process leading to the new Berlin definition and the change of ARDS epidemiology over 50 years since original Ashbaugh's definition of ARDS.

History of ARDS: the long path to define a syndrome - new acquisitions and limitations

The first description of ARDS probably belongs to Laennec, who defined it as "idiopathic pulmonary edema" in 1821 (4). The following century saw a number of traumatic injuries during the "big wars" period that eventually earned the definition of "wet lung" or "shock lung" to the unexplained lung edema (5,6). However, it was only in 1967 that Ashbaugh and colleagues termed, for the first time, "ARDS" a syndrome characterized by "acute onset of tachypnoea, hypoxaemia, and loss of compliance after a variety of stimuli" (7).

Ashbaugh et al. reported the presence of a specific clinical presentation seen in 12 adult patients and characterized by severe dyspnea and tachypnea, cyanosis not responsive to oxygen therapy, loss of lung compliance and presence of diffuse alveolar infiltration at chest X-ray evaluation, with a high mortality rate.

Since then, ARDS paradigm included the presence of a known risk factor for ARDS, severe hypoxemia despite high FiO2 delivery, bilateral pulmonary infiltrates and the exclusion of cardiogenic edema as a cause.

From the first ARDS definition, 50 years ago, different efforts have been dedicated to clarify the pathophysiology and the severity of this multifaceted syndrome.

In 1988, Murray and colleagues proposed a definition known as "expanded definition of ARDS". The authors took into account four different variables to which they assigned a score [0–4]: (I) the chest roentgenogram score, that describes the amount of pulmonary consolidation of the four quadrants; (II) the hypoxaemia stratified according to PaO_2/FiO_2 classes; (III) the PEEP level; and (IV) the compliance of the respiratory system. The final score, called "the Murray Lung Injury Score", is calculated as the sum of the single components score divided by the number of the accounted variables, and defines three categories: absence of lung injury (0), mild to moderate lung injury (1–2.5), and severe lung injury (>2.5) (8).

In 1994, the American-European Consensus Conference (AECC) defined acute lung injury (ALI) and ARDS as respiratory failure with: (I) acute onset; (II) presence of bilateral infiltrates at the chest X-ray; (III) pulmonary wedge pressure ≤18 mmHg or no clinical evidence of

high left atrial pressure (to rule out a cardiogenic cause of lung edema); and (IV) hypoxemia, independently of the PEEP level. The severity of hypoxia, defined the class of lung injury as ALI ($PaO_2/FiO_2 \leq 300$) or ARDS ($PaO_2/FiO_2 \leq 200$) (9).
Compared to the Murray Score, AECC definition of ARDS was characterized by the exclusion of a cardiogenic cause of edema, but it did not include the respiratory system compliance calculation at the diagnosis, and did not mention the PEEP level set.
Since respiratory failure due to ARDS is not just typical of the adult population but it is represented also in infants, AECC definition was clinically used either in adult or in pediatric critical care (10-12) to contextualize the grade of lung injury. However, oxygenation index (OI), proposed for the first time by Dr. Bartlett studying indications for ECMO in neonates with respiratory failure (13,14), is a commonly accepted indicator to describe lung injury in the pediatric population. OI is calculated as the product of mean airway pressure (in mmHg) × FiO_2 (in percent) × arterial partial pressure of oxygen (in mmHg).
In 2005, a group of experts from the University of Toronto proposed a formal consensus method to improve the AECC ARDS definition, using the Delphi technique (15). The Delphi technique consists in an individual survey of the participating panelists who anonymously receive group comments between iterations. The novelty of this approach included a clear definition of the acuity of the respiratory failure (<72 hours), the standardization of hypoxemia ($PaO_2/FiO_2 \leq 200$ setting a threshold of PEEP level ≥ 10 cmH_2O), the inclusion of the static respiratory system compliance (calculated with a tidal volume of 8 mL/kg of ideal body weight in a sedated patient with a

PEEP level ≥10 cmH_2O), and the presence of an ARDS predisposing factor (pulmonary versus extrapulmonary ARDS. Radiographic abnormalities introduced the concept of airspace disease involving ≥2 quadrants on frontal chest X-ray, and the role of echocardiography was mentioned to exclude a possible cardiogenic origin of the lung edema, under clinical indication.

In 2013, Villar and colleagues, proposed a refinement of the classification of severity of ARDS, aimed at assessing the ICU risk mortality, according to the PaO_2/FiO_2 ratio. The authors measured PaO_2/FiO_2 ratio at ARDS onset and 24 later, testing two different combinations of PEEP (≥5 and ≥10 cmH2O) and FiO_2 (≥0.5 and 1.0). The better ARDS risk stratification was obtained setting PEEP ≥10 cmH_2O and FiO2 ≥0.5 at 24 h after ARDS diagnosis, with mortality rates increasing from 17%, to 40.9%, to 58.1%, in mild, moderate and severe ARDS, respectively (16).

After 18 years of AECC definition, the need of a new definition of ARDS with more specific and generalizable criteria emerged.

Hence, the most recent revisited definition of ARDS was proposed by a “task force” endorsed by the European Society of intensive Care Medicine, and it is now known as the “Berlin definition” of ARDS (17).

Compared to the AECC definition, the ARDS Berlin definition clarified: (I) the acute onset, established within one week; (II) the characteristics of the bilateral lung infiltrates, on chest X-ray or CT scan; (III) the source of lung edema, without including pulmonary capillary wedge pressure cut-off; (IV) the standardization of the hypoxemia, calculated with a PEEP level ≥5 cmH_2O, and the

categorization of the lung injury into three grades of severity according to the PaO_2/FiO_2 ratio; furthermore, (V) if no predisposing condition was identified, as specified also in the Delphi consensus, an "objective" evaluation is mandate to rule out the cardiogenic origin of the lung edema. Berlin definition does not separate anymore, as the AECC definition of ARDS did, ALI and ARDS, unifying ARDS definition in a single entity graded into three classes of severity, according to the PaO_2/FiO_2 ratio and with a necessary minimum amount of PEEP (5 cmH_2O). This important step led not to overestimate patient hypoxemia just caused by lung atelectasis and then easily reversible with minimal PEEP (18). Consequently, ARDS definition could be applied to a less heterogeneous population, excluding patients with compromised oxygenation only in the absence of PEEP. The classification of ARDS into three increasing stages of severity—mild, moderate, and severe—according to the level of hypoxemia, significantly reflected an increased mortality rate (respectively: 27%, 95% CI, 24–30%; 32%, 95% CI, 29–34%; and 45%, 95% CI, 42–48%). Compared to AECC definition, predictive validity for mortality was significantly improved in the Berlin definition of ARDS (area under the ROC, 0.577, 95% CI, 0.561–0.593 versus 0.536, 95% CI, 0.520–0.553).

Berlin definition was proved to be adaptable also for pediatrics (19). De Luca et al., on behalf of the Respiratory Section of the European Society for Pediatric and Neonatal Intensive Care, in a retrospective, international, multicenter study of infants and early children with ALI or ARDS—according to the AECC definition—reported that the predictive validity for ARDS mortality using the Berlin definition was

confirmed and comparable to the results showed in adult population (17). This finding was mainly correlated to the introduction of the new category of “severe ARDS”, which demonstrated a higher mortality among the Berlin definition classes of ARDS. These results have been further recently confirmed by Barreira et al. (20).
Still, despite the contributions of several scientists, an entirely satisfactory definition of ARDS proofed to be an elusive goal.
The principal hindrance is due to the intrinsic nature of ARDS which is not a disease, with a univocal and straightforward trajectory of treatment and recovery, but a syndrome, composed of a multifaceted means of diagnosis and determined by different causes, with as many different clinical histories. Moreover, the pathological hallmark of ARDS (i.e., noncardiogenic pulmonary edema) cannot be easily identified by current clinical tool, and any clinical definition must rely on accessible proxies (e.g., hypoxaemia, chest X-ray).
The reliability of hypoxia definition is still controversial. Measurements of PaO_2 varies on time, PEEP level, and FiO_2 (16,21-24).
The chest X-ray, used to classify ARDS based on the presence of bilateral infiltrates, is not completely accurate, either for a relevant interobserver variability (25,26) or if compared to other diagnostic imaging tests, such as ultrasonography (27) and computed tomography (28). Promising diagnostic advances have been recently proposed, using ultrasound to rule out a cardiogenic source of lung edema and to predict ARDS in blunt trauma patients (29-31), and using low-dose chest CT to monitor and redirect the treatment strategy of the ventilator setting (32,33). Along with measurements of

SpO2/FiO2 by pulse-oximetry, in lack of PaO2/FiO2 measurements (34), lung ultrasound (35), can be extremely appealing in settings with limited ICU resources, as lately proposed by the Kigali modification of the Berlin definition (36).

Etiology of ARDS and ARDS phenotypes

ARDS is the result of a wide spectrum of different risk factors, which can be either local or systemic (37).

ARDS can be classified according to the origin of the inflammatory insult as direct lung insult or indirect lung injury.

The first one is commonly known as "pulmonary ARDS" (ARDSp), the second one as "extrapulmonary ARDS" (ARDSexp) (38,39) (Table 1).

Table 1. Main determinants of pulmonary and extrapulmonary ARDS

Pulmonary ARDS
Pneumonia
• Bacteria
• Virus
• Fungi
• Parasites
Aspiration
Toxic gases inhalation
Smoking
Non-protective ventilation (i.e., VILI)
Lung contusion/trauma
Thoracic surgery
Drowning
Pulmonary vasculitis
Fat embolism
Extrapulmonary ARDS
Non-pulmonary sepsis
Blood transfusions (i.e., TRALI)
Trauma
Pancreatitis

Drug reaction
Burns
Cardio-pulmonary bypass
Non-cardiogenic shock

Table 1 legend. ARDS, acute respiratory distress syndrome; VILI, ventilator induced lung injury; TRALI, transfusion related acute lung injury.

While pneumonia, extrapulmonary sepsis, and aspiration are the most frequent clinical risk factors for ARDS (40,41), chronic diseases such as obesity (42,43) and diabetes (44,45) have been associated to a lower occurrence of ARDS. The "obesity paradox", as called in a recent meta-analysis of the literature (42), is still hard to understand, due to the lack of a clear pathophysiologic mechanism behind these findings (46), and with some conflicting preclinical data (47). On the other side, instead, diabetes might be protective by means of a depressed immune response of the organism against an inflammatory insult (44). Among the modifiable risk factors for ARDS, alcohol abuse emerges (48,49) and impaired immune response involving alveolar macrophages is reported (50,51). Since the observation that positive cumulative fluid balance is independently associated to higher mortality rate in patients with lung injury (52), a number of study raised in order to assess the impact of conservative fluid management or active fluid removal on mortality in ARDS patients, but results are yet not conclusive (53,54).
High occurrence of ARDS has been linked also to demographic and environmental risk factors. These include older age (55), non-Caucasian race (56), defined genetic variants (57) and ozone exposure (58).

Some authors, studying patients with a number of predisposing conditions of ARDS and with associated risk factors for the development of ARDS, conceived and validated the acute lung injury prediction score (LIPS). However, the best LIPS score cut-off was able to predict ARDS with sub-optimal sensitivity (69%) and specificity (78%) (59).

Recently, thanks to an analysis of clinical and laboratory data from two large randomized clinical trials (RCTs)—the ARMA (60-62) and the ALVEOLI trials (63)—the ARDS Network identified two different phenotypes of ARDS. Phenotype 2—named hyperinflammatory subphenotype—is characterized by higher prevalence of inflammation, shock, sepsis and metabolic acidosis than phenotype 1.

The relevance of the ARDS subphenotypes classification showed not just a difference about clinical and laboratory data between the two clinical conditions, but more important, that subphenotype 2 recognizes a cohort of patients with worse clinical outcome, and with higher mortality, than phenotype 1 (64). A further analysis by the same group of the Fluid and Catheter Treatment Trial (FACTT) simplified the 2 phenotypes using a three-variable model (IL-8, bicarbonate and tumor necrosis factor receptor-1) that had a different answer in terms of fluid management strategy. In other words, ARDS subphenotypes can predict the severity of the disease and can direct the treatment choice (65).

Pathology of ARDS

As stated, diffuse alveolar damage (DAD) leading to high

permeability pulmonary edema is considered the histopathologic hallmark of ARDS (66-69). For this reason, while a “prefect” bedside definition of ARDS should capture all patients with DAD, without false positives, this is not always the case. This was evident since the first description by Ashbaugh and colleagues, later proved by the group of Vincent JL (70). More recently, these findings were further confirmed by Guerin and colleagues, studying patients with non-resolving ARDS (71).

The authors reported that DAD was markedly represented in non-resolving ARDS with no difference among the increasing level of ARDS severity, according to the Berlin definition. The presence of DAD, at the pathology examination, has also a relevant role to identify the clinical history of the disease.

In a recent meta-analysis of lung biopsy series for patients with ARDS, the presence of a pathologic pattern of DAD was associated with higher mortality compared to ARDS with no DAD (72).

ARDS is pathologically categorized into acute, subacute and chronic phase (73,74). The acute phase—exudative—(within 6 days) see the presence of either interstitial or alveolar edema, with acute inflammatory cells and red blood cells into the alveoli. Both endothelial and epithelial layers are damaged, and hyaline membranes develop in the alveoli. The subacute phase—proliferative—(between 7–14 days) shows the reabsorption of the edema, the proliferation of the alveolar epithelial type II cells and the fibroblastic infiltration with deposition of collagen fibers. The chronic stage—fibrotic—(following 14 days) presents the clearance of the neutrophils, the abundance of alveolar mononuclear cells and macrophages into the alveoli and

marked fibrosis, with a repairing process involving the alveolar epithelium.

ARDSp and ARDSexp are two different features of ARDS, not just in terms of etiology, but also regarding the characteristics of the lung lesions. Of note, ARDSp is characterized by the more pronounced alveolar collapse, fibrinous exudative material and edema of the alveolar walls, compared to ARDSexp (75). Furthermore, ARDSp has an increased collagen content, with a prevalence of the extracellular matrix remodeling (76).

Epidemiology

ARDS incidence—an underestimated syndrome

Since the beginning of the recognition of ARDS as defined entity, several studies tried to provide essential information about ARDS epidemiology. Most of them were constructed following the AECC definitions.

It is surprising the huge variability of ARDS incidence, including all ARDS categories, in various population-based studies (77) between different continents such as South America (10.1 per 100,000 person-years) (78), Europe, (17.9 per 100,000 person-years) (79), Australia (34 per 100,000 person-years) (80) and USA (78.9 per 100,000 person-years) (81) with a relevant geographic diversity. Furthermore, in countries of the same continent such as Europe, ARDS occurrence varies consistently, ranging from 10.6 per 100,000 person-years in Finland (82), to 17.9 per 100,000 person-years in Scandinavia (79), to 25.5 per 100,000 person-years in Spain (55). This is the case also for hospitalization based studies (77), which showed a ARDS proportion

ranging from 7.1% (83) to 12.5% (84) of incidence proportion of all ICU admissions in Europe to 19% in 14 ICUs of Ireland (85), among the admitted patients (Table 2).

Table 2. Main epidemiologic studies on ARDS incidence after AECC definition

First author, year of publication (reference)	Study period (years)	Incidence of all ARDS categories (per 100,000 person-years-population-based studies) or percentage (%, hospitalization based studies)	Incidence of moderate and severe ARDS categories (per 100,000 person-years-population-based studies) or percentage (%, hospitalization based studies)
Sigurdsson et al., 2013 (102)	1988–2010		3.65–9.63
Nolan et al., 1997 (101)	1990–1994		7.3–9.3
Luhr et al., 1999 (79)	1997	17.9	13.5
Bersten et al., 2002 (80)	1999	34	28
Brun-Buisson et al., 2004 (83)	1999	7.1% (of all ICU admissions)	6.1% (of all ICU admissions)
Rubenfeld et al., 2005 (81)	1999–2000	78.9	58.7
Manzano et al., 2005 (55)	2001	25.5	23
Sakr et al., 2005 (84)	2002	12.5% (of all ICU admissions), 19.1% (of all mechanically ventilated patients)	10.6% (of all ICU admissions), 16.5% (of all mechanically ventilated patients)
Li et al., 2011 (93)	2001–2008		81 (in 2001), 38.3 (in 2008)
The Irish Critical Care Trials Group, 2008 (85)	2006	19%	
Caser et al., 2014	2006–	10.1	6.3

(78)	2007		
Linko et al., 2009 (82)	2007	10.6	5
Villar et al., 2011 (91)	2008–2009		7.2
Bellani et al., 2016 (41)	2014	10.4% of all ICU admissions, 5.5 cases per ICU bed per year	

Table 2 legend. ARDS was defined using the Berlin definition nomenclature: all ARDS categories include mild, moderate and severe ARDS. AECC, American-European Consensus Conference; ARDS, acute respiratory distress syndrome.

Data incidence of ARDS in pediatric population shows less variability between different continents such as Europe [2.2 per 100,000 person-years in the Netherlands (86) and 3.9 per 100,000 person-years in Spain (87)] and Australia [2.6 per 100,000 person-years (88)], with the highest ARDS incidence in USA with 9.5 ARDS cases per 100,000 person-years (89). However, the less relevant geographic difference of ARDS incidence in children has to be interpreted considering the lower ARDS incidence in pediatrics compared to adult population.

Differences in availability of diagnostic methodologies, health resources, hospital admission practices and emergency medicine networks clearly impact on the recognition of critical diseases. In a careful overview on the global burden of critical illness in adults, Rubenfeld and coworkers observe how the availability of ICU beds differs between high-income countries and less advantaged countries. This disproportion might lead, therefore, to different filtering of acute critical diseases—such as ALI—due to different levels of care, and to a wide disproportion in incidence estimation (90).

Recent insights about the epidemiology of ARDS, according to the current Berlin definition, came from the LUNG SAFE study, an International, multicenter, prospective cohort study conducted in Intensive Care Units in 50 countries (41). Due to its design, the LUNG SAFE study cannot provide "population-based" estimates of ARDS incidence, or prevalence, but only ICU. ARDS occurrence was estimated to be 10.4% in all ICU admissions and in more than double (23.4%) among the mechanically ventilated patients. In a further analysis by country, the incidence of ARDS was the highest in Oceania, with 0.57 cases/ICU bed/year, followed by Europe, North America, Africa, South America and Asia, with the lowest ARDS occurrence of 0.27 cases/ICU bed/year. These findings are in line with previous epidemiologic studies (80,81,91-94) and might be interpreted in the light of the different distribution of ICU resources (95).

One of the most striking finding of the LUNG SAFE study was that of all ARDS patients, clinicians missed almost 40% of ARDS diagnosis, despite a specific online training on ARDS diagnosis, which was offered to all investigators. Even among severe ARDS, diagnosis was missed, in at least one patient out of 5. These results are in line with another study that estimated ARDS under-recognition by clinicians in up to 50% of cases, despite the accepted use of AECC definition and staff training (55). In LUNG SAFE, organizational and patient factors were reported to be associated with higher clinician recognition of ARDS in invasively ventilated patients. Among the first ones, the authors observed higher nurse-to-patient and physician-to-patient ratios. Patient variables associated with a lower ARDS under-diagnosis were younger patient age, lower predicted body weight,

higher non-pulmonary SOFA score, a lower PaO_2/FIO_2 ratio, and the presence of pneumonia, pancreatitis, neoplastic or immune or hematological disease, trauma at admission, absence of ARDS risk factors and concomitant presence heart failure.

ARDS clinical management

A second relevant finding of LUNG safe regards the therapeutic management of ARDS. Mechanical ventilation and therapeutic adjunctive measures to target ARDS are still not yet optimized, with significant potential future improvement. About 4 out of 5 patients were treated with a PEEP level below 12 cmH_2O. Plateau pressure, a well-known parameter of respiratory mechanics associated with mortality in ARDS patients (60), was measured in only 40.1% of the ARDS population. Among patients with ARDS, about 1 out of 3 patients with ARDS did not receive protective mechanical ventilation, with either a plateau pressure above 30 cmH2O or a tidal volume above 8 mL/kg of predicted body weight. Large tidal volumes are far from a diffuse and accepted clinical practice, but they mirror the results showed in two recent RCTs (96,97). Furthermore, data suggest that clinicians seem more incline to adjust FiO_2 than to increase PEEP to treat hypoxemia. Finally, adjunctive measures such as recruitment maneuvers and prone positioning were used in a minority of ARDS patients (20.9% and 7.9%, respectively) (41).

ARDS mortality—still a critical challenge

Mortality in ARDS patients is still high. The LUNG SAFE study reports a hospital mortality of 40%, with a significant increase across

the ARDS severity categories, in line with Berlin definition (34.9%, in mild ARDS; 40.3% in moderate ARDS; 46.1% in severe ARDS). These results proof that the Berlin definition of ARDS is an excellent predictor of outcomes in the studied population. In this context, Laffey and colleagues examined the predictors associated with outcome in a secondary analysis of the LUNG SAFE study. In 2,377 ARDS patients, who received mechanical ventilation, the authors observed that lower ventilation pressure (peak, plateau and driving), higher PEEP level, and lower respiratory rate confirm their validity as predictors of improved survival from ARDS (98). These results are in line with data from previously observed clinical trials (60,63,99,100). ARDS mortality decreased over the years probably also thanks to the improved therapeutic management of the ventilatory settings (60,63,99-101). In the early 90s, Nolan and colleagues reported a hospital mortality of 59% in moderate and severe ARDS in a population based study conducted in Australia (102). Brun-Buisson et al. described similar findings in the late 90s, in a hospitalization based study conducted in 78 ICUs in Europe, with a proportion of 57.9% even including mild ARDS (83). Since then, ARDS hospital mortality decreased to a stable level of about 40%, including all ARDS categories (25,84), and of about 45% considering moderate-severe ARDS (84,91,93). These findings are corroborated by mortality data of the recent LUNG SAFE study (41) *(Figure 1A,B).* Moreover, data from two large population based study in the northern European countries, analyzing patients with higher severity of ARDS (moderate and severe ARDS), report a 90-day long term mortality among 38–41.2% (79,103), and when overall ARDS is analyzed, Linko et al.

described a 90-day mortality of 47% (82). While in adult population ARDS has shown a clear trend of decrease in mortality over the last decades, these results are not univocally confirmed in pediatric population, as reported with conflicting results in two recent meta-analysis of the literature (104,105). Over the last few years, different RCTs have proposed promising results in terms of ARDS mortality improvement (53,106,107). Unfortunately, this enthusiasm has to be well weighted in sight of the study design (108,109). Enrolled subjects of RCT are rigorously selected, and the generalization of the results from a RCT might be deceptive if applied to the entire population.

Figure 1.

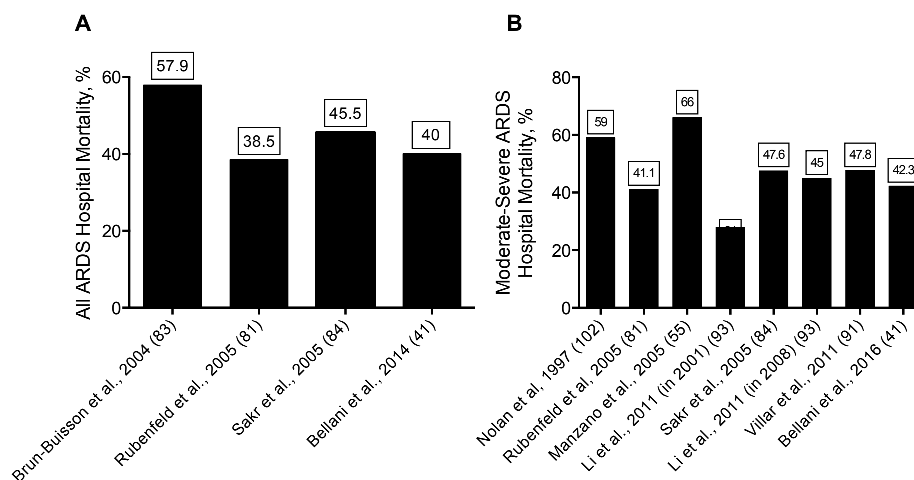

Figure 1 legend. Hospital mortality reported in the main epidemiological studies after AECC ARDS definition in all ARDS categories (mild, moderate and severe) (A) and in the subgroups of moderate-severe ARDS (B). On the X-axis, the studies are chronologically ordered based on the study period. In panel (B), moderate–severe ARDS hospital mortality in the study by Li et al. [2011] is reported in two different years of study, 2001 and 2008 (93).

The studies are reported as first author et al., year of publication (41,55,81,83,84,91,93,102). AECC, American-European Consensus Conference; ARDS, acute respiratory distress syndrome.

Conclusions

After 50 years of study, ARDS still looks nowadays a threatening enemy to defeat. Definition of ARDS has been improved over the years. Even so, the definition of hypoxemia in different settings and timing is debatable and the agreement about the optimal diagnostic imaging is yet to be reached. Studies on ARDS incidence consistently show that the disease is not rare, albeit often not recognized by clinicians. ARDS is undertreated and basic ventilator strategies are not yet standardly optimized, despite major advances in the management of mechanical ventilation and non-ventilatory strategies, aimed at preventing the VILI. Finally, ARDS mortality remains high. This suggests the need of a commonly accepted therapeutic strategy for ARDS, which should be prerogative of all the countries including the less developed ones.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Chapter 1. Introduction. Section B.

"The Safety and Efficiency of Addressing ARDS Using Stem Cell Therapies in Clinical Trials"

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Abstract

Acute Respiratory Distress Syndrome (ARDS) is a complex and debilitating disease of the lungs, which continues to have a high mortality rate and huge disease burden on patients. Incidence is rising, possibly due to greater awareness leading to more diagnoses rather than a change in the underlying rate. It arises from multiple etiologies, though pathogenic infection, termed pneumonia, is the most prevalent and widely studied. The distinct pathophysiology and rapid evolution of ARDS makes it uniquely challenging with regard to therapeutics development and, to date, no medicines are licensed for specific therapy. Antibiotics, ventilation, and other organ support remain intervention standards.

1.1. ARDS

Acute Respiratory Distress Syndrome (ARDS) is a complex and debilitating disease of the lungs, which continues to have a high mortality rate and huge disease burden on patients. Incidence is rising, possibly due to greater awareness leading to more diagnoses rather than a change in the underlying rate. It arises from multiple etiologies, though pathogenic infection, termed pneumonia, is the most prevalent and widely studied. The distinct pathophysiology and rapid evolution of ARDS makes it uniquely challenging with regard to therapeutics development and, to date, no medicines are licensed for specific therapy. Antibiotics, ventilation, and other organ support remain intervention standards.

1.2. Definition and Diagnosis

In 1967 Ashbaugh and colleagues [1] recognized a specific clinical pattern characterized by an acute onset of elevated respiratory rate, hypoxemia resistant to high FiO_2, bilateral lung infiltrates on chest X-ray in the absence of cardiogenic edema and the presence of a heterogeneous number of risk factors that can lead to the same syndrome [2]. The first formal definition of ARDS was developed at the American-European Consensus Committee in 1994 [3].

1. Acute onset.
1. Presence of bilateral infiltrates at the chest X-ray.
2. Pulmonary wedge pressure ≤ 18 mmHg or no clinical evidence of high left atrial pressure (to rule out a cardiogenic cause of lung edema).
3. Hypoxemia, regardless of the applied levels of positive end expiratory pressure.

The levels of hypoxemia were used to stratify the severity of lung injury as ALI ($PaO_2/FiO_2 \leq 300$) or ARDS ($PaO_2/FiO_2 \leq 200$). An updated and improved version was proposed in 2012 during a task force meeting of experts in Berlin, from which the last ARDS definition takes its name [4].

1. Rapid onset of symptoms that cannot be attributed to any underlying cause.
2. Bilateral infiltration of leukocytes from surrounding tissue to the airspace, as identified by chest X- ray.

3. Exclusion of hydrostatic causes of edema leading to respiratory failure.
4. Impaired blood oxygenation as assessed by arterial:alveolar oxygenation ratio (PaO_2/FiO_2), with relative levels denoting mild (≤300), moderate (≤200), or severe (≤100) ARDS.

Beyond initial diagnosis criteria, scoring systems have also been devised to assess degree of injury, including APACHE [5] and Murray [6] scales for adults and PRISM [7] and PIM [8] scales for pediatric patients. It was subsequently shown that the Berlin definition criteria of ARDS are adaptable also for the pediatric population [9, 10]. ARDS can arise from pneumonia, sepsis, and overaggressive ventilation strategies, while other less common causes include smoke inhalation, near-drowning, and poisoning [11, 12]. Only very recently has an accurate picture of in-hospital ARDS incidence been attained [13, 14], although prevalence as regards to the general population is still somewhat unclear.

1.3. ARDS Management

ARDS is an acute condition, generally arising within a week of an inciting event (e.g., pneumonia) essentially occurs and is resolved over a matter of days to weeks, and has a distinct acute hyperinflammatory phase [15, 16, 17]. The high and imminent mortality means issues such as eventual chronic fibrosis development may be secondary considerations compared to immediate restoration of lung function, specifically adequate blood oxygenation. Broad-spectrum antibiotics, given as early as possible where infection is

known or suspected to be present, as is support of gas exchange, usually via assisted ventilation or in more severe cases, if available, extra-corporeal membrane oxygenation (ECMO) support [18]. The core treatment of ARDS is based on supportive measures that primarily aim at gaining time to allow the antibiotic treatment or the patient immunologic system to defeat the primary cause of ARDS. Mechanical ventilation strategy includes: the use of low tidal volume ventilation, inspiratory pressure, higher positive end expiratory pressure and using prone positioning, and administering neuromuscular blockers in higher severity ARDS [19, 20, 21]. Current guidelines supporting protective mechanical ventilation is aimed at preventing the risk of ventilator-induced lung injury [22] in patients with ARDS. The pathophysiologic reason behind this is based on the concept of the "baby lung" [23], which has reduced compliance caused by the severe decrease of lung aerated volumes, and is defined based on CT scans over the course of initial ARDS diagnosis (0-7 days) and the subsequent fibroproliferative response (15–20 days) [24, 25]. It might also be argued that despite the era of protective mechanical ventilation [26], large tidal volumes are still fundamental of mechanical ventilation daily management [13], and adjunctive measures such as proning [27] and neuromuscular blockers [28], while proven to have a positive effect on outcome, have not been fully implemented yet [13].

Furthermore, ARDS mortality remains high, despite considerable advances in terms of antibiotic stewardship and antibiotic treatment options [29, 30, 31, 32] and fluid management [31, 33] to face up the two main risk factors leading to ARDS, pneumonia, and sepsis [13].

The multimodal nature of ARDS also necessitates a multimodal approach to treatment, which cannot be met with traditional small molecule or recombinant protein medicines. A wide variety of anti-inflammatory pharmacologicals, proteins, and antibodies have demonstrated promise in the laboratory but have failed in clinical trial (reviewed in [34]). Of relevance here is the fact that injury and repair responses are intimately linked phenomena at the cell signaling and transcriptional level, and blanket inhibition of inflammation may delay or even prevent essential regenerative processes that restore lung tissue to normal function [35, 36, 37]. An idealistic treatment of ARDS should target multiple mechanisms and biologic pathways instead of aiming at a single exclusive target. This hypothesis is supported by: (1) the heterogeneity of the mechanisms involved in the lung injury, (2) decades of negative randomized clinical trials with pharmacologic and other therapies and (3) the new upcoming evidences about the role of biologically distinct pathways and response to treatment in specific subsets of ARDS patients – as recently observed by the identification of specific endotypes and phenotypes [38, 39, 40].

In light of these considerations, cell-based therapies with mesenchymal stem/stromal cells (MSCs) have been proposed as novel therapeutics in the treatment of ARDS, due to their broad immunomodulatory effect during inflammation, their enhancement of host defense through antimicrobial mechanisms, and their lung healing potential through the activation of repairing mechanisms [41].

1.4. Epidemiology

ARDS occurrence remains as high as 10.4% of all ICU admissions, rising up to 23.4% of mechanically ventilated patients. ARDS still appears to be an under-recognized syndrome, with up to 40% as recently reported in the Large observational study to Understand the Global impact of Severe Acute respiratory FailurE (LUNG SAFE) study [13]. Furthermore, ARDS mortality is still estimated as high as 40%.

1.5. Pathology

The pathophysiologic hallmark of ARDS is the well-known diffuse alveolar damage (DAD), which leads to the characteristic protein-rich nonhydrostatic pulmonary edema during ARDS [33, 42, 43, 44]. Considering that a match among clinical signs and pathology findings is far from being perfect, it is relevant that DAD is highly associated to the category of nonresolving ARDS [45] and that DAD predicts higher mortality in ARDS compared to patients with non-DAD ARDS [46]. Excess alveolar fluid blocks gas exchange at the alveolar epithelial surface, while surfactant inactivation by infiltrating albumin and other substances [47, 48, 49] damages fluid tension leading to alveolar collapse. Even for patients undergoing ventilation, eventually ventilation/perfusion (V/Q) mismatch occurs where oxygen delivered to the pulmonary space fails to reach the bloodstream and there is impairment of CO_2 clearance, leading to systemic hypoxemia and hypercapnia.

At the cellular and molecular level, the initial phase of ARDS arising from pulmonary infection involves resident pulmonary macrophage

defenses being overcome, and the epithelial cell layer lining the alveolus begins to produce proinflammatory cytokines [50]. These signals initiate the recruitment of circulating leukocytes, including neutrophils, macrophages, and B-cells, to migrate up a chemokine concentration gradient to the lung tissue, where these cells release matrix metalloproteinases that enzymatically digest connective tissue facilitating entry to the airspace [51]. Recruited infiltrating cells produce a wide range of noxious substances including superoxide radicals [52, 53, 54, 55, 56, 57, 58], leukotrienes [59, 60, 61, 62, 63, 64], and antimicrobial peptides [65, 66, 67] and further cytokine cocktails in an attempt to destroy the infectious agents. In ARDS, a section of these substances is uncontrolled and induce damage to the lung tissue itself leading to fluid buildup and surfactant loss. Finally as the alveoli fill with liquid and the integrity of the epithelial and endothelial barrier is compromised, gas exchange deteriorates and hypoxemia and hypercapnia result. In later phases of ARDS during systemic inflammatory response syndrome (SIRS), patients may suffer from functional immune-suppression and have heightened susceptibility to additional infection [68, 69, 70]. While ARDS presents an acute onset after the exposure to the causing factor, its evolving process starts with acute exudation and infiltrates of acute inflammatory cells into the alveoli within 1 week. During the second week a subacute deposition of collagen fibers are produced by fibroblasts. The syndrome typically resolves through a chronic stage characterized by alveolar macrophage infiltration into the alveoli and a fibrotic repairing process of the lung parenchyma [11].

1.6. MSCs for ARDS: A Promising Potential Therapy

MSCs have generated interest for a wide range of regenerative medicine applications and appear to have broad immunomodulatory properties rendering them attractive for autoimmune and other inflammatory disorders. Initial safety has been amply demonstrated [71, 72], although some questions remain over hypersensitivity to repeat dosing in non-ARDS models which will only be answered over time [73, 74]. As further conditions are likely to have MSCs licenses as a medicinal therapeutic, this will be of added relevance where MSC therapy may be employed in the same patient for sequential separate disease instances.

MSCs accumulate in the lungs initially after IV administration [75, 76, 77] and can remain viable there for up to 24 h [78] after which this time MSCs disappear, suggesting any therapeutic effect has already been conferred to the host; however, it is not clear what happens to MSCSs after they have left the lungs. Interestingly, recent stem cell therapy research in other diseases has indicated the lung is crucial to licensing of MSC to ultimately allow their beneficial effects [79, 80, 81], suggesting an immunomodulatory effect that outlasts the MSC's presence in the body.

The MSC's responsiveness to the injury milieu [82, 83, 84, 85] and diverse range of effects on multiple pathological and repair processes has made them, theoretically, an ideal candidate for ARDS interventional studies [86] (Fig. 1.1). Indeed, many of the leukocyte subpopulations involved in ARDS pathology have been shown to have direct interaction with MSCs [87, 88, 89], while direct antibacterial activity [65] is to be considered an added bonus. Also, the fact that

most patients with ARDS require tracheal intubation to permit support of lung function opens up the possibility of direct delivery to the lung airspace of MSC or MSC derivatives [90], although since patients are also almost certain to have IV access obtained and there have been, to date, no demonstrated efficacy advantages observed in delivery of MSC intra-tracheally over IV [91] in ARDS models, this remains a point of urgent future investigation.

Fig. 1.1

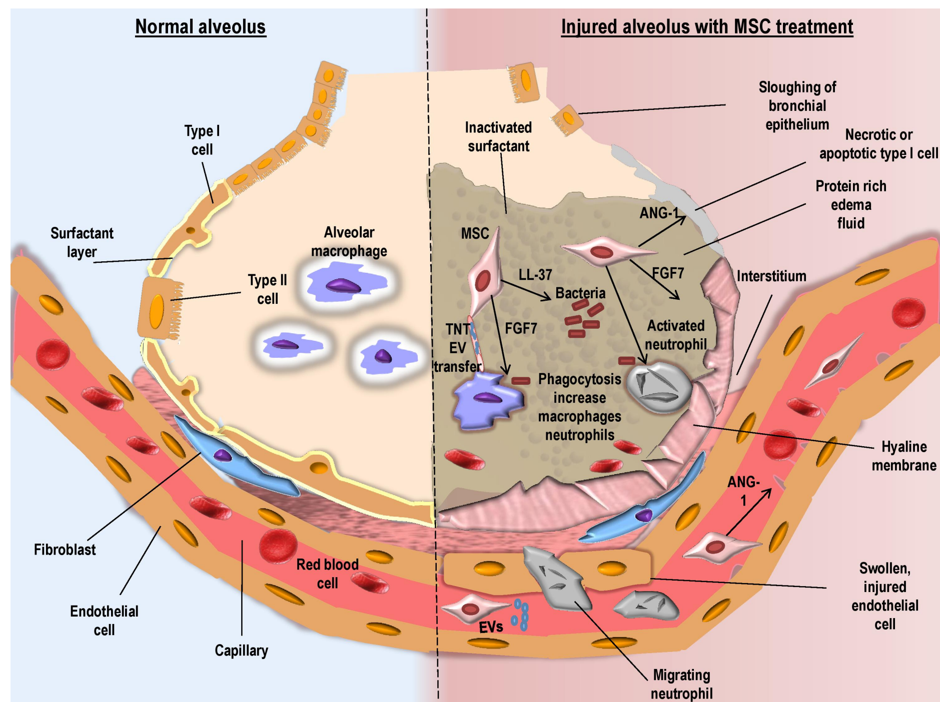

Figure 1.1 legend. Possible mechanisms of action of the MSC in the ARDS alveolus and surrounding vasculature. Left side: healthy alveolus. Right side: during injury, protein-rich edema fluid and inflammatory cells permeate the alveolus. MSCs have been

demonstrated to alleviate the pathophysiological symptoms of ARDS through the secretion of paracrine factors, cell-to-cell contact and mitochondrial transfer (TNT and EV release). Indirect and direct contact of MSCs has been shown to reduce the permeability of alveolar epithelium and increase fluid clearance. Through the secretion of ANG-1, endothelial and epithelial repair is increased. Reduction of neutrophil migration also improves endothelial and epithelial barriers. Release of KGF promotes an increase in alveolar fluid clearance. Bacteria clearance is achieved through the direct release of the antimicrobial peptide LL-37 or through increased phagocytosis by neutrophils and macrophages mediated by the release of cytokines including FGF-7 or by transfer of micro vesicles through TNTs. Mitochondrial transfer to epithelial cells also increases surfactant release. A few mechanisms of action of MSCS in ARDS have been displayed in this diagram. *EVs* extracellular vesicles, *TNT* Tunnelling Nanotubules, *ANG-1* angiopoietin-1, *PGE2* prostaglandin E2, *KGF* keratinocyte growth factor, *LL-37* peptide β-cathelicidin.

Inviting as MSC therapy may appear, the timecourse and whole body nature of ARDS demand a specific set of considerations around preparation, storage, and administration to be resolved before deployment of MSCs to the clinic. Translation of experimental findings from animal models to the patient are also problematic, with uncertainty regarding dose scaling and testing of the human MSCs destined for patients in nonhuman models, where DNA/RNA sequence and protein/ligand binding incompatibilities warrant extra attention.

1.7. Ex Vivo Human Lung Models

In a first report in 2009, Lee et al. explored the potential role of allogenic human MSCs in the treatment of ARDS induced by *E. coli* endotoxin in an ex vivo perfused human lung [92]. The authors administered allogeneic human MSCs or MSCs-derived conditioned medium at 1 h after the injury induction. Fluid balance was normalized by the decrease of the extravascular lung water, restoring the alveolar fluid clearance (AFC) and by improvement of lung endothelial barrier permeability. The alveolar epithelial fluid transport was in part coordinated by the keratinocyte growth factor (KGF), secreted by the MSCs, which restored the correct function of the amiloride-dependent sodium transport.

Some years later, it was observed that clinical-grade MSCs, administered via the lung perfusate or directly into the right middle lobe, could decrease neutrophil influx and inflammation, effectively cleared bacteria, confirming the contributing role of the KGF, and restored the clearance of the alveolar fluid, with a relevant improvement of the lung histology [93]. In 2014, it was observed in a follow-on study that intravenous administration of clinical-grade allogenic human MSCs could increase the AFC at 4 h. The role of KGF in the AFC was confirmed by the study of a neutralizing antibody of KGF that could decrease the AFC activity [94]. Recently, the same group explored the effects of microvesicles (MVs) released by human mesenchymal stem cells in their established ex vivo human lung perfusion model of bacterial pneumonia. The investigators reported positive results highlighting the beneficial effects of MSC MVs in increasing lung antibody forming cells, in decreasing the lung

permeability, and improving the bacterial clearance, particularly when MSCs were pretreated before isolation of MVs with a Toll-like receptor 3 agonist, polyinosinic:polycytidylic acid (Poly(I:C)) [95].

1.8. Mechanistic Considerations for Clinical Therapy

Allogenic MSCs have the ability to avoid detection of the immune system and it is assumed that this evasion is due to the low expression of the major histocompatibility complexes (MHC) I and II, while MSCs also do not express CD80 and CD86 which are identified T-cell stimulators [96]. Therefore autologous MSC administration is considered a viable therapeutic option as the likelihood of an immune response is extremely low.

The routes of administration will influence the MSCs ability to differentiate, their immunogenic effect, and ultimately their survival [97]. Some studies have contradicted the MSC's proposed ability to evade immunological detection. MHC II protein expression analysis on MSCs has been shown to be higher than originally documented [98, 99, 100]. In vivo studies have also shown that allogenic MSCs are not immune privileged and have the potential to cause an immune response, while other research has contradicted findings and stated MSCs are immune privileged [101, 102, 103, 104].

MSC efficacy has been demonstrated in multiple preclinical models of ARDS [90, 93, 94, 105, 106, 107, 108, 109, 110, 111], while MSC products including conditioned medium and extracellular vesicles have also shown promise [91, 94, 107, 112, 113, 114, 115, 116, 117]. These are interesting in that they avoid safety and cryostorage issues associated with whole cell delivery and may be more compatible with

direct delivery to the airspace by nebulizer. MSCs have been shown to reduce inflammation and improve bacterial clearance [107] through direct antimicrobial peptide release such as LL-37 and indirectly through the modulation of phagocytic activity in BAL monocytes [108] as well as alveolar resident monocytes specifically [93].
More recently, MSCs have been documented to protect from injury via direct interaction through tunneling nanotubules (TNT) or formation of connexin 43 gap junctions [118, 119, 120, 121, 122, 123, 124, 125]. It has been demonstrated that mitochondrial transfer from MSCs to alveolar epithelial cells improved survival after endotoxin injury [118], while in a rat model of COPD, iPS-derived MSC mitochondrial transfer to bronchial epithelial cells was also observed [126]. MSCs have been shown to transfer mitochondria to macrophages in vitro and in vivo, improving macrophage function and enhancing phagocytosis [127]. In a mouse model of *E. coli*-induced pneumonia it was reported that therapeutic effect was dependent on transfer of MSC mitochondria to alveolar macrophages through TNT, enhancing antimicrobial activity and phagocytosis [128]. While it is unclear whether the mechanism of action in vivo is due to an enhancement of a normal mitochondrial function or restoration of dysfunctional mitochondria, there is some evidence for damaged mitochondrion, e.g., downregulated NDUFB8 (complex I) and ATP synthase (complex V), in ARDS meaning the latter is a distinct possibility [129, 130].

1.9. Production Considerations for Clinical Cell Therapies

For ARDS, autologous stem cell therapy is not an option as there is

insufficient time to isolate and expand patient MSCs while the rapid onset nature of ARDS demands a cryopreserved MSC product. Cryoprotective agents are used to protect the cellular components from crystal formation and osmotic shock and membrane damage during the slow freezing process, preserving the fine structures of cells [131]. For clinical applications, MSCs are typically frozen to at least −150 °C at a controlled rate of 1–5 °C per minute in 5% or 10% dimethyl sulfoxide in an electrolyte solution and added protein, typically human serum albumin [132]. Despite extensive optimization, the process can cause damage and affect cell viability [133, 134, 135] and inadequate insight into how MSCs function after systemic infusion remains an issue [134, 136, 137, 138, 139].
Freeze-thawed MSCs, in comparison to cells harvested from continuous cultures, have diminished immunomodulatory properties as well as a reduced responsiveness to proinflammatory cytokines [140]. The immunomodulatory effects of MSCs is affected by cryopreservation, launching a heat shock protein response [141]. In vivo experiments have shown that cryopreserved are less well tolerated. In a clinical application where predominant indications included graft versus host disease (GvHD) and tissue injury in hemorrhagic cystitis, therapeutic properties of freeze-thawed and freshly harvested MSCs were compared. A 100% response rate was observed in patients treated with fresh cells at a low passage compared to patients treated with cryopreserved freeze-thawed cells at a higher passage, with cryopreserved MSCs eliminated faster by compliment after exposure to recipient blood [140]. The thawing process can damage cell surface proteins and this abnormality attracts the binding

of complement initiating clearance by phagocytosis [142, 143, 144]. After complement exposure, there is an 80% decrease in cell viability in cryopreserved cells compared to a 50% decrease in fresh MSCs [141, 145]. However activation of complement may not be negative as recognition of opsonized MSCs are hypothesized to induce an M2 phenotype, producing anti-inflammatory mediators [140].
Macrophages can display various phenotypes, with the two being described as M1 and M2. M1 phenotypes are generated by the classical pathway [146, 147] and produce abundant inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-12, and reactive oxygen species. M2 phenotypes are generally activated by the alternative pathway and express a variety of lectins, protein, and scavenger receptors [146, 147, 148].
While various research have demonstrated that umbilical cord (UC) [149, 150, 151, 152] and adipose tissue (AT)-derived [153] MSCs have a faster population doubling time than bone marrow (BM) sourced, attaining human-sized doses of the order of 10^9 cells is still a daunting task. Additionally, there is currently no data published on whether there is an upper limit on MSC population doubling that still retains therapeutic efficacy. Kern et al. compared the senescence ratio of AT-MSC to BM-MSC and found that BM-MSC had a growth threshold of passage 7, whereas AT-MSC had a threshold of 8 [154], but efficacy itself may be lost long before senescence arises. In addition, MSCs isolated from patients with advanced age [155, 156], diabetes [157], rheumatoid arthritis [158], or indeed ARDS itself [159] have decreased activity, including lower regenerative and differential potential and therefore autologous MSC therapy in

patients with significant chronic comorbidities may not be a promising approach in any case. Downregulation of inflammatory marker receptors may render MSCs isolated from such patients less responsive to the injury microenvironment and hence of lower overall therapeutic value [146, 147, 159].
Beyond the conventional MSC therapeutics, human embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) as new cell types have also been investigated in immunoregulation and have shown encouraging results [160, 161, 162, 163]. iPSCs are immunomodulatory in a mouse model of allergic inflammation [164], while their systemic administration inhibited serum levels of IgE and TH2 cytokines (IL-4, IL-5 or IL-13) with better survival and engraftment rate after transplantation compared to adult tissue derived MSCs [163, 165]. There are also, variations in age related to DNA methylation levels, which is correlated to differential abilities, with ESC-derived iPSC having a higher proliferation and regenerative capacity [166, 167]. However, caution is still required as genetic abnormalities remain an area of concern in iPSCs [170]. Clinical studies using iPSCs found some cells in the study contained genetic abnormalities, and were consequently not used. Cells taken from elderly patients to be reprogrammed for administration can come with increased risks of genetic abnormalities [170], demonstrating the need for screening of cells before infusion if autologous cells are ever to be used in elderly patients.

1.10. Clinical Trials - Demonstrating Safety in ARDS

Clinical trials utilizing MSCs for ARDS patients are in their infancy

and focused on safety, tolerability and feasibility concerns. Tolerability and feasibility concerns. We are far from being able to claim MSC therapy is a viable option for ARDS. However, during the last decade, promising preclinical evidence supports the hypothesis of a potential benefit in treating ARDS patients with MSCs [171]. Despite the many studies into the potential clinical benefit of MSCs in ARDS that have been proposed over the last years and posted on clinicaltrials.gov , most of them are still currently ongoing or they lack a status update. Furthermore, few studies have disclosed the initial safety results (Table 1.1). The first clinical trial where MSCs were used to treat ARDS was reported in 2014 (NCT01902082), where Zheng and coworkers administered 1×10^6 AT-MSCs/kg of body weight or saline in a 1:1 fashion in 12 patients with moderate and severe ARDS. The investigators reported that the administration of allogeneic MSCs was feasible and safe with no infusion toxicities or serious adverse events related to MSCs in the treatment group and with no differences in terms of adverse events and biomarkers of lung injury between the MSCs and the placebo group [172].

Table 1.1

List of clinical trials reported on clinicaltrials.gov about mesenchymal stem/stromal cells in ARDS—current status and key findings.

Study	NCT	Reference/url	Design	MSC type	Dose	Population	Status	Findings
Mesenchymal Stem Cells (MSCs) for Treatment of Acute Respiratory Distress Syndrome	02804945	https://clinicaltrials.gov/ct2/show/results/NCT02804945	Phase 2 – open label	Allogeneic Human Mesenchymal Stem Cells (hMSCs)	3×10^6 cells/kg one time on day 1 over about 1-2 hours	ARDS $PaO_2/FiO_2 \leq 200$	Recruiting	In progress

(ARD) in Patients with Malignancies								
Adipose-derived Mesenchymal Stem Cells in Acute Respiratory Distress Syndrome	01902082	https://clinicaltrials.gov/ct2/show/NCT01902082	Phase 1 – "Triple blinded" (Participant, Care Provider, Investigator)	Allogeneic adipose-derived hMSCs	$1x10^6$ cells/kg body weight within 48 hours of enrollment	ARDS $PaO_2/FiO_2 < 200$	Unknown	Published: no infusion toxicities or serious adverse events related to MSCs administration
Human Umbilical Cord Mesenchymal Stem Cells (MSCs) Therapy in ARDS (ARDS)	03608592	https://clinicaltrials.gov/ct2/show/NCT03608592	Early phase 1	Umbilical cord derived mesenchymal stem cells (UCMSCs)	$60x10^6$ cells suspended in 100ml normal saline after randomization in 30-60 minutes	ARDS $PaO_2/FiO_2 < 200$	Not yet recruiting	In progress
Human Mesenchymal Stem Cells for Acute Respiratory Distress Syndrome	01775774	https://clinicaltrials.gov/ct2/show/NCT01775774	Phase 1 – open label	Allogeneic Bone Marrow-Derived hMSCs	3 cohorts with 3 subjects/cohort who receive doses of 1, 5 and $10x10^6$ cells/kg predicted body weight (PBW)	ARDS $PaO_2/FiO_2 < 200$	Completed	Published: No pre-specified infusion-associated events or treatment-related adverse events
Human Mesenchymal Stem Cells for Acute Respiratory Distress Syndrome (START)	02097641	https://clinicaltrials.gov/ct2/show/NCT02097641	Phase 2 – Triple Masking (Participant, Care Provider, Investigator)	Allogeneic Bone Marrow-derived hMSCs	A single dose of $10x10^6$ cells/kg PBW over approximately 60-80 minutes	ARDS $PaO_2/FiO_2 < 200$	Completed	Published: No predefined MSC-related
Mesenchymal Stem Cells for Multiple Organ Failure after Cardiac Surgery	03552848	https://clinicaltrials.gov/ct2/show/NCT03552848	Pilot – open label	Mesenchymal stem (stromal) cells (MSC)	$1x10^6$ cells/kg of body weight intravenously once every 4 days for 4 times	Failure > 2 organs and: 1. In case of ventilation $PaO_2/FiO_2 < 100$ 100% oxygen demand 2. Sequential organ failure assessment (SOFA) ≥ 10, multiple organ dysfunction (MOD) ≥ 10	Not yet recruiting	In progress
Mesenchymal Stem Cell in Patients with Acute Severe Respiratory Failure (STELLAR)	02112500	https://clinicaltrials.gov/ct2/show/NCT02112500	Phase 2 – open label	Bone marrow derived mesenchymal stem cells	Not reported	ARDS $PaO_2/FiO_2 < 200$ Not clear if AHRF or ARDS?	Unknown	
Repair of Acute Respiratory	03042143	https://clinicaltrials.gov/ct2/s	Phase 1 dose escalation pilot	Orbcel-C (Human umbilical cord	Dose not specified -	ARDS $PaO_2/FiO_2 \leq 27k$	Recruiting	In progress

Distress Syndrome by Stromal Cell Administration (REALIST)		how/NCT030 42143	study / Phase 2 - Quadruple Masking (Participant, Care Provider, Investigator, Outcomes Assessor)	derived CD362 enriched MSCs)	Maximum tolerated dose from the phase 1 – single infusion	Pa		
Human Umbilical-Cord-Derived Mesenchymal Stem Cell Therapy in Acute Lung Injury (UCMSC-ALI)	02444455	https://clinicaltrials.gov/ct2/show/NCT024 44455	Phase 1/2 - open label	Umbilical-cord-derived mesenchymal stem cell (UC-MSC)	5×10^5/kg once a day, a total of three times - Maximum tolerated dosage without side effects	ARDS Oxygenation index:200 < $PaO_2/FiO_2 \leq 300$ mmHg	Unknown	
Using Human Menstrual Blood Cells to Treat Acute Lung Injury Caused by H7N9 Bird Flu Virus Infection	02095444	https://clinicaltrials.gov/ct2/show/NCT020 95444	Phase 1/2 – open label	Menstrual blood stem cells	$1\sim10\times10^7$ cells/kg, 2 times a week, 2 weeks for infusion	H7N9 infection and critical lung tissue injury	Unknown	
A Phase 1/2 Study to Assess MultiStem Therapy in Acute Respiratory Distress Syndrome (MUST-ARDS)	02611609	https://clinicaltrials.gov/ct2/show/NCT026 11609	Phase 1/2 – Quadruple Masking (Participant, Care Provider, Investigator, Outcomes Assessor)	MultiStem	Low versus High MultiStem dose	Moderate to Severe ARDS	Recruiting	Active, not recruiting
Human Umbilical-Cord-Derived Mesenchymal Stem Cell Therapy in Paraquat Poisoning Induced Lung Injury (UCMSC-PQLI)	02444858	https://clinicaltrials.gov/ct2/show/NCT024 44858	Phase 1/2 – Single Masking (Participant)	Umbilical-cord-derived mesenchymal stem cell (UC-MSC)	5×10^5 cells/kg, once a day, a total of three times	Paraquat induced lung injury	Unknown	
Treatment of Severe Acute Respiratory Distress Syndrome with Allogeneic Bone Marrow-derived Mesenchymal Stromal Cells	02215811	https://clinicaltrials.gov/ct2/show/NCT022 15811	Phase 1 – open label	Allogenic bone marrow-derived mesenchymal stromal cells (BM-MSC)	Not reported	ECMO patients with viral induced ARDS	Unknown	
A Pilot Study Using Placenta Derived Decidual Stromal Cells for Toxicity and Inflammation with Special	02175303	https://clinicaltrials.gov/ct2/show/NCT021 75303	Phase 1/2 – open label	Decidual stromal cell therapy	1×10^6 cells/kg, at one or more occasions at weekly intervals dependent on clinical response	Unclear criteria including: - Acute Lung Injury - Decidual Stromal Cells - Stem Cell Transplantation	Unknown	

Focus to the Allogeneic Hematopoietic Cell Transplantation Setting						- Inflammation		

In 2015, Wilson J.G. and coworkers tested the safety of BM-MSCs in a multicenter phase 1b dose-escalation study in patients with moderate-severe ARDS, with PaO_2/FiO_2 less than 200 mm Hg, a positive end- expiratory pressure (PEEP) $\geq$ 8 cmH_2O, and bilateral infiltrates at the frontal chest X-ray (NCT01775774). Nine patients were enrolled and three groups of three received three doses of MSCs intravenously (1, 5, or 10 $\times$ 10^6 MSC/kg ideal body weight). The investigators reported no significant difference in biomarkers of inflammation (IL-6, IL-8), lung epithelial (receptor for advanced glycation end products - RAGE) and endothelial injury (Ang-PT2) among the groups. MSC administration was safe and the authors reported neither infusion-associated events nor serious adverse events. The viability of the MSCs infused ranged from 50–63% [173].
The same investigators recently reported the findings of a double-blind multicenter randomized phase 2a clinical trial testing the safety of BM-MSCs versus placebo in ventilated patients with moderate-severe ARDS, with PaO_2/FiO_2 < 27 kPa and PEEP $\geq$8 cmH_2O. Patients randomly received in a 2:1 fashion 1 × 10 BM-MSCs/kg ideal body weight or placebo. The primary objective of this investigation was the safety of MSCs in an intention to treat analysis (NCT02097641). No patient in the MSCs treatment group experienced any adverse respiratory or hemodynamic events. The treatment group had higher APACHE III score, minute ventilation, and PEEP compared to placebo. No statistically significant 28-day mortality

difference was observed among treatment and placebo group, even after adjustment with the APACHE III score, while a trend in a lower number of ICU-free days to day 28 was reported in the treatment group compared to placebo. Of importance, there was higher absolute 28-day and 60-day mortality in the MSC group, although it is unclear at this point if there is any clinical significance to this result or if it was related to variability in MSC viability on administration or some other quality issue.

Furthermore, a higher severity of illness at baseline - quantified by the SOFA and APACHE III scores - was present in the MSC group compared to the placebo group and mortality in the MSC group and in the placebo group was lower and higher than anticipated, respectively. However, this RCT was not powered for efficacy, as per the Food and Drug Administration mandate to clearly demonstrate safety before targeting lung oxygenation or compliance as in a phase 2b trial. The viability of the MSCs infused ranged from 36–85% [71].

The range in MSC viability was unanticipated and only discovered after study completion. The authors reported a significantly higher MSCs viability after centrifugation when MSCs were thawed compared to when the cells were washed to remove dimethyl sulfoxide during preparation. Based on these findings the investigators conducted a post-hoc analysis and observed that plasma angiopoietin-2 levels in the intermediate and highest tertiles of MSCs viability were significantly lower in the MSCs treatment group at 6 h after administration compared to placebo, and albeit nonsignificantly, the oxygenation improved at day 2.

These results suggest that the administration of MSCs with a high

viability is required to target an improvement in efficacy. Recent experimental data on the comparison of different cell products reports that fresh BM-MSCs are 14% more viable compared to cryopreserved ones [174]. Furthermore, delivery of MSC immediately upon thawing instead of thawing and washing the MSCs could enhance MSC viability, as observed by Matthay MA et al [71]. This is an unusual finding, as washing of MSC in physiological buffer has not been considered traditionally to have any impact on viability, and warrants further investigation.

Simonson and colleagues reported data on the clinical outcomes of two patients with severe ARDS who received allogenic BM-MSCs. MSC administration was safe and no adverse events were reported during infusion. The investigators reported a decrease of plasma and BAL proinflammatory cytokines, chemokines, miRNAs, and biomarkers of epithelial apoptosis and alveolar-capillary fluid leakage. One patient developed pneumonia 5 days after cell administration, which resolved after antibiotic therapy and the patient was subsequently extubated 4 weeks after MSCs administration. The second patient was extubated 12 days later [92].

Very recently, Athersys disclosed in a press release the positive results for the MUST-ARDS study about MultiStem® Cell Therapy in patients with moderate-severe ARDS (NCT02611609). After an initial dose confirmation phase (*n* = 6), Athersys confirmed the tolerability and the safety profile of the MultiStem® treatment (*n* = 20) with no adverse events during administration, and lower levels of inflammatory biomarkers compared to the control group (*n* = 10). Furthermore, despite the study was not powered for efficacy

outcomes, MultiStem® cell therapy was associated with better short term prognosis, as shown by a lower mortality rate (25% versus 40%), higher ventilator-free (12.9 versus 9.2) and ICUfree days (10.3 versus 8.1) compared to control. Further findings will be unveiled at end of the collection of the 1-year follow-up data, as aimed according to the study design. (http://www.athersys.com/news-releases/news- release-details/athersys-announces-positive-results-its-exploratory-clinical). All the studies currently ongoing in the field of MSCs and ARDS are safety studies (Phase 1, 2, 1/2) (Table 12.1). At the moment there are still additional issues that need to be overcome: (1) improvement of MSCs bioavailability by the optimization of the cell preparation and storage [71]; (2) the modulation of the microenvironment [175]; (3) the characterization of the specific phenotypes/endotypes of ARDS potentially more suitable to respond to cell therapy [39, 40]. This might enhance the likelihood of success in subsequent efficacy (Phase 3) studies.

1.11. Future Directions

1.11.1. Patient Stratification

ARDS is classified by the Berlin definition into different severity categories, according to the degree of hypoxemia, and each associated with increasing mortality rates. However, other evidence suggests that: (1) either the etiology (i.e. pulmonary versus extrapulmonary ARDS) [176, 177, 178, 179], or (2) the macroscopic ARDS presentation at radiological imaging [180] or (3) the levels of different inflammatory biomarkers contributing to different biological patterns of ARDS might play a key role in stratifying the outcome of this

syndrome [181]. Pulmonary ARDS was associated with longer total ventilation time and longer ICU stay compared to extrapulmonary ARDS [182]. ARDS patients with a higher epithelial injury, as observed by higher levels of soluble form of the receptor for advanced glycation end product (sRAGE), showed a specific nonfocal CT lung pattern, which was associated with higher mortality compared to the focal pattern [180].

The ARDS Network proposed a novel classification of ARDS with two distinct subphenotypes, which included different clinical and laboratory characteristics [39]. Interestingly, in a secondary analysis of the ARMA [26, 183, 184] and the ALVEOLI trials [185], the investigators could identify a specific pattern of ARDS that the investigators named hyperinflammatory subphenotype, phenotype 2. Phenotype 2 showed higher plasma concentrations of inflammatory biomarkers greater prevalence of vasopressor use and lower serum bicarbonate concentrations than phenotype 1. The hyperinflammatory subphenotype could differentiate a subgroup of patients with a higher mortality rate.

In light of the heterogeneity of ARDS, attempts have been made to optimize treatment regimens [175], and stratification parameters are emerging among recipients of MSC therapy which may be of relevance to ARDS patients [186].

1.11.2. Large-Scale Cell Manufacture

As detailed earlier, generation of human-sized doses of GMP quality MSCs, for the numbers of patients needed for large-scale clinical trials (and subsequent clinical therapy), is not a trivial undertaking.

Preclinical studies typically use between 1×10^6 and 10×10^6 MSCs per kg of bodyweight or ideal bodyweight of the patient, and clinical trials have been designed with these doses in mind. In light of the observed relatively low cell viability in ongoing trials, production of the order of 10^9 MSC may be required to reach the upper doses when allowance for dead cells is calculated. Coupled with the lack of information with regard to passage or population doubling at which efficacy is lost, this will likely necessitate pooled donor batches to enter the MSC isolation and production process. Considerable work is being concentrated on this area both academically and industrially to optimize and automate, including the utilization of xeno-free media that allays fears of contamination with viruses or other as yet unknown contaminating factors.

The preponderance of preclinical work with MSCs has involved freshly harvested MSCs and this will remain an impractical and unlikely therapeutic for the clinic. Despite the development of rapid shipping solutions from manufacturing facility to clinical site, and research into supportive media to extend the MSCs' effective lifespan in suspension prior to administration, it is probable that a cryopreserved MSC will become the choice in the long term. Cryopreservation at the clinical site or expedited transport from manufacturing facilities will be required, but it remains to be determined what further equipment such as centrifuges and viability assessment assays will be needed to prepare the MSC dose and allow quality control prior to administration to the patient.

1.11.3. Lack of Clearly Defined Mechanism of Action

Despite a myriad of possible effector mechanisms by which the MSC may alleviate ARDS severity, including secreted antimicrobials, cytokines, extracellular vesicles, and other factors, and the observed influence MSCs have on leukocytes, it has remained difficult to ascertain which of these mechanisms are of importance to the MSCs' efficacy. Indeed several studies that have sought to replicate the various mechanisms proposed through administration of MSC-produced factors have failed or not reproduced the efficacy of the MSC itself, indicating critical gaps in our knowledge of MSC action in ARDS and suggesting interpretation of unsuccessful or even successful follow on clinical trial will be difficult. If the mechanism remains unknown, then interactions with other drugs or comorbidities will always be unpredictable.

1.11.4. Lack of MSC Potency Assay

Related to both production and mechanism, a critical limiting factor in successful deployment of MSC therapy to the clinic is the lack of defined assays to accurately predict MSC potency in the ARDS patient. Many cell manufacturers and research groups have proposed small, easily quantified molecules such as aldehyde dehydrogenase (ALDH) [187] or indoleamine 2,3-dioxygenase (IDO) [188], which correlated well with in vitro tests such as T-cell expansion inhibition or in vivo tests in ARDS animal models. However, as the MSC's mechanism of action in ARDS remain unclear, these factors can only be considered correlative and not conclusive proof of likely efficacy in the human patient.

1.11.5. Beyond the MSC?

Determining the mechanism(s) of action of the MSC specifically, however, will lead to us a question: do we need the cell at all? A suite of effectors produced by MSC cultures, or indeed by similar cell types engineered to replicate or improve upon the MSC secretome while being more open to manipulation and expansion, could replace cell therapy entirely. Also, as alluded to already, these factors will be likely easier to analyze, store, and deliver than the MSC they are derived from. Presuming cell-contact dependent mechanisms such as TNTs are the sole means underlying the MSC's efficacy in ARDS, we may ultimately see an MSC product cocktail available in stable, off-the-shelf format that can be delivered IV or intra-tracheally by nebulizer that will reproduce the efficacy initially demonstrated with the IV-delivered cryopreserved whole cell.

1.12. Conclusions

ARDS has been a stubbornly challenging syndrome to address clinically for decades. Despite gradual improvement in supportive care for the patient, specific therapies have proven elusive. The MSC is an exciting prospect, as it is a real paradigm shift from traditional approaches, due to its ability to respond to the level and nature of injury, having both direct and immunomodulatory properties, and a multimodal mechanism of action that targets multiple pathologies seen in the ARDS patient. Issues around dosing, MSC production, and potency reproducibility remain but are being addressed. We look forward to the conclusion of the many current and planned clinical

trials to determine the true therapeutic potential of MSCs for those suffering from this devastating disease.

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Chapter 1. Introduction. Section C.

Scope of the thesis

Chapter 1

This chapter will introduce the thesis work by two different sections: the first one will explain the core features of Acute Respiratory Distress Syndrome (ARDS), a type of respiratory failure mainly caused by pneumonia; the second section will expand on the characteristics and role of mesenchymal stem cells in the treatment of ARDS and their potential applications in patients and clinical trials.

Chapter 2

In the current chapter, we described the study of the characteristics of growth of *S.aureus* and we identified the conditions to obtain the bacteria at late log early phase for the potential application for *in-vivo* infection studies.

Chapter 3

In this chapter, we will evaluate the characteristics of UC-MSCs after preactivation compared to naïve UC-MSCs and the role of conditioned media from stem cells on a line on a human monocytic cell line. We will explore the cytokine expression of UC-MSC and THP-1 cells, cell viability after injury and the effect of the stem cell conditioned media on phagocytosis and on the repair of a model of lung injury in vitro.

Chapter 4

In this chapter, we will evaluate the potential of UC-MSCs to reduce the acute lung injury in an *in-vivo* model of pulmonary ARDS induced

by *Staphylococcus Aureus.* As first, we will establish a model of pulmonary ARDS using *S. Aureus* Newman, a gram positive bacterium obtained from a clinical isolate. Secondly, we will study the role of cell therapy in 2 different series of animals. In the first one we will compare the role of naïve versus preactivated UC-MSCs in the reduction of lung injury *S. aureus* mediated; in series 2, we will explore the potential of low dose preactivated MSCs. The role of UC-MSCs will be evaluated on physiology variables, histology samples, biomarkers in bronchoalveolar lavage and phagocytosis.

Chapter 5

In this chapter, we discussed the results of 3 recently published manuscripts that provide insights into current and potential future approaches – including cellular therapy - to the treatment of sepsis, a major leading cause of ARDS.

Chapter 6

In this chapter we summarized the thesis work, described the conclusions and future perspectives.

Chapter 2.

"Preactivated Umbilical Cord-Mesenchymal Stem Cells reduce lung injury and bacterial load in *Staphylococcus Aureus* induced pulmonary ARDS."

In vitro section – microbiology work

Manuscript in preparation

2.1 Introduction

Staphylococcus is a Gram-positive, extracellular bacterium that causes both superficial and invasive infections. The bacterium is capable of causing a range of illnesses including sepsis and bacteremia (Aureus and Lowy, 1998). The bacteria is one of the most frequently isolated bacteria in clinical settings (Dantes et al., 2013). Proteins which are located on the cell wall of the bacteria aid in pathogenesis (*Staphylococci in Human Disease*, 2009; Foster *et al.*, 2014)**.** The proteins interact with host cells extracellular matrix molecules namely fibronectin and collagen as well as B cell receptors (Kim et al. 2010). This interaction enables the bacteria to invade the host cell (Kim *et al.*, 2010; Foster *et al.*, 2014). Through clumping factor A (a major virulence factor of *S.aureus)* adhesion factor, this promotes binding adherence to blood clots and biomaterials (McDevitt *et al.*, 1994; Moreillon *et al.*, 1995). Adherence to blood clots is a contributing factor in the pathogenesis of sepsis. The ability to bind to biomaterial increase the likelihood of patients contracting an infection when undergoing mechanical ventilation. Both areas of interest in this work. As one of the major causes of ARDS is sepsis and a major treatment for these patients is mechanical ventilation. *S. aureus* is a leading cause of community and hospital acquired infections worldwide (Adhikari *et al.*, 2012) and models of pneumonia have already been established in mice (McElroy *et al.*, 1999) It is a clinically relevant bacteria which makes it an ideal choice in selecting a bacteria for a gram positive lung injury model.

2.1.1 Aims

To culture *S. aureus* Newman bacteria and identify conditions to obtain the bacteria at late log early phase for *in-vivo* infection studies.

2.2 Materials and Methods

2.2.1 Bacteria

The S. Aureus Newman used in these experiments was kindly provided by Timothy Forster, Professor Emeritus of Molecular Microbiology at the Trinity College of Dublin. *S. aureus* Newman was originally obtained from a clinical isolate (Duthie ES, Lorenz LL, 1952, J Gen Microbiol; Kneuper H, 2014, Molecular microbiology; J Bacteriol. 2008 Jan;190(1):300-10; Bae T. 2006, Mol Microbiology) and is a well-established S. Aureus strain able to induce lethal pneumonia in mice (Kohler J, Microbes and Infection, 2011; Ragle BE, 2010, Microbial agents and chemotherapy; Infection and Immunity, 2007, Wardenburg JB; Adhikari RP PLOSone 2012; Inoshima 2011 Nature Medicine).

2.2.2 Storage

Bacteria were cultured onto Baird parker agar plates using streak for isolation procedure to obtain single colonies. Briefly, the inoculum is steaked out over the ager surface to thin out or condense the bacteria. Some individual cells are separated and spread out as the original bacteria is diluted by streaking it over successive quadrants. By the fourth quadrant discrete colony forming units (CFU) will be observed. The agar plates were inverted and incubated at 37°C for 24 hr. After 24 hr Microbank beads (MicrobankTM, Pro Lab Diagnostics Inc.)

were used for storage. Using an inoculation loop a single colony was taken from the fourth quadrant of the agar plate and placed into the microbank. The lid was placed on and the inoculated microbank was inverted a few times. The liquid in the microbank was then removed and the microbank was then stored at -80°C for long term storage. Several stock microbanks were prepared for this project.

2.2.3 Agar and Broth Preparation

This project used two types of culture media; solid form agar and liquid form broth. The agar used for S. aureus was Baird Parker (Oxoid Product: CM0275). Baird parker is a selective agar for staphylococci species, and is differential between *S. aureus* and *epidermidis*. Sodium pyruvate is added as a selective growth stimulant. *S. aureus* colonies appear as a differential brown black shiny color with a clear halo due to the ingredient egg yolk tellurite (Oxoid Product (SR0054) in the media that is able to differentiate the species of *Staphylococcus*. The broth used was tryptone soy broth (TSB) (LabM Product: LAB004, Tryptone Soy Broth TSB Lancashire, UK). TSB broth is generally used to cultivate aerobic bacteria. It is a general purpose media used to grow bacteria with high nutritional requirements.

2.2.4 Preparation of media

Baird parker media was prepared by adding 63g of dehydrated powder to one litre of distilled water the solution was then autoclaved at 121°C for 15 minutes. The agar was then cooled to 50°C and 50ml of Egg Yolk Tellurite Emulsion was then aseptically added. The solution

was mixed well before pouring 15mL into sterile petri dishes. The agar was left at room temperature to cool and solidify. TSB agar was prepared by adding 30g of dehydrated powder to one litre of distilled water the solution was then autoclaved at 121°C for 15 minutes. The broth was allowed to cool before mixing and adding to culture vessels

2.2.5 S.aureus growth curve

For all experiments a bead was taken from the microbank stocks. The bead was placed into an aerated cultivation tube containing 100 mL of TSB. The suspension was incubated at 37°C and stirred at 180 rpm in a rotary incubator. At one hour intervals 200 µL samples were taken from the suspension. 200 µL of fresh broth was added as not to hinder growth conditions with sample collection. 100 µL of samples was used for serial dilutions and plate count. Bacteria at varying dilutions were spreads over agar plates aseptically. The agar plates were inverted and incubated at 37°C for 24 hr after which colonies were counted and recorded. The other 100µL was used for OD readings at 600nm against reagent blank.

2.2.6 The Gram stain

The Gram stain, namely one of the most important staining techniques in microbiology was used in this project to confirm identification of the bacteria isolate and to ensure no contaminants were present. The procedure differentiates bacteria into two different classifications; Gram positive and Gram negative. The method involves staining the bacteria with two dyes in series. The bacteria that retain the initial crystal stain are Gram positive/purple. Whereas those that are

decolorized and stain the subsequent stain are said to be Gram negative. The retention of the dyes is based on the thickness of the bacteria peptidoglycan layer.

2.2.7 Gram stain procedure

Inoculating loops were sterilized by placing into the Bunsen flame until red hot and allowed to cool before inoculation as this would damage the bacteria. A loop of sterile water was placed onto a clean slide. A small amount of a colony was taken from an agar plate and used to make an even suspension on the slide. The inoculating loop was used to spread the sample out onto the slide. The sample was then set to air dry. Using a clamp, the slide was spread over the flame of a Bunsen to heat fix the bacteria onto the slide. Starting with a clean slide, transfer a loopful of the broth culture to the center of the slide. The slides were then placed on a staining rack and covered in crystal violet solution for 30 seconds. The slide was then washed with water to remove any unbound stain. The slid was then immersed in Gram iodine for 1 minute. The slide was then washed with water to remove any remaining solution. 95% alcohol was then washed over the slide to decolorize any unbound stain. The slide was then washed with water again to remove any remaining solution. The slide was then immersed in safranin counterstain for 1 minute. The slide was then washed in water and blotted dry for observation using a microscope at 20-40 X lens.

2.3. Results

2.3.1. Confirmation of *S. aureus* Newman isolate

The Gram stain is essentially a qualitative assessment of a bacterium identity. After Gram stain under microscope observation, the clinical isolate appeared as a Gram negative cocci shaped bacteria. No other contaminating bacteria appeared to be present. To further confirm this the isolate grew in brown/ black colonies on Baird parker media as seen in image.

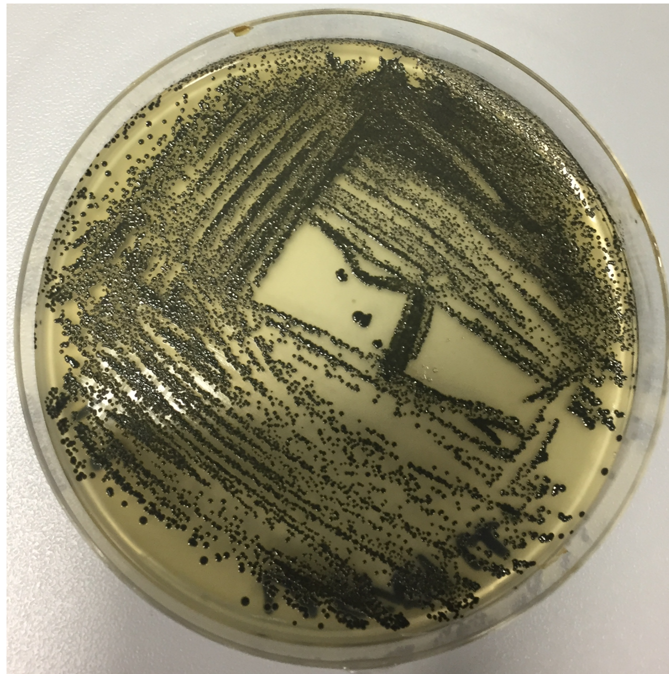

Figure 1. Colonies of *S.aureus* Newman on Baird Parker media.

Growth curve of *S. aureus* Newman

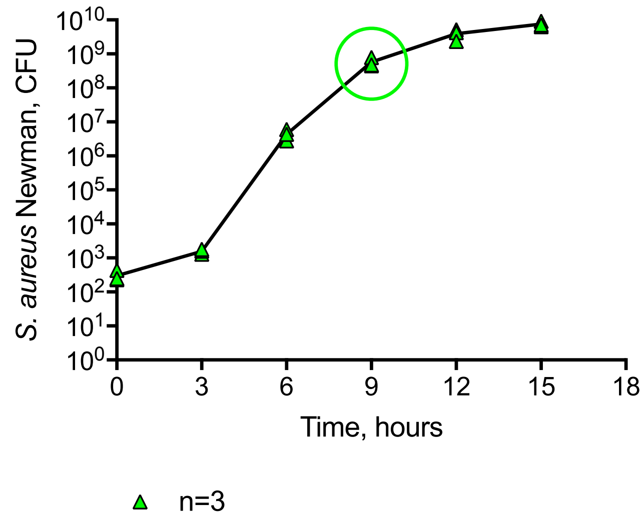

Figure 2. Growth curve of *S.aureus* Newman over 18 hours determined by CFU count.

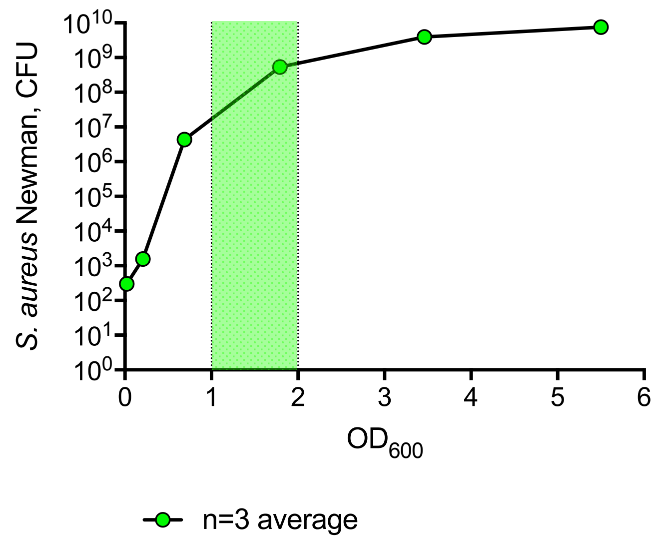

Figure 3. CFU count of S. aureus Newman correlated to OD readings

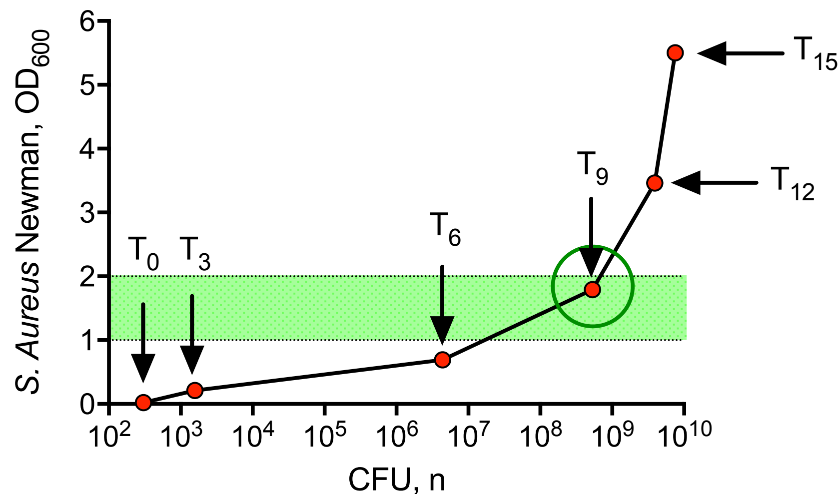

Figure 4. Optical density measurement of *S.aureus* Newman correlated to CFU count

2.4. Discussion

One of the main aims of this project was to establish a model of gram positive pneumonia *in-vivo. S. aureus* is a pathogen associated with life threatening sepsis and pneumonia (Foster, 2004).

After obtaining the isolate, a final confirmation was required in order to fully confirm as firstly the bacteria was *S. aureus* Newman and secondly no contaminating bacteria were present. In a clinical setting, more elaborate molecular and biochemical tests are required to identify bacteria. In this scenario the isolate was obtained from a trusted laboratory and was already characterized, thus a simple Gram stain and selective culture techniques were carried out. *S.aureus* is a Gram-positive cocci shaped bacteria and the Gram stain re-affirmed this. When the isolated was cultured on Baird parker media, the

bacteria grew in brown/black colonies characteristic of *S.aureus*. When this identification and lack of contaminants was confirmed, stocks of the bacteria were prepared in microbank stocks. This was done for ease of access for future experiments and more importantly to ensure the bacteria did not become 'lab trained' and loose virulence factors or pathogenicity.

Bacterial growth is an increase in cell number in the population which occurs through growth and division. The growth is usually dependent on growth conditions (Finkel, 2006). For in-vivo injury studies, bacteria were required to be in late log early stationary phase. This is because when bacteria enter the stationary phase they often activated stress defense mechanisms which increase virulence and affect survival (Bryan *et al.*, 2016). The model was required to be moderate to severe short term. If the bacteria were intubated during log or early log phase, more time would have been required to induce infection. It must also be indicated that the bacteria will be taken from culture broth with the only competition the bacteria encounters are self population. It will then be intubated into a rodent model with an innate immune system ready for this invasion. Thus to ensure an infection is achieved, the bacteria must be in a phase with adequate virulence factors and at a number capable of inducing an infection. These experiments determined that at the conditions described the bacteria will reach late log stationary phase at 9 hours. The experiments also determined the concentration of bacteria present at 9 hours. For *in-vivo* injury the bacteria dose could be altered to intubate each animal with the same concentration of bacteria at the same phase of growth

cultured under the same conditions. This ensured two things consistency among animals and the best possibility of infection.

2.5 Conclusion

These experiments confirmed the isolate was *S.aureus* Newman and optimal growth conditions. More importantly the time taken for the bacteria to reach stationary phase was quantified.

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Chapter 3.

"Preactivated Umbilical Cord-Mesenchymal Stem Cells reduce lung injury and bacterial load in *Staphylococcus Aureus* induced pulmonary ARDS."

In vitro section – cell work

Manuscript in preparation

3.1 Introduction:

Mesenchymal stem cells have potent immunosuppressive and immunomodulatory properties. They are currently being investigated for their clinical application as a treatment for an array of illnesses including ARDS which has reached the clinical trial stage of research (Matthay et al., 2019). The immunomodulatory and antimicrobial potential of MSCs is still under intense investigation, with ability confirmed but exact mechanisms of how they exert these effects not fully understood. Current research is focusing on analysis such as intracellular signalling and genomic testing. Other investigations are examining how stem cells respond differently in injurious environments (Leburel *et al.,* 2017 and Islam *et al.,* 2010). MSCs modulate the innate immune response and promote the generation of regulatory cells (Németh *et al.*, 2009; Oh *et al.*, 2012; Ko *et al.*, 2016). MSCs after administration also promote innate immune regulatory cells in the lungs (Németh *et al.*, 2009). Unfortunately MSCs administration does not always have a positive effect (Islam *et al.,* 2010). MSCs have been found to elicit immunomodulatory properties after exposure to inflammatory stimuli such as IFN-Y, IL-1β and TNF-α (Krampera *et al.*, 2006; Ren *et al.*, 2008). This work focused on determining firstly the cytokine expression profiles of MSCs after administration of each cytokine individually and in combination. Next to predict the effects of the MSCs on immune cells, conditioned media from these treatments was used to determine the effects on macrophages. These effects included cytokine expression, percentage phagocytosis and finally to determine if the conditioned media could

reduce apoptosis after injury. The alveolar epithelium is heavily involved in the pulmonary response to acute lung injury (Matthay, Ware and Zimmerman, 2012). A549 cells were injured with two types of insult inflammatory (IL -1β) and chemical (H_20_2), cells were then incubated with conditioned media. To assess affects inflammation levels were measured via IL-8 ELISA and viability after chemical injury. These tests give a foundational basis on the possible effects of primed MSCs in an lung injury model.

3.2 Aims:

- To determine the effects of inflammatory cytokines individually and in combination on the cytokine release profiles of umbilical cord stem cells
- To assess if conditioned media from primed and naive MSCs has any effect on macrophages
- To establish if conditioned media from primed and naïve MSCs has the ability to reduce injury *in-vitro*

3.3 Methods and Materials:

3.3.1 Cell Culture

3.3.1.1 Pulmonary Type II Alveolar Cell Line

Pulmonary type II alveolar A549 cells are adenocarcinomic human alveolar basal epithelial cells. They are categorised under the squamous subdivision of epithelial cells. The cells were received by

NUI Galway. The cells were cryopreserved at a passage of 90. The cells were used at passage 91-97 for *in-vitro* experiments. Cells were cultured and expanded in 175cm^2 tissue culture flasks (SARSTEDT AG & Co., Numbrecht, Germany). The growth medium used was RPMI growth medium (RPMI-164, Sigma-Aldrich, St.Louis, MO, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/ml) and streptomycin (100 μg/ml) solution (Sigma-Aldrich). The cells were subcultured with 0.035% trypsin-0.05 mM ethylenediamine tetra acetic acid (EDTA; GIBCO. Invitrogen Corporation, NY, USA). A549 cells were seeded at 6x10^4 cells/cm^2 in 96 well flat bottomed tissue culture plates (SARSTEDT) for all assays carried out.

3.3.1.2 Human Monocytic (THP-1) Cells

THP-1 is a human monocytic suspension cell line derived from the peripheral blood of an acute monocytic leukaemia patient. THP-1 cells were received by NUI Galway. The cells were cryopreserved at a passage of 20. Cells were cultured and expanded in 175cm^2 tissue culture flasks (Sarstedt AG & Co., Numbrecht, Germany). The growth medium used was RPMI growth medium (RPMI-164, Sigma-Aldrich, St.Louis, MO, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/ml) and streptomycin (100 μg/ml) solution (Sigma-Aldrich). For differentiation into macrophages, the cell was treated with phorbol 12-myristate 13-acetate (PMA) at a concentration of 100ng/ml. THP-1 cells were

seeded at $6x10^4$ cells/cm^2 in 96 well flat bottomed tissue culture plates (Sarstedt) for all assays carried out.

3.3.1.3 Mesenchymal Stem Cells

The cells were cryopreserved at a passage of 1. Cells were cultured and expanded in $175cm^2$ tissue culture flasks (Sarstedt T AG & Co., Numbrecht, Germany). The growth medium used was MEM-alpha growth medium (GIBCO. Invitrogen Corporation, NY, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/ml) and streptomycin (100 μg/ml) solution (Sigma-Aldrich). Fibroblast growth factor (fgf) (Peprotech US, NJ, USA) was used at a concentration of 10ng/ml. The cells were subcultured with 0.035% trypsin-0.05 mM ethylenediamine tetra acetic acid (EDTA; GIBCO. Invitrogen Corporation, NY, USA).

3.3.1.4 Cell Passaging

Before passaging, cells we viewed using white light microscopy for phenotypic changes to ensure continued health and viability as per standard aseptic and cell culture practice. Monolayers were formed in the tissue culture flasks. Cell lines were passaged 2/3 times a week, depending on doubling times. Cells were washed with sterile phosphate-buffered saline (PBS).

Detachment occurred using 0.05% trypsin-EDTA. Once detached the cell suspension was diluted to 10mL of media and centrifuged at 400 x g for 5 min. The cell pellet was then re-suspended in the growth

medium. Cells were counted and seeded to new tissue culture vessels at the appropriate seeding density.

3.3.1.5 Cell Treatment

96 well plates were used for all Biochemical endpoint assays. Only the inner 60 wells of the plate were seeded to avoid unnecessary evaporation and inaccuracies. Once seeded, the outer wells were filled with 100µl incomplete sterile media in TC hood and placed in an incubator overnight to adhere. It was necessary for the cells to have reached 70% confluency in the 96 well plates before treatment. For all assays, MSCs, A549s, and THP-1 cells were seeded in 96 well plates for 24h in 5% CO_2 at 37^0C. After 24 hrs culture media was removed and fresh media containing dilutions of test substance and controls were added for another 24 hrs at the same incubation conditions. The final volume of media per well was 100 µl.

3.3.2 Biochemical Endpoint Assays

3.3.2.1 MTT cytotoxicity assay

Yellow 3-(4, 5-dimethylthiazoyl-2-yl)-2, 5 diphenyltetrazolium bromide salt (MTT, Sigma) is metabolised by cells to a purple formazan product yielding a media change from yellow to blue/purple. This metabolism occurs inside the mitochondria of cells, which acts as an indicator of correct mitochondrial action and in turn cell viability. The assay was carried out as per manufacturers' instructions.

0.5 mg/mL MTT was dissolved in complete media and added to the cells for 3 hrs at 37^oC. 100µL DMSO was added to lyse the cells and solubilise the formazan. The absorbance of each well was read at 570 nm after a 60second shake performed by the Synergy™ HT Multi-Mode Microplate Reader (BioTek, USA).

3.3.2.2 Hydrogen peroxide (H_2O_2) cell injury

A549 cells were grown to 80% confluence. THP-1 cells were pretreated with PMA for 72 hrs before exposure. Cells were then treated with various doses of H_2O_2 ranging from 0.001 to 10 mM. This was done to determine the optimal dose to injure cells. For assays cells were injured with the chosen dose of H_2O_2 and also simultaneously treated with conditioned media from MSC cells either naïve or treated with cytokines. After injury and treatment, the plates were incubated at 5% CO_2 at 37^0C for 24hrs. MTT assay was performed on A549 cells to determine if cells survived H_2O_2 treatment and were thus protected by conditioned media. THP-1 cells were stained with annexin V-FITC antibodies. The percentage of apoptotic cells was quantified by flow cytometry.

3.3.2.3 Enzyme Linked Immunosorbent Assay

Human Sandwich ELISAs were used for detection of cytokine expression from the supernatants of in-vitro cell culture experiments. The sandwich ELISAs were purchased from R_ND Systems. Samples were analysed according to the manufacturer's instructions. Results

were graphed as pictogram per millilitre. When high quantities were secreted results were graphed at nanogram per millilitre.

3.3.2.4 Phagocytosis Assays

THP-1 cells were treated with conditioned with conditioned media from MSC cells either naïve or treated with cytokines. After 24 hrs cells were incubated with alexafluo9r labeled *E. coli* BioParticles® (Invitrogen, Germany) were reconstituted at 20 mg/ml in PBS and coated opsonizing reagent (OpsR, Invitrogen, Germany) in the dark for 2 hours. After incubaton the cells were washed and The percentage of phagocytosis was quantified by flow cytometry.

3.3.2.5 A549 IL-1 Injury

A549 cells were treated grown to 80% confluence in 96 well plates. Cells were then treated with 10ng/mL of recombinant human interleukin-1 beta (rh IL-1b / IL1F2) (ImmunoTools, Gladiolenweg 2, Germany). Cells were simultaneously treated with conditioned media MSC cells either naïve or treated with cytokines. Plates were then incubated for 24 hrs. at 5% CO_2 at 37^0C. After incubation supernatants were removed for ELISAs for IL-8 secretion analysis.

3.3.2.6 Statistical Analysis

The distribution of all data was tested for normality using the Kolmogorov-Smirnov test. Results are expressed as mean (± SEM). Continuous data were analysed with one-way Analysis of Variance (ANOVA) corrected for multiple comparisons by controlling the false discovery. A p value of <0.05 (two-tailed) was deemed statistically significant. Data were analysed using Graphpad Prism (Version 7.0a, La Jolla, USA).

3.4 Results:

3.4.1 Cytokine Release Profile of MSCs after administration of cytokines

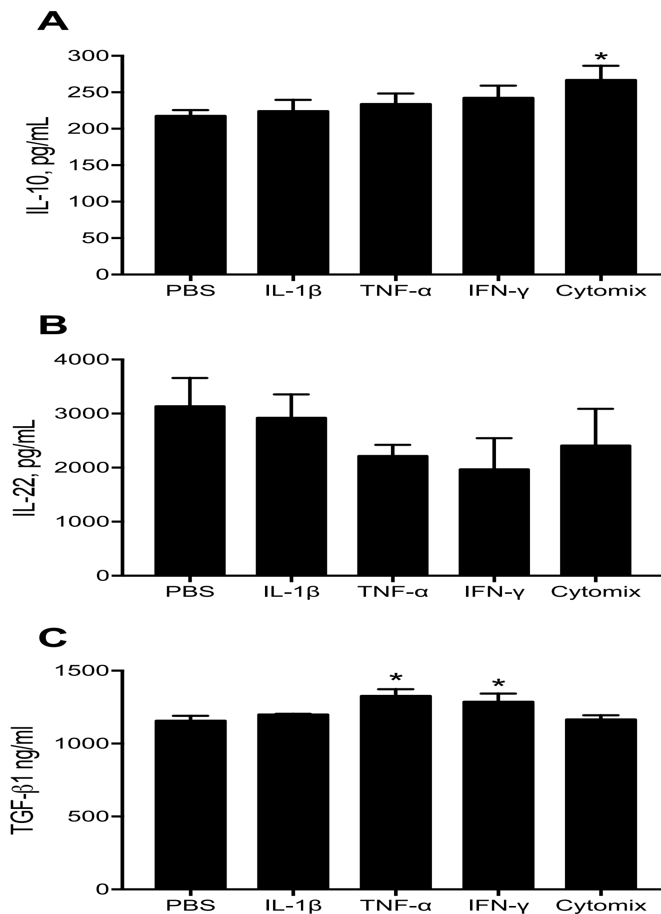


Figure 3.1: The effect of inflammatory cytokines on the secretion of anti-inflammatory markers in MSCs.

ELISA analysis shows that cytomix significantly increases IL-10 secretion in MSCs (Panel A). None of the treatments had any significance on the secretion of IL-22 (Panel B). TNF-α and IFN-Y significantly increased the secretion of TGF-β1 from MSCs (Panel C). All measurements were compared to respective PBS controls.

* $p<0.05$ versus PBS.

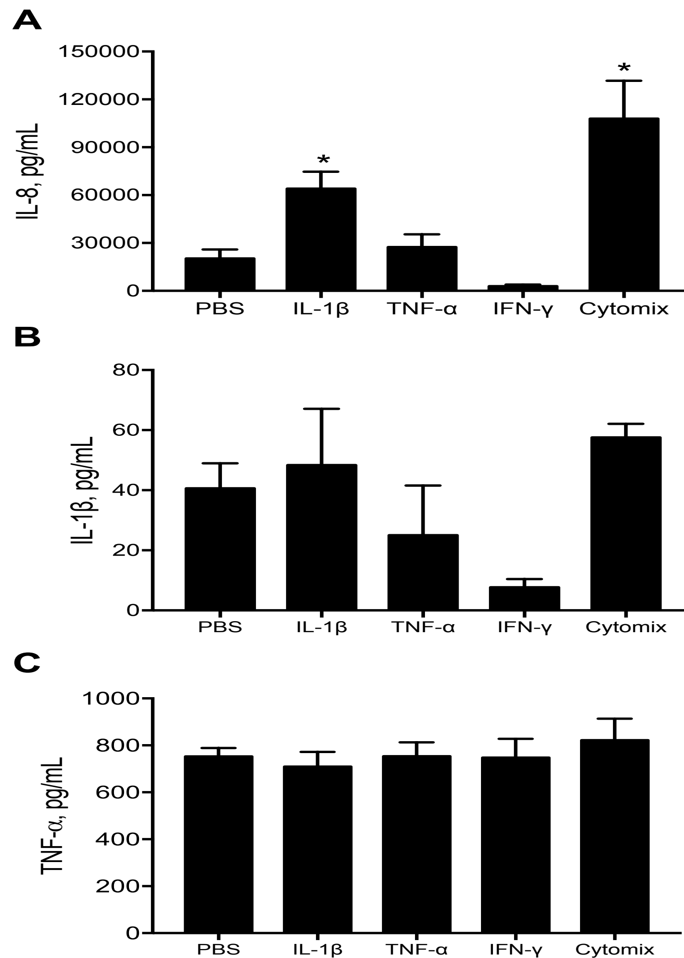
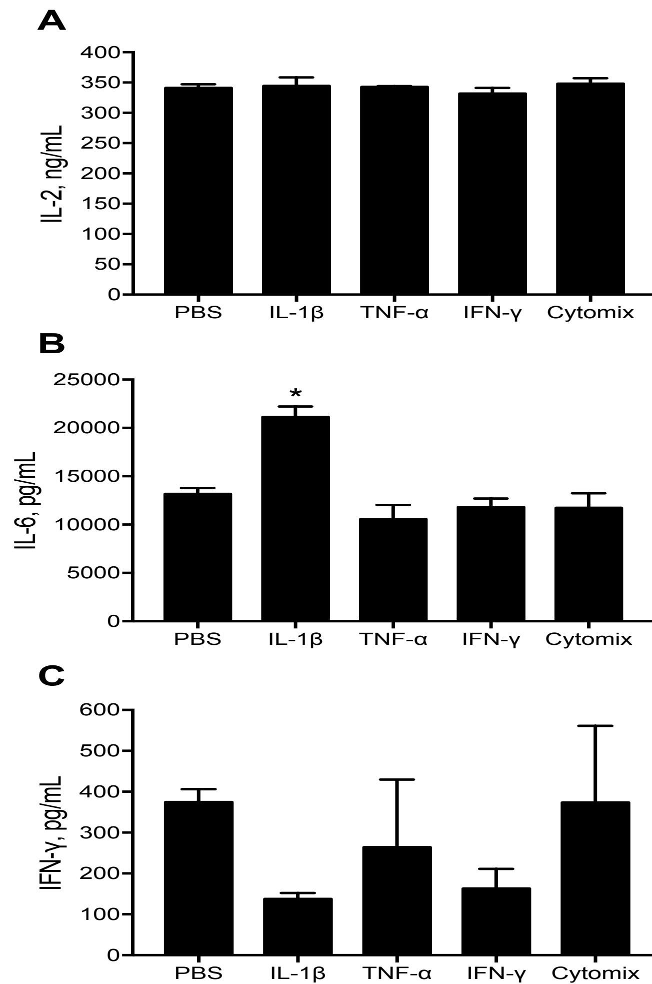


Figure 3.2 The effect of inflammatory cytokines on the secretion of pro-inflammatory markers in MSCs.

ELISA analysis shows that cytomix and IL-1β significantly increases IL-8 secretion in MSCs (Panel A). None of the treatments had any significance on the secretion of IL-1β (Panel B). and TNF-α (Panel C). from MSCs. All measurements were compared to respective PBS controls. * $p<0.05$ versus PBS.



3.3 The effect of inflammatory cytokines on the secretion of cytokines in MSCs.

ELISA analysis shows that IL-1β significantly increases IL-6 secretion in MSCs (Panel B). None of the treatments had any significance on the secretion of IL-2 (Panel A). and IFN-Y (Panel C). from MSCs. All measurements were compared to respective PBS controls.* $p<0.05$ versus PBS.

3.4.2 Effect of Conditioned media from primed and naïve MSCs on THP-1 cells

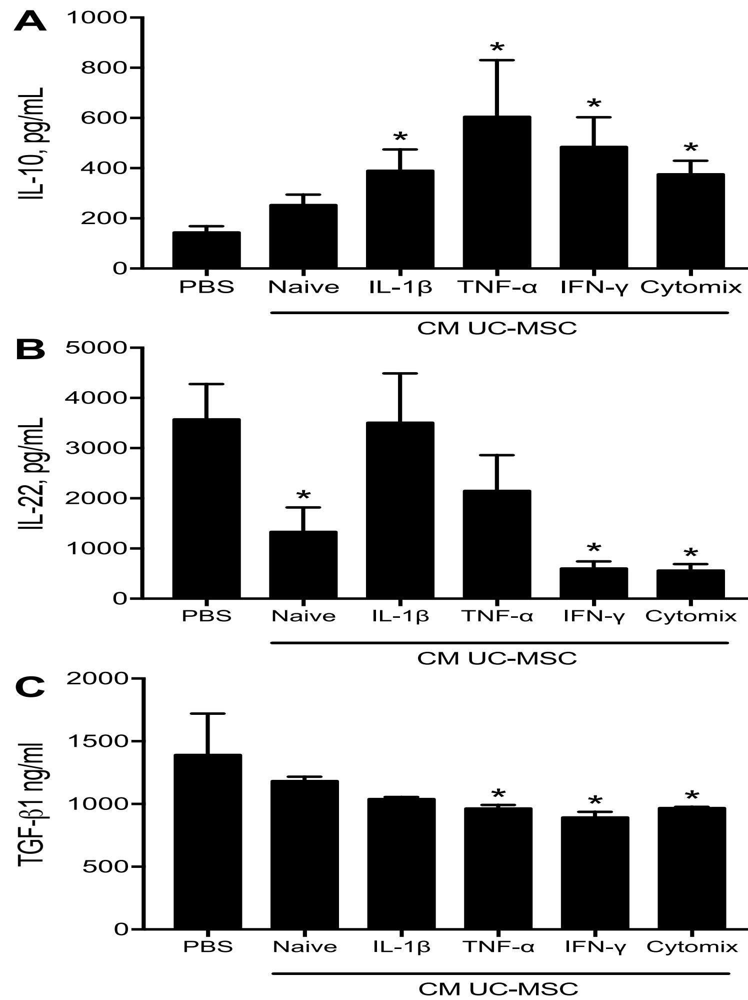


Figure 3.4: The effect of conditioned media from MSCs primed with different cytokines on the secretion of anti-inflammatory markers in a human monocytic cell line THP-1

ELISA analysis shows that all samples of conditioned media from primed MSCs increase the secretion of IL-10 (Panel A). Naïve, IFN-Y and cytomix primed conditioned media significantly reduce the secretion of IL-22 (Panel B). TNF-α, IFN-Y and cytomix primed conditioned media significantly reduced the secretion of TGF-β1 from MSCs (Panel C). All measurements were compared to respective PBS controls. * $p<0.05$ versus PBS.

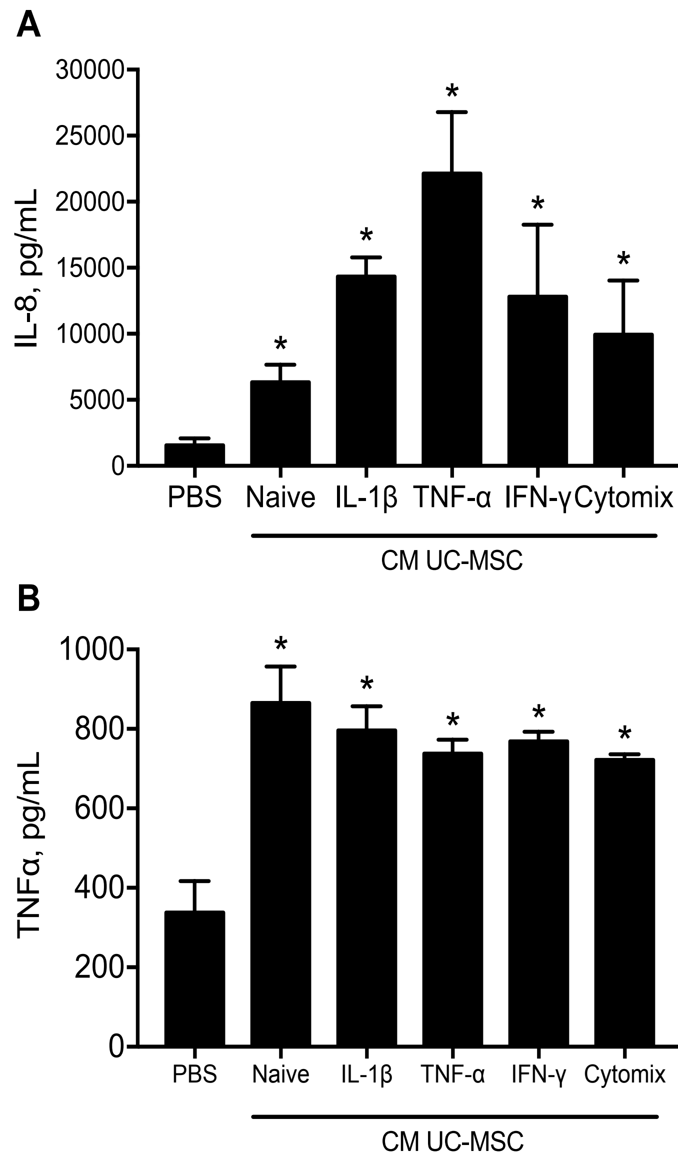


Figure 3.5 The effect of conditioned media from MSCs primed with different cytokines on the secretion of inflammatory markers in a human monocytic cell line THP-1

ELISA analysis shows that all samples of conditioned media from naïve and primed MSCs increase the secretion of IL-8 (Panel A) and TNF-α, (Panel B). All measurements were compared to respective PBS controls. * $p<0.05$ versus PBS.

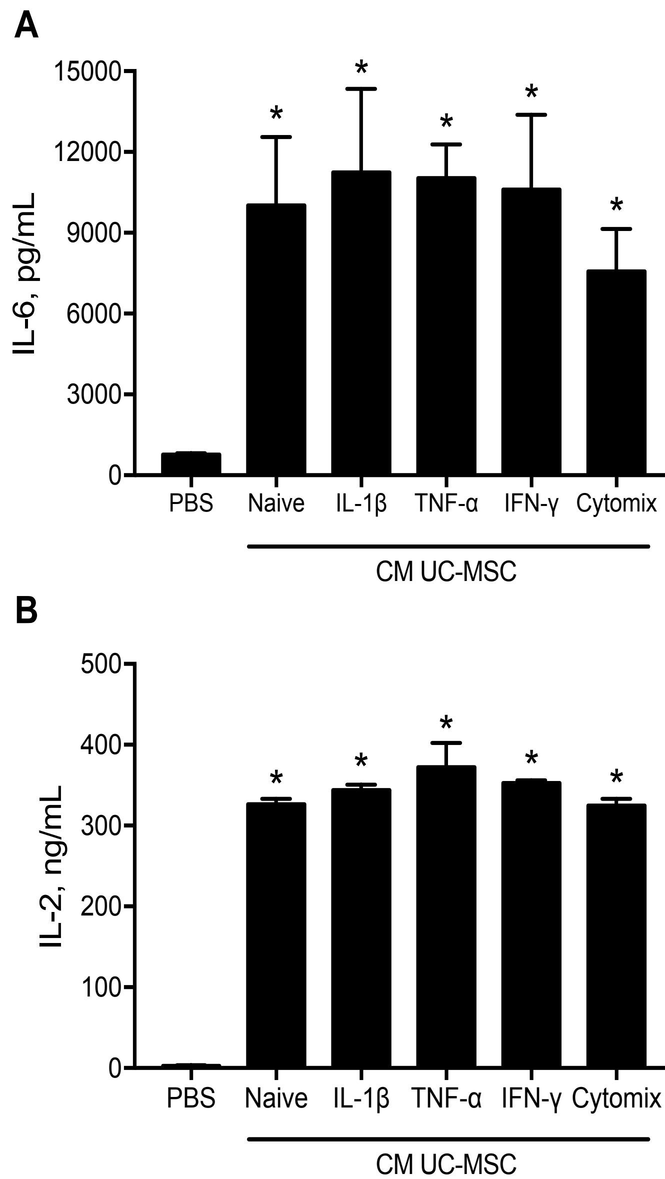


Figure 3.6 The effect of conditioned media from MSCs primed with different cytokines on the secretion of cytokines in a human monocytic cell line THP-1.

ELISA analysis shows that all samples of conditioned media from naïve and primed MSCs increase the secretion of IL-6 (Panel A) and IL-2 (Panel B). All measurements were compared to respective PBS controls. * p<0.05 versus PBS.

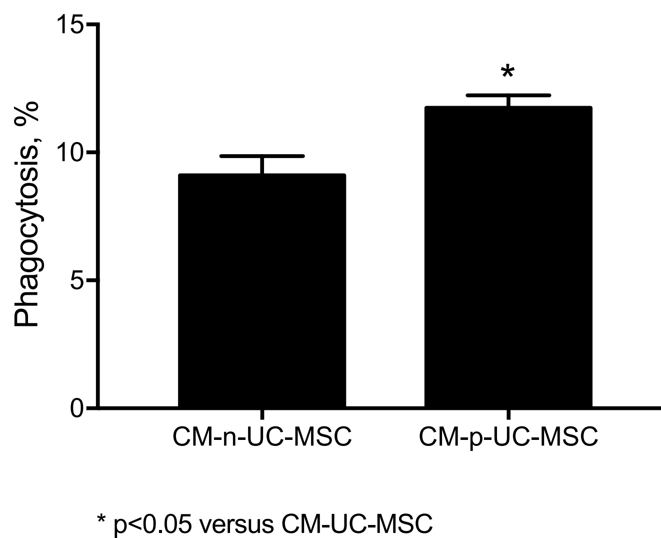


Figure 3.7 : The effect of conditioned media from naïve and Preactivated MSCs on percentage phagocytosis of THP-1 cells measured using flow cytometry.

Analysis of percentage phagocytosis a human monocytic cell line after treatment of conditioned media from naïve and cytomix primed MSCs demonstrated that THP-1 treated with primed conditioned media had a significantly higher percentage phagocytosis with respect to conditioned media form naïve cells.

3.4.3 Effect of conditioned media from primed MSCs on inflammatory and chemical injury

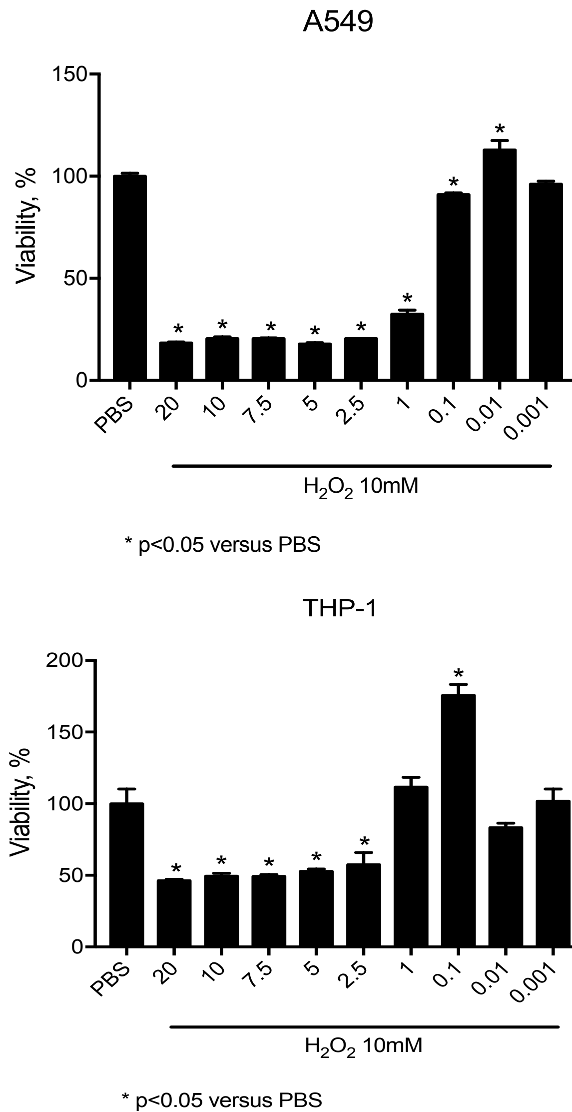


Figure 3.8: Effect of H_20_2 on A549 (Panel A) and THP-1 cells (Panel B) measured using MTT.

MTT analysis demonstrated the effect of H_20_2 on A549 cells and THP-1. Analysis showed that doses ranging from 0.1-20 mM significantly reduced the viability of A549 cells. Doses ranging from 2.5 – 20 mM

had a significant effect on the viability of THP-1 cells. 0.1mM had a proliferative effect in both cell lines. * $p<0.05$ versus PBS.

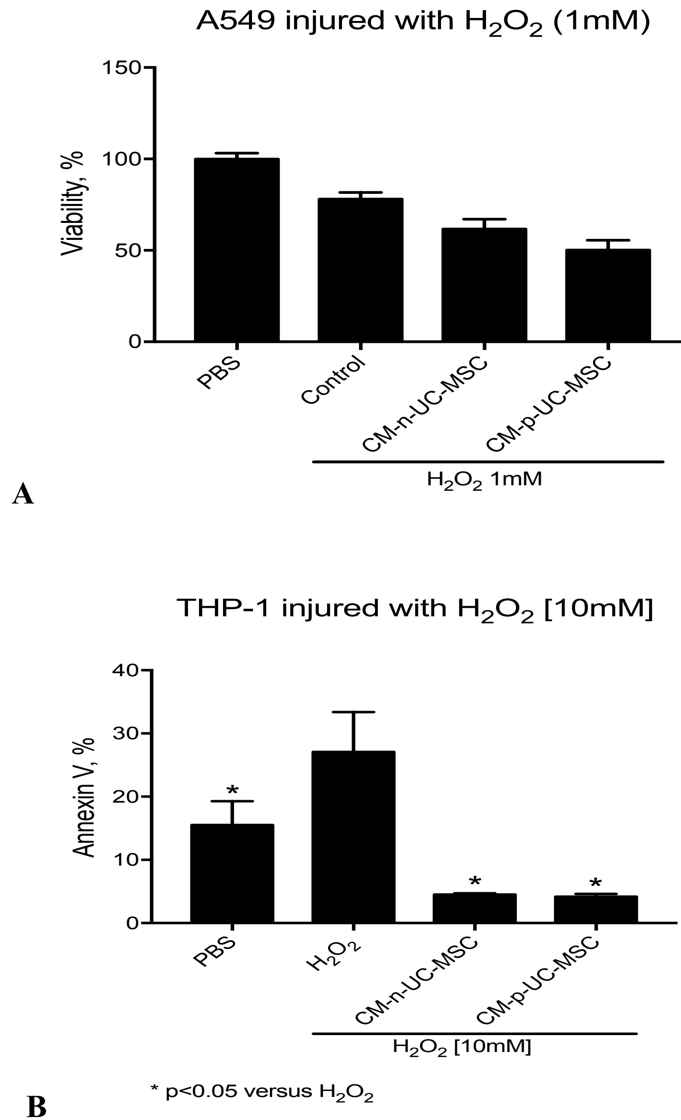
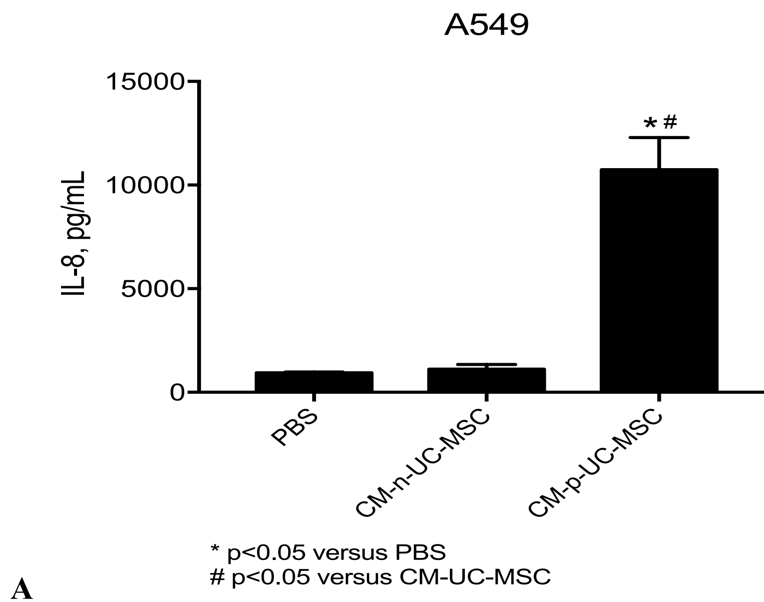
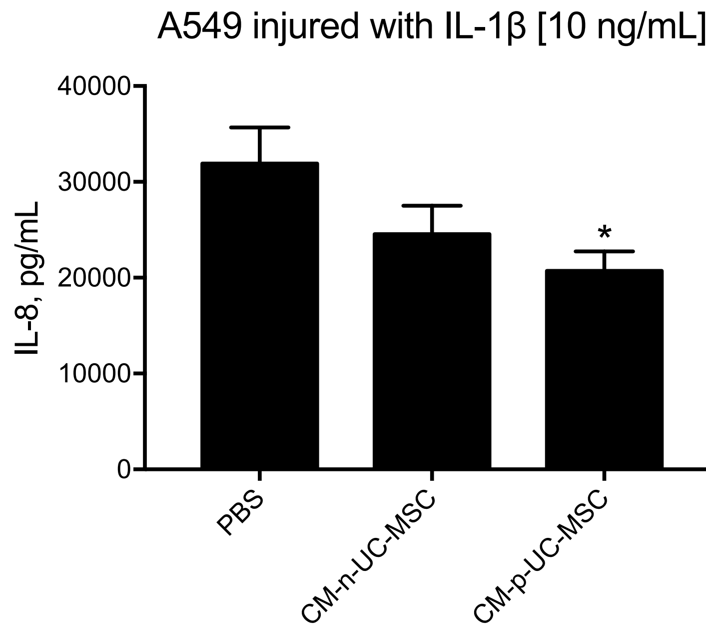


Figure 3.9: The effect of conditioned media from naïve and preactivated MSCs on A549 (Panel A) and THP-1 (Panel B) cells after chemical injury

MTT analysis demonstrated that conditioned media had no effect on the viability of A549 cells after H2O2 injury (Panel A). Analysis of Annexin V showed that conditioned media from MSCs significantly reduced apoptosis in THP-1 after injury (Panel B) with H2O with respect to injury. Panel A, * $p<0.05$ versus PBS; Panel B, * $p<0.05$ versus H_2O_2 injury.



A



B

Figure 3.10 : Meaurement of IL-8 using ELISA after incubation with conditioned media (Panel A) and after injury with IL-1β (Panel B).

A549 cells were injured with IL-1β and simultaneously treated with conditioned media (CM) from naïve and preactivated MSCs IL-8 was then measured by ELISA 48 hrs after an injury to determine the inflammatory response. Results show that CM from preactivated UC-MSCs significantly increased IL-8 compared to naïve cells and PBS before injury. In contrast, in the presence of injury induced on A549 by IL-1beta, CM from cytomix primed UC-MSCs significantly decreasd the levels of IL-8 compared to PBS.

3.5 Discussion

3.5.1 Priming of MSCs using inflammatory cytokines

In inflammatory environments MSCs elicit immunomodulatory properties. Therefore in theory by priming MSCs with inflammatory cytokines an inflammatory milieu is imitated. The hypothesis is that this inflammatory replica will provoke MSCs into their immunomodulatory state and increase therapeutic effect. Previous work has demonstrated that priming MSCs with IFN-Y, IL-1β and TNF-α can enhance their immunomodulatory capacity (English *et al.*, 2007; Schu *et al.*, 2012).

The functionality of MSCs depends on the secretion of cytokines, chemokines and growth factors. Cytokine measurement gives an indication of how the cell is responding and the signals the cells are releasing as a result of pre-activation. For this reason, a multitude of pro and anti-inflammatory cytokines was measured using ELISA in the supernatants of MSCs. Results show that cytomix significantly increases IL-10 secretion in MSCs (Figure 3.1 Panel A). TNF-α and IFN-Y significantly increased the secretion of TGF-β1 from MSCs (Figure 3.1 Panel C). Cytomix and IL-1β significantly increased IL-8 secretion in MSCs (Figure 3.2 Panel A). IL-1β significantly increases IL-6 secretion (Figure 3.3 Panel B. The treatments did not have a significant effect on the secretion of IL-22 (Figure 3.1 Panel B) IL-1β (Figure 3.2 Panel B), TNF-α (Figure 3.2 Panel C), IL-2 (Figure 3.3 Panel A), and IFN-Y (Figure 3.3 Panel C).

These results are mixed. The cytokine release profile is mixed, for example cytomix induces the secretion of IL-10 and IL-8.

Chemokines are secreted by MSCs and have a chemo attractive effect. IL-8 is a chemokine secreted by MSCs which is of an inflammatory nature as it recruits immune cells. IL-10 is a cytokine secreted by MSCs which suppresses macrophages and neutrophils (Xing *et al.,* 1998**,** Mocelin *et al.,* 2005) which are overactivated in conditions such as ARDS. Th cytokine has also been demonstrated to have anti-inflammatory effects on other cells such as dendritic and T-cells (Opal and Depalo 2000**,** Yang *et al.,* 2009). Certain activating substances can induce IL-10 secretion from MSCs (Shi *et al*., 2010, Dazzi and Krampera 2011).

Cytomix had no effect on IL-6 secretion where as IL-1β significantly increases IL-6 secretion. MSCs can spontaneously secrete IL-6 and after activation by IL-1, TNF-α and IFNγ (Dazzi and Krampera 2011, Xing *et al.,* 1998**,** Ivano-Todorova *et al.,* 2009).When secreted by MSCs IL-6 has been shown to regulate the immune response and inflammation (Kishimoto 2010, Yun *et al.,* 2012). In conjunction with IL-1 and TNF-α, IL-6 assists in the recruitment of neutrophils, switch from neutrophil to macrophage inflammation as well as the secretion of acute-phase proteins. IL-6 suppresses the secretion of pro-inflammatory cytokines IL-1, TNF-α and Interferon gamma (Opal and Depalo 2000, Steensberg *et al.,* 2003). Which is interesting as none of these cytokines were expressed after priming with any of the agents.

Liopcalin 2 is a protein involved in the innate immune response. It limits bacterial growth by sequestrating iron. It binds to bacterial siderophores and limits iron used by bacteria. It has been reported that lipocalin 2 is needed for effect host defence against pulmonary infection (Chan *et al.,* 2009). Studies have reported that IL-1β and TNF-α have the ability to upregulate cellular production of lipocalin 2 with TNF-α had the strongest ability to upregulate lipocalin 21 production (Gupta *et al.,* 2012, Shen *et al.,* 2005). TGF-β is one of the most prominent immune-modulatory cytokines produced by MSCs. Like IL-10 and IL-6 it is a pleiotropic cytokine, regulating multiple functions including proliferation, differentiation and immune responses (Li *et al.,* 2006) both TNF-α and IFN-Y significantly increased the secretion of TGF-β1.

3.5.2 Effect of Conditioned media from primed and naïve MSCs on THP-1 cells

The human monocytic cell line THP-1 is used extensively for studying monocyte and macrophage differentiation and function (Tsuchiya *et al.* 1980). THP-1 macrophages differentiated with phorbol myristate acetate (PMA) were used as an *in-vitro* model as these cells share many characteristics of human macrophages. (Auwerx 1991, Asseffa *et al.* 1993). Like stem cells in response to being exposed to inflammatory stimuli, macrophages secrete cytokines. Cytokines can be either of an inflammatory profile or an anti-inflammatory profile

ELISA analysis shows that all samples of conditioned media from primed MSCs increase the secretion of IL-10 (Figure 3.4 Panel A). Naïve, IFN-Y and cytomix primed conditioned media significantly reduce the secretion of IL-22 (Figure 3.4 Panel B). TNF-α, IFN-Y and cytomix primed conditioned media significantly reduced the secretion of TGF-β1 from MSCs (Figure 3.4 Panel C). All conditioned media samples from naïve and primed MSCs increase the secretion of IL-8 (Figure 3.5 Panel A), TNF-α, (Figure 3.5 Panel B) IL-6 (Figure 3.6 Panel A) and IL-2 (Figure 3.6 Panel B).

These results indicate again that primed media has a varied effect on macrophages. IL-10 a potent anti-inflammatory markers is increased whereas IL-8 a potent anti-inflammatory marker is also increased. In the air space of the lungs, alveolar macrophages during ARDS secrete IL-1, IL-6. IL-8, IL-10 and TNF-α to stimulate chemotaxis and attract neutrophils which adhere to the injured epithelium and migrate towards the interstitium (Streiter *et al.* 1999, Wheeler and Bernard 1999, McGrath *et al.* 2011). When macrophages are co-cultured with stem cells, macrophages showed an increase in anti-inflammatory activity (Sukho *et al.* 2018). MSCs have been shown to secrete factors which upregulate the secretion of IL-10 in macrophages (Eggenhofer *et al.,* 2014).

Tumour Necrosis factor (TNF) is known to stimulate the acute phase of the immune response and is one of the first cytokines to be released in response to a pathogen. TNF, induces vasodilation which benefits neutrophil and monocyte infiltration in ARDS as well as recruiting

inflammatory cells to the site of injury. (Beutler 1999, Arango Duque and Descoteaux 2014). TNF-α is an essential molecule in ARDS as it promotes the secretion of IL-1, IL-6 and IL-8 from other cells in an inflammatory response (Fiers 1991). Taken together, these cytokines will activate endothelial cells which increase the surface adhesion molecules. Studies have shown that TNF- α stimulates lung liquid clearance (Tillie-Leblond *et al.* 2002). Inflammatory signalling IL-6 recruits monocytes to the inflammatory or injury site **(**Hurst *et al.* 2001). TGF-β1 moderates the activity of cytokines TNF-α and IL-1β (Defrance *et al.* 1992) IL-22 has a direct effect on epithelial barriers, including those on the lungs (Whittington *et al.* 2004).

These difference in effects may be occurring as MSCs are primed for an inflammatory milieu, thus sending out an array of mixed signals (as observed in section 3.5.1) modulating as well as activating the macrophage. Priming MSCs could lead to the generation of a population of a mixed phenotype of macrophages such as M1 and M2 or inflammatory and anti-inflammatory.

Phagocytosis assays show (Figure 3.7) that conditioned media from MSCs primed with cytomix increase phagocytosis in THP-1 It is believed that an anti-inflammatory M2 phenotype is induced in macrophages by MSC mediated immunomodulation in ARDS and sepsis. MSCs also enhance bacterial killing and phagocytosis, which

is mostly associated with M1 phenotype. (Németh *et al.* 2009, Mei *et al.* 2010).

3.5.3 Effect of conditioned media from primed MSCs on inflammatory and chemical injury

3.5.3.1 A549 cell injury

There are many functions of the alveolar epithelium including, removal of alveolar oedema fluid via ion secretion, surfactant secretion and prevention of translocation of cytokines and bacteria in to circulation (Wang *et al.*, 2007). ARDS is pathologically characterised by injury to the lung parenchyma, which is partially due to the immune response (Laffey and Matthay 2017). Restoration of the pulmonary endothelial and epithelial cells is required to resume normal lung function

Mesenchymal stem cells have been shown to interact with alveolar type II cell (Fang *et al.*, 2015). Affects observed were mediated through the secretion of angiopoietin-1 (Fang *et al.*, 2010). Fang et al., 2010 injured A549 cells with cytomix and measured cell permeability restoration. Findings show that allogenic MSCs restored epithelial protein permeability. The group also state that these findings were mediated by the secretion of the paracrine-soluble factor Ang1 by the MSCs. This effect was also mediated in part through the suppression of NFκB activity. Schwede et al., 2018 also found that MSCs reversed inflammatory genes expressed by A549 after injury with cytomix.

This experiment was important as it has been previously shown that, an inflammatory environment induced by endotoxins, caused a reduction in surfactant production and alveolar fluid clearance *in-vivo* both major functions of alveolar epithelial cells (Gupta *et al.*, 2007; Fang *et al.*, 2010). ATII cells also play a role in host defence through cytokine and chemokine expression (Thorley *et al.*, 2007) Thus, eepithelial cells of the lungs contribute to both innate and acquired immune responses through the release of transmitting agents (Martin and Wallace 2006).

If the inciting cause of ARDS is by an infectious substance, inflammatory markers are secreted by epithelial cells which attenuated the immune response making increasing injury. In this work A549 cells were insulted with IL-1β at a concentration of 10ng/mL. Cell monolayers were then subsequently treated with PBS, conditioned media from naïve MSCs and MSCs preactivated with cytomix for 24hrs. After 24hrs cells were washed and fresh media was placed on cells. After which IL-8 was measured a marker of inflammation mediated by NFκB. This chemokines is measured to determined if treatments had the ability to alleviate the immune response after injury. Results show that, IL-8 was significantly reduced (Figure 3.10).

3.5.3.2 H202 injury

Reactive oxygen species (ROS) are produced as intermediates in metabolic pathways. ROS species play an essential role in the progression of inflammation as they are generated by PMNs and cause endothelial dysfunction and tissue injury. Oxidative stress thus leads to the opening of inter-endothelial junction and this promotes the migration of inflammatory cells across the endothelial barrier (Mittal et al 2014).

In pathological conditions such ARDS, ROS may be higher (Marabini et al. 2011) resulting in lung oxidative stress. H202 was used in these *in-vitro* tests as it induces oxidative stress. H_2O_2 generates hydroxyl radicals which are unstable ROS (Valavanidis et al., 2013). Figure 3.8 demonstrates the effect of H_20_2 at varying doses on A549 (Panel A) and THP-1 cells (Panel B) measured using MTT. This assay was carried out to determine the optimal dose to treat A549 and THP-1 cells. Analysis showed that doses ranging from 0.1-20 mM significantly reduced the viability of A549 cells. Doses ranging from 2.5 – 20 mM had a significant effect on the viability of THP-1 cells. As a reduction in viability at roughly 50% observed in A549 was found to be 1mM and with THP-1 10mM these doses were chosen for injury assays.

To mimic *in-vivo* scenarios cells were treated with H_2O_2 to induce oxidative stress as H_2O_2 induces oxidative stress. To determine if naïve and pre-activated cell conditioned medium displayed

protective effects against oxidative stress, injured cells were simultaneously treated with conditioned media and viability was measured using MTT after 24 hours. As no significant increase in viability was observed in A549 cells after injury and subsequent treatment with conditioned media it suggests that primed MSCs do not display this protective effect *in-vitro* (Figure 3.9 Panel A). Analysis of Annexin V showed that conditioned media from MSCs significantly reduced apoptosis in THP-1 after injury (Figure 3.9 Panel B). with H2O with respect to injury eliciting a protective effect after injury.

3.6 Summary

These *in-vitro* experiments were carried out to determine the cellular effects of cytokines alone or in combination on MSCs. These effects were broadened to THP-1 cells and injury model by treating cells with conditioned media from primed and unprimed cells. Results show that priming elicits a mixed response in MSCs and this effect is also observed in THP-1 cells. Conditioned media was also shown to have protective effects in inflammatory and chemical injury.

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Chapter 4.

"Preactivated Umbilical Cord-Mesenchymal Stem Cells reduce lung injury and bacterial load in *Staphylococcus Aureus* induced pulmonary ARDS."

In vivo section – animal work

Manuscript in preparation

4.1 Introduction

The ability of hMSCs to reduce the severity of lung injury has been demonstrated in various preclinical models of lung injury from both non-infectious (Ortiz et al. 2003; Curley et al. 2012) and infectious causes (Ionescu et al. 2012; Mei et al. 2010; Krasnodembskaya et al. 2012) MSCs elicit theses effects in different ways including the decrease of endothelial and epithelial permeability increasing fluid clearance through mitochondrial trans, interactions with macrophages either through the secretion of soluble mediators or through tunneling nanotubules (TNT) and other immune cells. MSCs have been shown to possess antimicrobial properties in part through the secretion of the anti-microbial peptide LL-37. (Keane et al. 2017)

Experimental data about the therapeutic role of MSCs has been widely described in infective models of pulmonary ARDS induced by Gram negative bacteria in small and large animals (Asmussen et al. 2014; Devaney et al. 2015; Curley et al. 2017; Perlee et al., 2019; Asami et al. 2018; Gupta et al., 2018; Mao et al., 2015; Gupta et a;. 2012**)**.
In contrast, very limited information is described in the literature about the therapeutic potential of MSCs in preclinical models of infective pulmonary ARDS induced by Gram positive bacteria.

S.aureus is the most common clinical isolate among gram positive bacteria in nosocomial pneumonia, therefore it is object of our preclinical investigation (Jones RN, 2010; Diekema et al., 2019; Koulenti et al., 2017; Tong et al., 2015**).**

We here aimed to establish a relevant preclinical model of *S. aureus* induced ARDS using the strain Newman, which was originally obtained from a clinical isolate (Duthie et al., 1952; Kneuper et al., 2014; Baba et al., 2008; Bae et al., 2006) and well established to induce lethal pneumonia in mice (Kohler et al., 2011; Ragle et al., 2010; Wardenburg et al., 2007; Adhikari et al., 2012; Inoshima et al., 2011).

Among different available options of hMSCs, umbilical cord mesenchymal stem cells (UC-MSCs) are an ideal source for cell therapy due to their accessibility, pain-free collection, expansion capacity and number of early passages compared to MSCs obtained from other sources (Hua et al., 2013; Kern et al., 2006; Fong et al., 2012**).**

As per these characteristics we wished to explore the role of MSCs in the treatment of a model of pulmonary ARDS induced by the development of bilateral pneumonia in rats using a direct intratracheal instillation of *S. aureus* Newman.

Lung microenvironment during injury determines the behavior of MSCs, these effects can be none, beneficial or detrimental. Islam et al., 2010 found that beneficial effects of MSCs are determined by lung micro-environment. In another study of microenviroment, MSCs were found to be inhibitory towards healthy PBMCs and supportive towards endotoxin injured PBMCs. This effect was enhances by pre-culturing the MSCs with a combination of low/short dose endotoxin or LPS **(**Leburel et al., 2017**)**. Furthermore, over the last decade, a model of lung injury in vitro has been established using cytomix, a mixture

of IL-1β, TNF-α, and IFN-γ (50 ng/ml). hMSCs demonstrated to be a therapeutic resource in the injury resolution and in the gene regulation (Fang et al., 2010; Fang et al., 2015; Schwede et al., 2018**).**
MSCs were thus primed with cytomix before administration. This was done to determine if the MSCs exposed to an inflammatory signal would it alter how they behaved in a lung injury model.

To determine the effects of soluble factors secreted by MSCs *in-vitro* injury assays were carried out to determine if any beneficial or detrimental effect was observed. ELISAs were also carried out on supernatants after exposure of MSCs to cytomix to determine the cytokine expression profile as preliminary assessment of inflammatory or anti-inflammatory behavior (Islam et al., 2019) determined that administration of MSCs carrying human genes for IL-10 could potentially protect the lung. We found that, after pre-activation of cytomix MSCs expressed significantly higher levels of IL-10 in respect to PBS controls potentially predicting a beneficial outcome *in-vivo*. We wished to further explore whether the priming of UC-MSCs using cytomix could implement the resolution of the lung injury in a live bacterial pneumonia-induced ARDS.

4.1.1. In-vivo study aims

The aims of this chapter are to determine whether;

1. *S. aureus* Newman can induce a model pulmonary ARDS in rats;
2. Naïve and preactivated UC-MSCs can prevent ALI induced by *S. aureus* Newman;

3. Low dose preactivated UC-MSCs can prevent ALI induced by *S. aureus* Newman.

4.1.2. Key objectives

- To develop a model of *in-vivo* gram positive lung injury using a clinical isolate of *Staphylococcus aureus* in rats
- To determine whether UC-MSCs with or without preactivation with cytomix can reduce a model of pulmonary ARDS induced by *S. aureus* (Series 1)
- To determine whether, after the results obtained in Series 1, low dose of preactivated UC-MSCs can decrease the lung injury in an induced *S. aureus* model of pulmonary ARDS (Series 2).

4.1.3. The overall hypothesis

The overall hypothesis is that mesenchymal stem cells isolated from umbilical cord primed with cytomix will demonstrate anti-microbial and immune-modulating effects in an *in-vivo* model of lung injury induced by *S.aureus,* a gram-positive bacterium obtained from a clinical isolate.

4.2 Methods

4.2.1. Approval and Ethical Issues

All experiments were conducted under license from the Department of Health and Children, Ireland. Approval was received from the Animal Care Research Ethics Committee of National University of Ireland,

Galway. In vivo experiments were carried out using specific-pathogen-free adult male CD® Sprague Dawley rats (Charles River Laboratories, Kent, United Kingdom) with a weight ranging 350-450g.

4.2.2. Animal Health and Distress Scoring

Animal welfare was regulated according to the principle of the 3 Rs (reduction, refinement, replacement). All in-vivo experiments were carried out following the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines (Kilkenny et al., 2010**).**

Before in vivo experiments and after experimental procedures, animals were continuously monitored using distress scoring to assess their health status and to check the presence of distress, pain, morbidity or mortality, and courses of action were performed as per distress sheet scoring recommendations. Distress scoring was performed before the injury and for
48 hours following injury and treatment administration and it was approved by the Health Products Regulatory Authority of Ireland.

Injury Model

4.2.3 Oro-Tracheal Intubation

Animals underwent anaesthesia by isoflurane inhalation gas anaesthesia system
(3-5% isoflurane in 100% O_2, oxygen flow rate 2L/min).

After check and confirmation of the depth of anaesthesia by paw clamp, the tail vein was cannulated using a 22G IV catheter (BD Insyte®; Becton Dickinson Ltd., Oxford, United Kingdom).
Orotracheal intubation was performed as previously described (Kastl et al., 2004). The animals were placed on an oblique metal plate and the position was maintained by clasping a non-absorbable thread around the upper incisors.
Laryngoscopy was performed and vocal cords were visualized using an otoscope. Therefore, a guide wire was introduced into the trachea under direct vision. The guide wire was maintained in place, the otoscope was carefully removed in order to avoid its displacement and a 14G IV cannula was positioned over the guide wire and inserted into the trachea. The guide wire was then removed and the correct position of the cannula was assessed as first by the presence of water vapor on a glass slide at each breath and confirmed, immediately after, by connecting the ventilator and inspecting the movement of the thorax and absence of stomach distention.

4.2.4 S. aureus preparation

For all experiments a bead was taken from the microbank stocks. The bead (bead #1) was placed into an aerated cultivation tube containing 30 mL of TSB. The suspension was incubated at 37°C and stirred at 180 rpm in a rotary incubator. At 9 hour (late log phase) 1 mL of TSB was collected from the stock and centrifugated. The supernantant was removed and the pellet was resuspended in 1 mL of phosphate-buffered saline (Ratio TSB to PBS, 1:1). Optical density at 600 nM against reagent blank. Another mL from the original stock underwent

the same process and it was used to quantify CFU count using serial dilution and plate count. Bacteria at varying dilutions were spreads over agar plates aseptically. The agar plates were inverted and incubated at 37°C for 24 hr after which colonies were counted and recorded. A second bead was then taken from the microbank stock and processed under the same experimental condition. At 9 hours, based on the quantified CFU count of bead #1, TSB broth from bead #2 was centrifugated, the pellet was resuspended in PBS in order to obtain a concentation of proximally $5x10^8$ CFU count in 300 microL of PBS.

4.2.5. *S. aureus* instillation

After the confirmation of the depth of anaesthesia by paw clamp following induction of anaesthesia with isoflurane, and the correct placement of the cannula into the trachea, a suspension of 300 µl PBS with *S. aureus* Newman was instilled by a 14 G cannula into the trachea under direct vision.

A preliminary series of experiments was performed in order to establish the bacterial load of intra-tracheal *S. aureus* required to produce a severe lung injury over a 48 hour period. An inoculum of proximally 5×10^8 CFU of *S. aureus* produced a severe ALI over a 48 hour period, compared with controls inoculated with the same volume of sterile PBS with no added bacteria (sham animals).

After bacterial instillation, the animals were allowed to recover for one hour and randomized to treatment within 2 hours.

4.2.6 Experimental design

Following *S. aureus* instillation, animals were entered into one of the following two animal series:

Series 1 compared the efficacy of human naïve versus preactivated UC-MSCs therapy in attenuating *S. aureus* induced pulmonary ARDS. Animals were randomized within 2 hour post injury to intravenous administration of: (i) vehicle (PBS, 1000µL); (ii) $1x10^7$ UC-MSCs/kg (Naive); or (iii) $1x10^7$ cytomix primed UC-MSCs/kg (Cytomix).

Series 2 evaluated the lowest effective preactivated MSC doses. Animals were randomized within 2 hour post injury to intravenous administration of: (i) vehicle (PBS, 1000µL); (ii) $5x10^6$ cytomix primed UC-MSCs/kg or (iii) $2x10^6$ cytomix primed UC-MSCs/kg.

4.2.7 Injury assessment and euthanasia

After the start of the experiment and following intra-tracheal instillation of *S. aureus* and IV hMSCs administration, animals were monitored according to their distress scoring for 48 hours. Animals were singly contained in separated ventilated cages. At 48 hours post injury induction, animals underwent anaesthesia with subcutaneous injection of 0,4 mg/kg medetomidine (Domitor; Vetoquinol, Ireland) and 60 mg/kg ketamine (Ketalar; Pfizer, Ireland).

Intravenous access was obtained and secured via rat tail vein using a 22 G cannula.

Pre-tracheal fur was removed, and pre-tracheal muscles and fascia were blunt dissected with arterial forceps to expose the trachea. The trachea was engraved between the 4th and 5th tracheal rings, and a tracheostomy tube (2-mm internal diameter) was inserted and secured. Right sided pre-tracheal muscles and strap muscles of the neck were

moved aside on the right. The right sided carotid artery was put under direct vision and the vagus nerve and adherent sheath were dissected from all the visible tract of the carotid artery. An intra-arterial access was put in place uing a 22-gauge cannula (Becton Dickinson, Cowley, United Kingdom).
The arterial cannula was connected to an arterial line for blood sampling and for direct measurement of the arterial pressure waveform (Biopac systems, Ltd, MP36).
The tracheostomy tube was connected immediately after to a small animal ventilator (CWE SAR 830 AP, CWE Inc, Pennsylvania, USA) and animals were ventilated using medical air (FiO_2 0.21), respiratory rate 80 breaths/min, tidal volume of 6 mL/kg, I:E ratio of 1:1, and positive end expiratory pressure of 2 cmH_2O.
Anesthesia was maintained with repeated intravenous boli of alfaxalone (Alfaxan; Jurox, Ireland) and Cisatracurium besylate 0.5mg/kg (GlaxoSmithKline, Dublin, Ireland) was administered intravenously to induce muscle relaxation.
To minimize lung derecruitment, a recruitment manoeuvre consisting of positive end-expiratory pressure 15 cmH_2O for 20 breaths was applied at the start of the protocol and every 5 minutes throughout the protocol.
All animals were ventilated with medical air for 20 minutes using these settings. Depth of anesthesia was assessed every 10 min by monitoring the cardiovascular response to paw clamp. Body temperature was maintained at 36–37.5°C by using a thermostatically controlled blanket system (Harvard Apparatus, Holliston, MA) and confirmed with an indwelling rectal temperature probe.

Systemic arterial pressure, peak airway pressures and temperature were continuously measured. After 20 minutes, an arterial blood sample (100 microlitre) was drawn for blood gas analysis (ABL 90; Radiometer, Copenhagen, Denmark) and static inflation lung compliance measured. Incremental 1-ml volumes of room air were injected via the tracheostomy tube, and the pressure attained 3 s after each injection was measured, until a total volume of 5 ml was injected (Laffey et al., 2004). Animals were then ventilated for a further 15 minutes with an inspired gas mixture of $FiO_2 = 1.0$ and arterial blood samples were again drawn for blood gas analysis.
At the end of the treatment protocol, heparin (400 IU/kg; CP Pharmaceuticals, Wrexham, United Kingdom) was administered intravenously, and the animals were then euthanized by exsanguination.

Tissue sampling and assays

4.2.8 Tissue Sampling and Assays

A thoracotomy was performed immediately postmortem. The heart-lung block was removed from the thorax. The following analyses were performed.

4.2.9 Wet:dry ratio

The right upper lobe was isolated, excised and weighted (wet), and placed in a 40°C
oven. After 72 hours the same lobe was re-weighted (dry) for calculation of wet:dry ratio.

4.2.10 Bronchoalveolar lavage collection

Bronchoalveolar lavage (BAL) collection was performed as
previously described
(Higgins et al., 2009).
15ml of sterile NaCl 0.9% was injected via the tracheostomy into
lungs, in
5ml increments. These 5ml increments were allowed to return into a
15ml
conical tube. 20 microlitres of BAL were used undiluted for total cell
count,
and 150 microlitres were used for preparation of slides for differential
cell count. Remaining BAL fluid was centrifuged at 1500g for 15
minutes. The supernatant was collected and stored at -80°C for later
analysis.

4.2.11 Total cell count

Total cell numbers per milliliter of BAL fluid were counted.
Each sample of undiluted BAL was counted twice for total cell count.
A standard Neubauer hemocytometer counting chamber was used,
counting cells in the four 1/25 sq. mm corners plus the middle square
in the central square. 10 microL of BAL
fluid were pipetted underneath the haemocytometer coverslip. In order
to obtain total cells per ml the total count was divided by 5 and
multiplied by $1x10^4$.

4.2.12 Differential cell count

150 microliters undiluted BAL fluid was pipetted into the funnel of a cytospin cartridge (Thermo-Fisher Scientific). The cartridge, along with a glass slide, were spun for 10 minutes at 200 rpm in the cytospin centrifuge (Thermo-Fisher Scientific). Cells were transferred to the glass slide, and fluid became adherent to the blotting paper on the underside of the cytospin cartridge. Slides were then stained using the Diff-Quik method. Slides were immersed 6 times in methanol, 5 times in eosin, and 3 times in methylene blue and allowed to dry. To obtain the differential cell count, 300
inflammatory cells (macrophages, neutrophils, lymphocytes or eosinophils) were identified in three different regions of the slide, and the number of neutrophils in each count was noted. An average of the neutrophil count in the 3 different fields was calculated to give the percentage neutrophil count. The total cell count was used to calculate the number of neutrophils per ml of BAL.

4.2.13 Protein assay

A commercially available protein assay kit - Micro BCA™ Protein assay
kit (Pierce, Rockford, IL, USA) was used. Each sample was examined in triplicate. Due to the high concentration of protein in the BAL, we first made a 1:2 dilution of each sample in PBS. We first prepared standards as outlined in kit instructions to give a working range of protein concentrations from 20-2000 μg/ml. The Working Reagent (WR) was prepared by mixing 50 parts of BCA™ Reagent A
with 1 part of BCA™ Reagent B (50:1, Reagent A:B). We pipetted 25 μl of each

standard or sample replicate into a microplate well (working range =
20-2,000
µg/ml). We then added 200 µl of the WR to each well and mixed the
plate
thoroughly on a plate shaker for 30 seconds. The plate was then
covered and
incubated at 37°C for 30 minutes before being cooled to room
temperature. The
absorbance of the samples was then measured at 595nm on a Wallace
plate
reader.

4.2.14 Enzyme-linked immunosorbent assay

We used commercially available enzyme linked immunoassay kits
from R&D
Systems Europe Ltd, Abingdon Science Park, Abingdon, OX14 3NB
United
Kingdom. Nunc (Denmark) 96 well flat bottomed ELISA
(MAXISORB) plates were firstly coated with the binding antibody
and coating buffer at 100 µl per well, wrapped in parafilm and left to
incubate at 40°C overnight. Plates were then subjected to three washes
per well of 300 µl PBS Tween. Blocking buffer of 1% BSA, 100 µl
was added to each well and left for one hour at room temperature. A
standard
curve set of samples was constructed with 400 µl neat IL-1 beta, TNF-
a, IL-6, IL-10, KGF, or CINC1 at the top end of the range and 7 serial
half dilutions of this then made with 1% BSA as the diluents added to

the plate . Samples were then added in triplicate to the remaining well diluted in a 1:2 dilution. 1% BAL was used in duplicate as a blank control. The plates were then incubated for 2hrs at room termperature. Plates were washed after this incubation strep as previously described. A 1 in 400 dilution of detection antibody in reagent diluent was added to each well and left to incubate for 2hrs at room temperature. After incubation the plates were washed again as previously described. Strep –Hrp conjugate at a dilution of 1:40 in reagent diluent was added to each well and incubated at room temperature for 20 minutes. The plate was then washed again. 100 µl of TMB was added to each well and the plate was incubated in the dark for 20 minutes. Finally dark 50 µl of stop solution (1M H_2SO_4) was added to each well and the plate read at the Wallace plate reader. Absorbance readings at 450nm for 0.1 secondsand 550nm for 0.5 seconds were taken.

Histology

4.2.15 Preparation of lung tissue

The left lung was isolated and fixed for morphometric examination as previously described **(**Laffey et al., 2004; Howell et al., 2003**).** The pulmonary artery was cannulated, the left atrium was incised, and the pulmonary circulation was perfused with normal saline at a constant hydrostatic pressure of 35 cm H_2O until the left atrial effluent was clear of blood. The right lung was ligated and removed to be used for further rt-PCR and Western Blot analysis.

The left lung was then inflated through the tracheal catheter using paraformaldehyde (4% wt/vol) in phosphate-buffered saline (300 mOsmol) at a

pressure of 25 cm H_2O. Paraformaldehyde was then instilled through the pulmonary artery catheter at a pressure of 62.5 cmH_2O. The left atrium was then tied off to prevent pulmonary venous inflow into the atrium, creating a constant distending pressure across the pulmonary vasculature, and maximally distending the pulmonary vessels. After 30 min, the pulmonary artery and trachea were ligated, and the lung was stored in paraformaldehyde for 24 h and then embedded in paraffin wax. After fixation, the vertical axis of the left lung was identified. The lung was cut into six equal sections 4 mm thick perpendicular to this axis using a sharp blade.

4.2.16 Paraffin embedding

After sectioning the paraformaldehyde fixed lung, the sections were placed in an
appropriately labelled histoprocessing cassette (Sigma). Also included in the cassette was a pencil inscribed label detailing the animal and section number (A to E from the top to the bottom). The sections were then placed in a histoprocessor (ASP 300 histoprocessor, Leica Microsystems, Wetzlar, Germany) on the factory preset "routine overnight" program. The following morning the cassettes were removed
from the processor and the sections embedded in paraffin wax using a Heated Paraffin Embedding Module (Leica). Each section was orientated in a transverse manner, with its top as the cutting edge. Sections were allowed to set on a cold plate before slide preparation.

4.2.17 Slide preparation

Sections were cut at 7 μm using a microtome (Leica RM2235 Microtome (Leica).)
Sections were carefully placed onto the surface of water maintained at 40°C. Sections were examined on the water surface for imperfections before being lifted onto Superfrost Plus microscope slides (Thermo Scientific) and allowed to drain. Sections were then stored at room temperature overnight before staining using Haematoxylin and Eosin. It is important to note that all labeling of slides was performed in a blinded manner.

4.2.18 Hematoxalin and Eosin Histological Staining

The following morning the slides were then put through two baths of xylene (Cat # 305756G, VWR International Limited, Poole, BH15 ITD, England) and graded alcohols (Cat # 1.00983.2500, Merck KGaA, 64271, Darmstadt, Germany) (100%, 90% and 70% respectively) to deparaffinize and rehydrate the tissue, and then rinsed for 2 minutes with running tap water.
The slides were then placed in a bath of hematoxylin for 6 minutes and rinsed for 4 minutes through running tap water. Sections were checked for adequate staining at this point. Eosin staining occurred for 2 minutes followed by dehydration through 50%, 70%, 95% and 100% alcohol baths for 30 seconds each.
The slides were next placed in a bath of eosin (Cat # HT110232, Sigma-Aldrich Ireland Ltd. Dublin, Ireland) for 2 minutes. They were immersed in two baths of xylene, 15 minutes each. Finally, the slides were then mounted in DPX (VWR International, Orion Business Campus, Northwest Business Park, Ballycoolin, Dublin 15, Ireland)

and coverslipped (Thermo Scientific Thickness: Nr. 1). The DPX mountant was allowed to spread between the sections and the cover slip and the slide was then left overnight at room temperature. Slides prepared in this manner were stored finally at room temperature in plastic slide boxes and protected from light.

4.2.19 Stereological Analysis

Quantitative stereological technique was performed as previously described (Hopkins et al., 2001).

Slides prepared as described above were viewed at a 20X magnification under a

microscope and using a bright field view (Leica BioSystem, Aperio digital pathologyslide scanner, ScanScope, Leica Microsystems Srl, Milano, Italy). Two fields of view from each slide were chosen at random and digitised using a digital camera (Olympus BX61, Mason technologies, Milan, Italy). Images were stored in eight-bit (256 level) format. The grid reference, i.e. X and Y grid coordinates for each image was recorded by referencing the scale attached to the microscope (200 micrometer). A 100 point counting grid was overlaid on each image in ImageJ imaging software package (Version 1.52k, Wayne Rashband, national Institute of Health, USA). Care was taken to ensure that the software was set to 10X magnification. Once this grid was superimposed over the image, a touch count was performed. At each of 100 intersection points on the grid, a record was taken for each of the following: acinar tissue, non-acinar tissue and airspace. The intraacinar tissue was defined as all tissues within the gas exchange portion of the lung, i.e., respiratory bronchioles, alveolar

ducts, alveolar sacs, and alveoli, including blood vessels contained within their walls. The intraacinar airspace was defined as all airspaces within the lumen of respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. Intersections of the grid were manually counted for airspace, acinar and non-acinar tissue. Non-acinar tissue was subtracted from the overall tissue to calculate percentage alveolar tissue.

4.2.20 Mesenchymal Stem Cell Techniques

Human umbilicord stem cells were provided by Tissue Regeneration Therapeutics Ltd, Toronto, Canada.

The cells were cryopreserved at a passage of 1. Cells were cultured and expanded in 175cm^2 tissue culture flasks (Sarstedt T AG & Co., Numbrecht, Germany). The growth medium used was MEM-alpha growth medium (GIBCO. Invitrogen Corporation, NY, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/ml) and streptomycin (100 µg/ml) solution (Sigma-Aldrich). Fibroblast growth factor (fgf) (Peprotech US, NJ, USA) was used at a concentration of 10ng/ml. When cells reached the desired confluence, cells were washed twice in Dulbecco Phosphate buffer saline (DPBS) the medium was then replaced with culture medium containing either PBS or cytomix. Cells were treated for 24hrs. After 24hrs cells were washed in PBS and sub-cultured with 0.035% trypsin-0.05 mM ethylenediamine tetra acetic acid (EDTA; GIBCO. Invitrogen Corporation, NY, USA). Cells at required dose were centrifuged at 1500 RPM and resuspended in PBS for I.V administration.

4.2.21 Data Analysis

All analyses were performed using graphpad Prism (Version 7.0a, La Jolla, USA).

The distribution of all data was tested for normality using the Kolmogorov-Smirnov test. Results are expressed as mean (± SEM) or as median (interquartile range) as appropriate. In vitro data were analyzed with one-way Analysis of Variance (ANOVA) corrected for multiple comparisons by controlling the false discovery rat. In vivo data were analyzed with one-way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls test for post-hoc analysis. Data of sham animals were represented in graphs as reference of healthy animal. A p value of <0.05 (two-tailed) was deemed statistically significant. Data were analyzed using Graphpad Prism (Version 7.0a, La Jolla, USA).

4.3 Results

4.3.1 Establishing a new model of Gram positive pulmonary ARDS using *S. aureus* Newman to induce bilateral pneumonia.

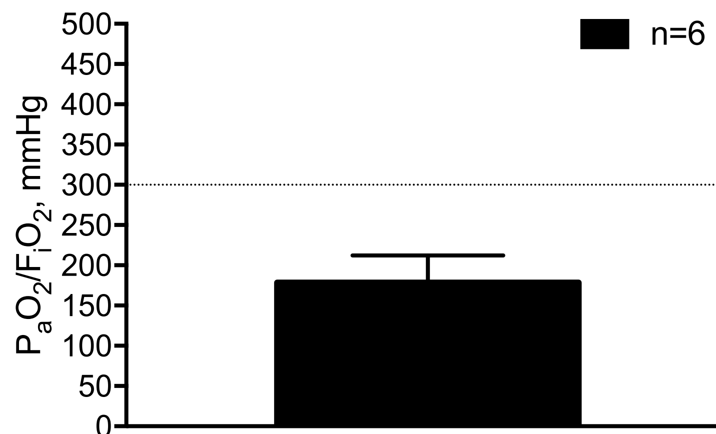

Figure 1. Preliminary experiments were performed to determine the bacterial load of intratracheal *S. aureus* required to produce a lung injury over a 48 h period.

The intratrachel injection of proximally $5x10^8$ CFU of S. aureus Newman induced a model of moderate-severe ARDS (PaO_2/FiO_2<200 mmHg) at 48h. All animals survived (n=6).

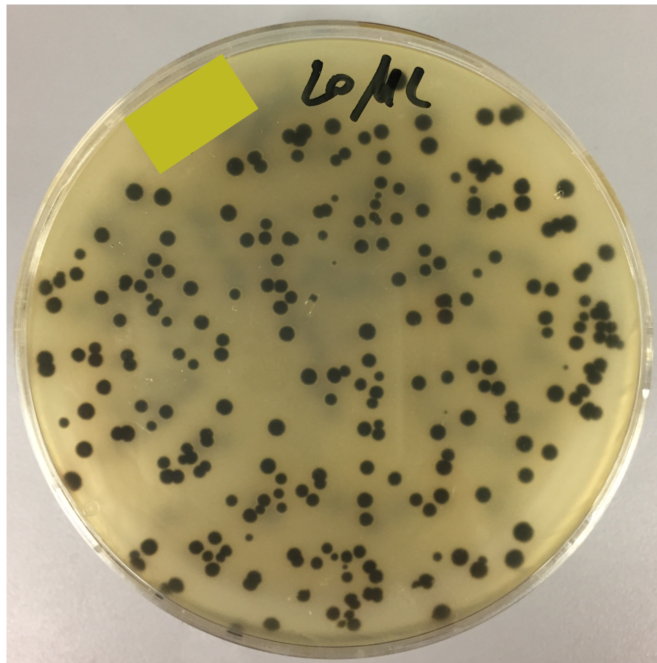

Figure 2. Representitive image of *S. aureus* Newman colonie in BAL of animals treated with $5x10^8$ CFU of S. aureus Newman confirming the physiological measurement of poor oxygenation of Figure 1.

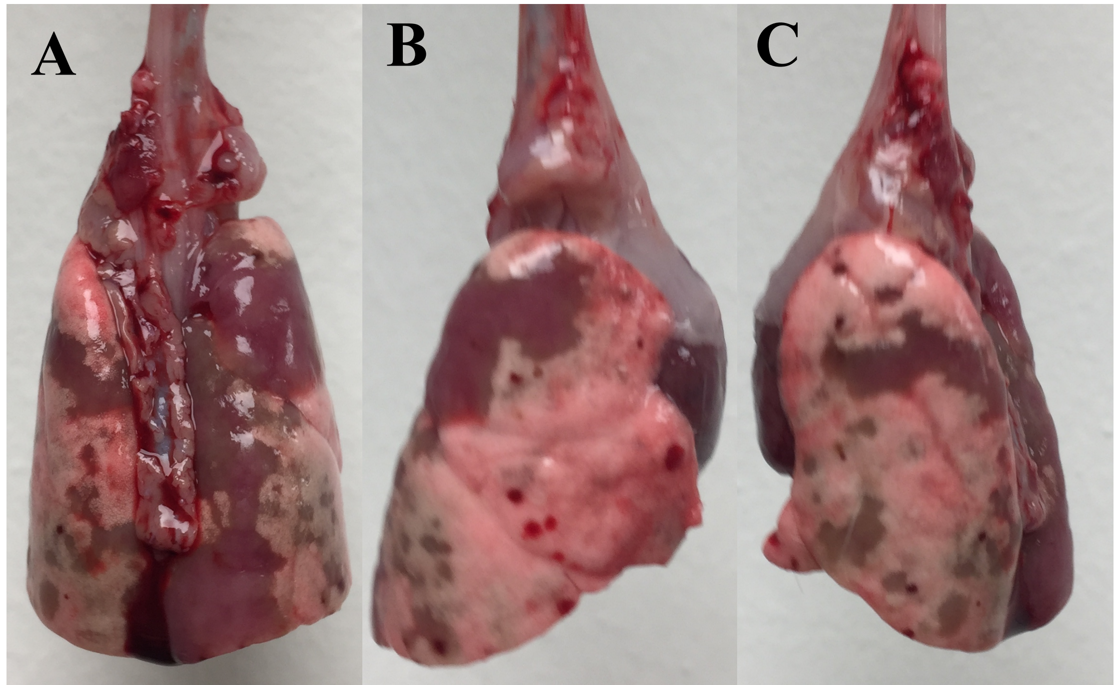

Figure 3. This a representative image of the lung macroscopy after the intratracheal instillation of $5x10^8$ CFU of *S. aureus* Newman confirming the presence of bilateral infiltrates and collapse. A, view of both lungs; B, view of the right lung; C, view of the left lung.

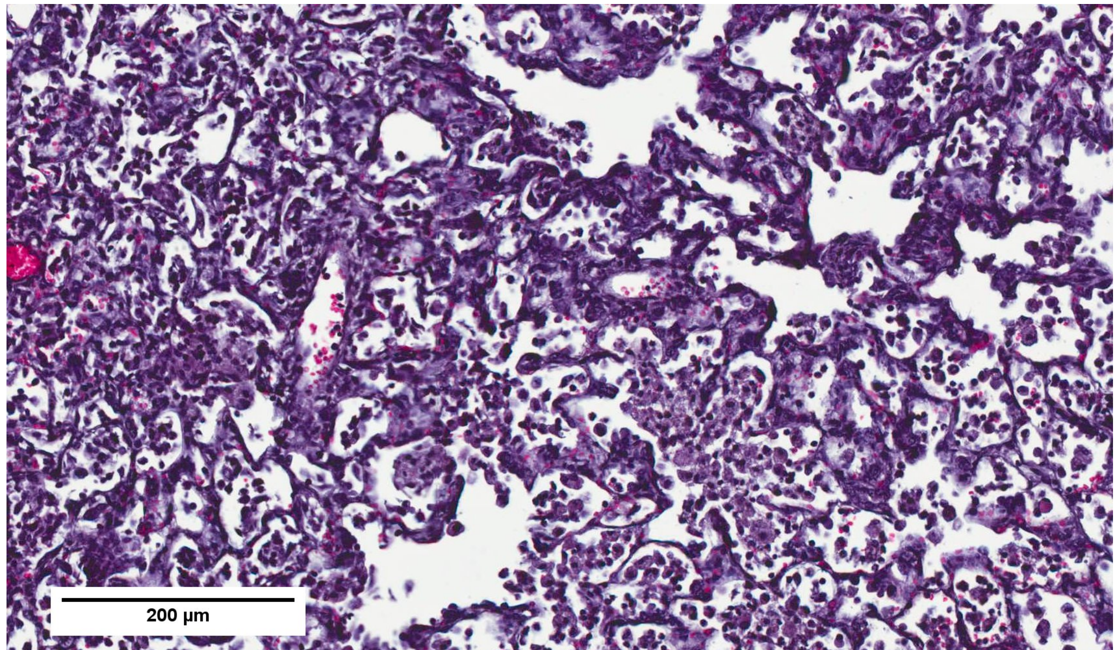

Figure 4. This a representative image of an histology lung section at 48h after the intratracheal instillation of $5x10^8$ CFU of *S. aureus* Newman in rats confirming the thickening of the interstistial septa (tissue consolidation), the reduction of the alveolar spaces and relevant presence of white blood cell infiltrates in the alveoli.

4.3.2 Series 1: To determine whether UC-MSCs with or without preactivation with cytomix can reduce the lung injury in a model of pulmonary ARDS induced by *S. aureus*.

4.3.2.1. Assessment of lung injury: physiological measurements

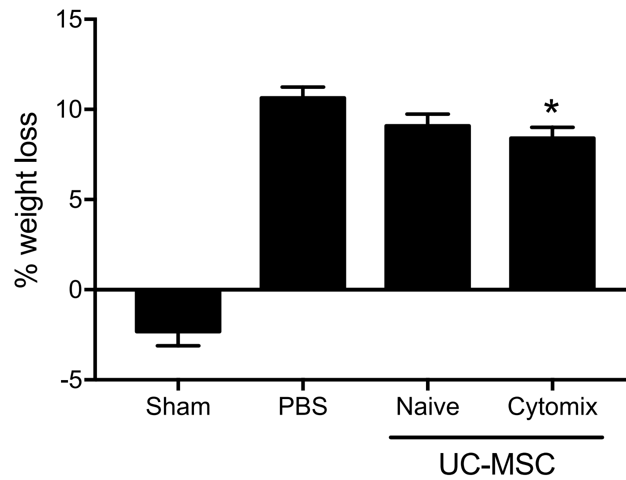

Figure 5. Animal weight loss at 48h. Preactivated UC-MSCs prevented animal weight loss over 48 hours compared to PBS. *$p<0.05$ vs PBS; n=6-9. Data as mean ± SEM.

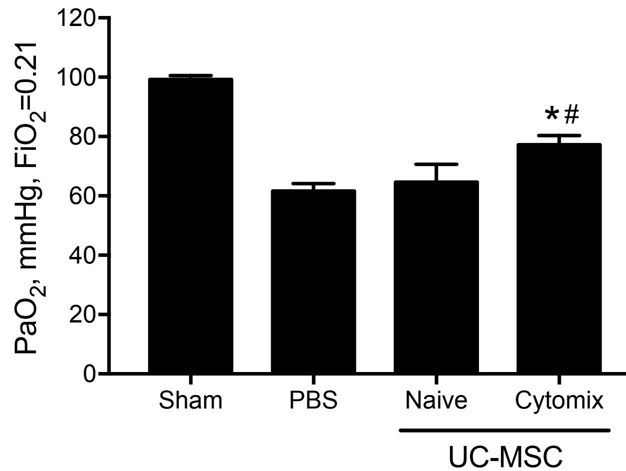

Figure 7. Animal oxygenation at room air at 48h post injury. The oxygenation in the animal group treated with preactivated UC-MSCs was significantly higher compared to both groups of animals that

received PBS or naïve UC-MSCs. *p<0.05 vs PBS; #p<0.05 vs Naïve. n=6-9. Data as mean ± SEM.

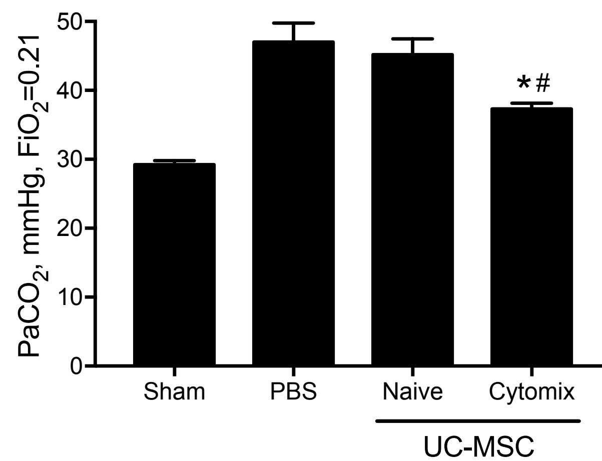

Figure 8. Animal arterial CO_2 clearance at 48h with room air. The treatment with preactivated UC-MSCs led to a better clearance of arterial carbon dioxide, compared to PBS and naïve UC-MSCs treated animals. *p<0.05 vs PBS; #p<0.05 vs Naïve. n=6-9. Data as mean ± SEM.

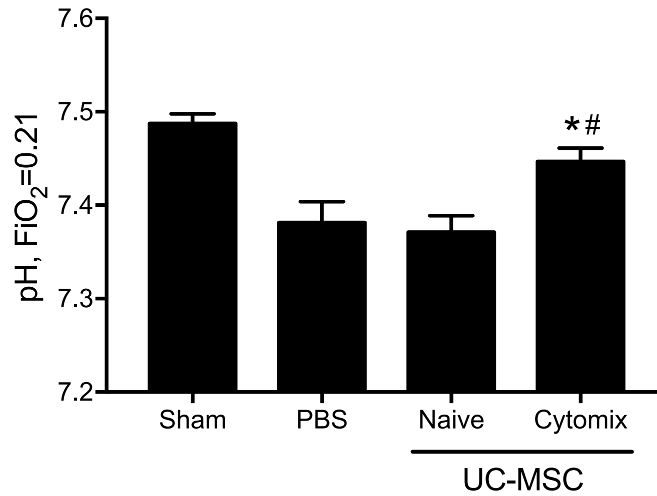

Figure 9. Animal arterial pH at 48h with room air. Preactivated UC-MSCs improved arterial pH compared to PBS and naïve UC-MSCs treated animals. *$p<0.05$ vs PBS; #$p<0.05$ vs Naïve. n=6-9. Data as mean ± SEM.

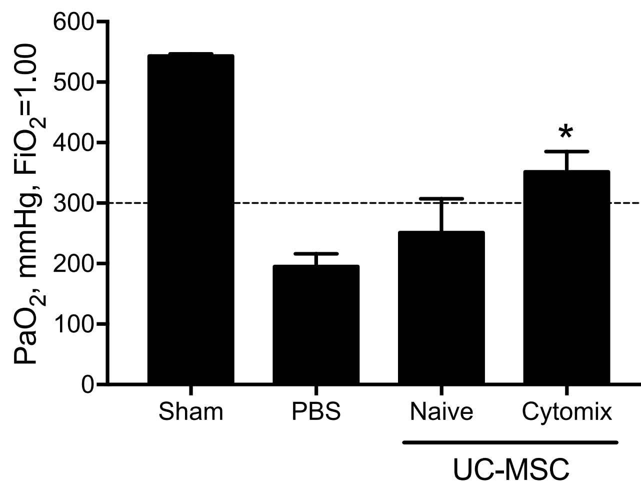

Figure 10. Animal oxygenation at 48h with FiO_2=1. Preactivated UC-MSCs prevented lung injury according to PaO_2/FiO_2 criteria compared to PBS treated animals. The dashed-line indicate threshold

of oxygenation criteria for ALI in a clinical setting. *p<0.05 vs PBS. n=6-9. Data as mean ± SEM.

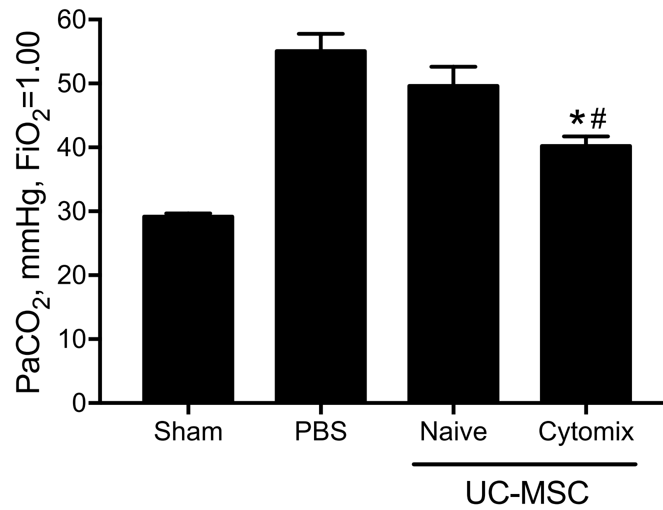

Figure 11. Animal arterial pCO_2 clearance at 48h with FiO_2=1.
The treatment with preactivated UC-MSCs led to a better clearance of arterial carbon dioxide at FiO_2=1.0, compared to PBS and naïve UC-MSCs treated animals. *p<0.05 vs PBS; #p<0.05 vs Naïve. n=6-9. Data as mean ± SEM.

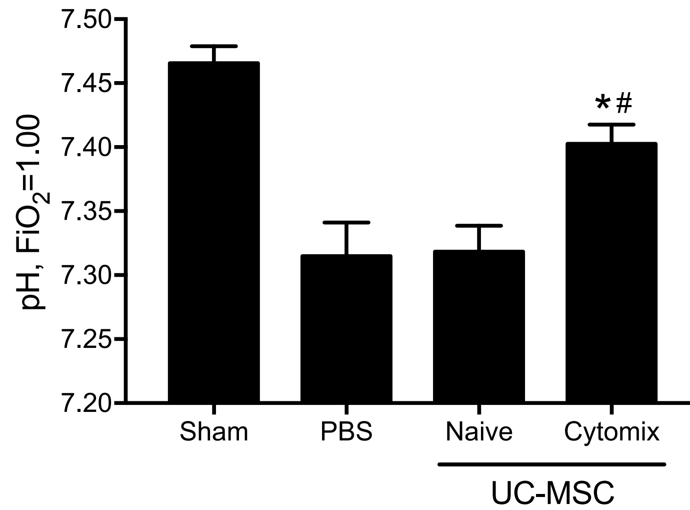

Figure 12. Animal arterial pH at 48h with FiO_2=1. Preactivated UC-MSCs improved arterial pH compared to PBS and naïve UC-MSCs treated animals at FiO_2=0.2. *$p<0.05$ vs PBS; #$p<0.05$ vs Naïve. n=6-9. Data as mean ± SEM.

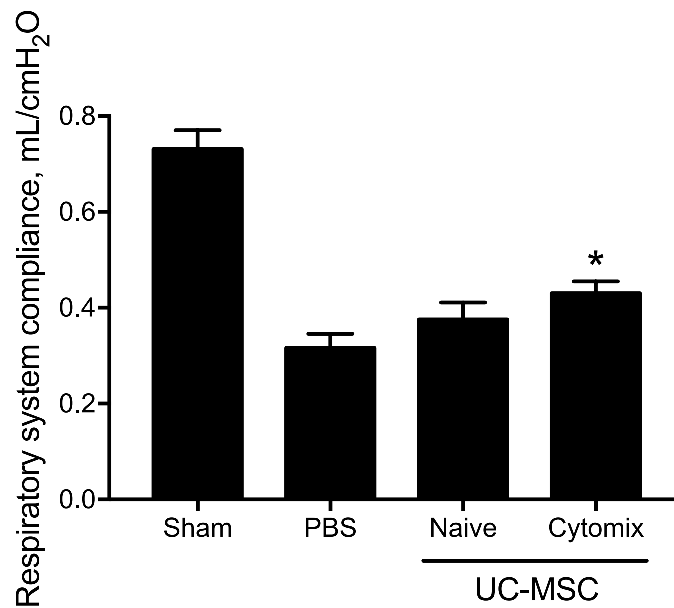

Figure 13. Respiratory mechanics at 48h post injury. The treatment with UC-MSCs primed with cytomix the respiratory system compliance - measured in static conditions - compared to PBS treated animals. *p<0.05 vs PBS. n=6-9. Data as mean ± SEM.

4.3.2.2. Ex-vivo analysis: wet to dry ratio

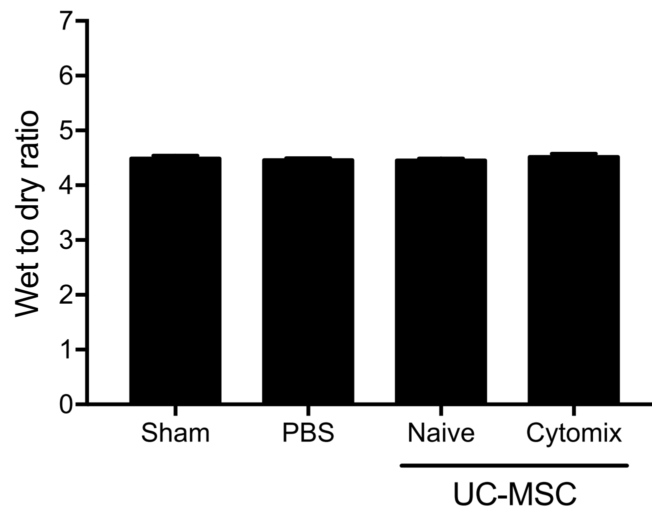

Figure 6. Lung wet to dry analysis. The wet to dry ratio did not differ among the experimental groups. n=6-9. Data as mean ± SEM.

4.3.2.3. Ex-vivo analysis: BAL

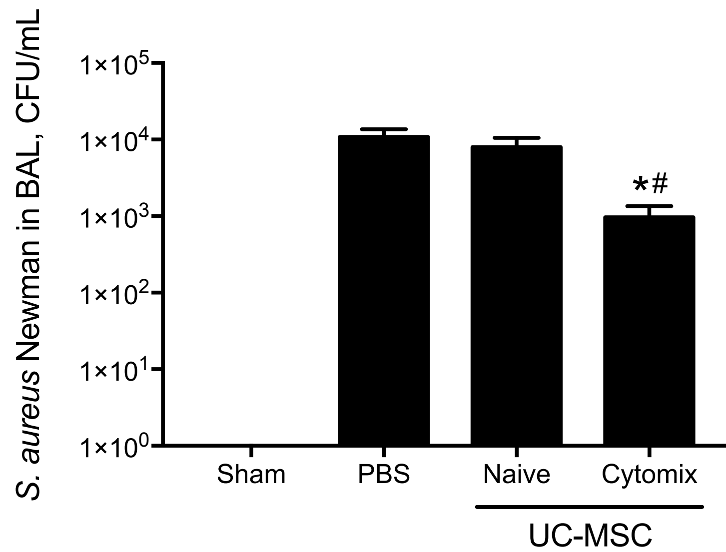

Figure 14. ***S. aureus*** **CFU count in BAL.** The treatment with preactivated UC-MSCs increased the bacterial clearance as demonstrated by significantly lower CFU count in BAL compared to the levels of CFU in PBS and naïve UC-MSCs treated animals. *$p<0.05$ vs PBS. #$p<0.05$ vs Naïve. n=6-9. Data as mean ± SEM.

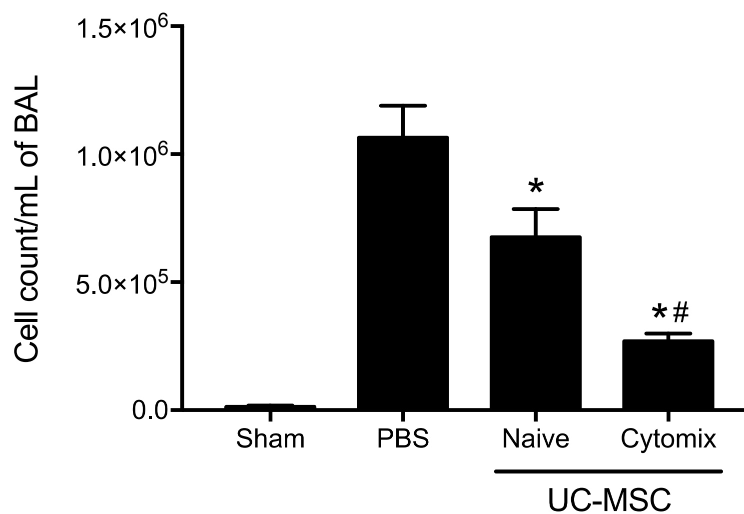

Figure 14. White blood cell count in BAL. The treatment with both preactivated and naïve UC-MSCs significantly decreased the cell count in BAL compared to PBS. UC-MSCs primed with cytomix further decreased the cell count in BAL compared to naïve UC-MSCs. *p<0.05 vs PBS. #p<0.05 vs Naïve. n=6-9. Data as mean ± SEM.

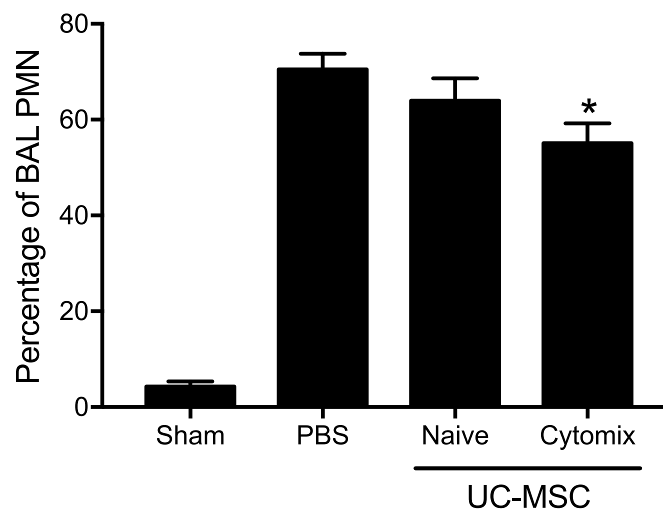

Figure 15. Differential cell count in BAL. The analysis of the differential cell count demonstrated that cytomix preactivated UC-MSCs decreased the percentage of polymorphonuclear leukocytes in BAL compared to PBS treated animals. *p<0.05 vs PBS. n=6-9. Data as mean ± SEM.

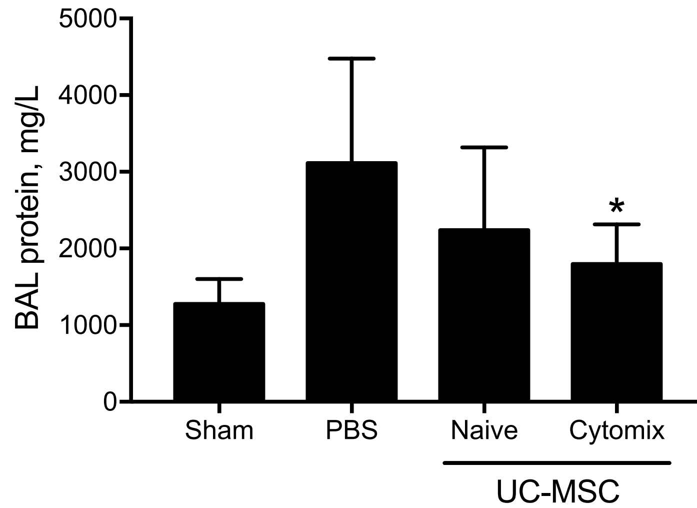

Figure 16. Quantification of BAL protein levels ex-vivo. The treatment with UC-MSCs primed with cytomix decreased the protein level in BAL compared to the animals treated with PBS. *p<0.05 vs PBS. n=6-9. Data as mean ± SEM.

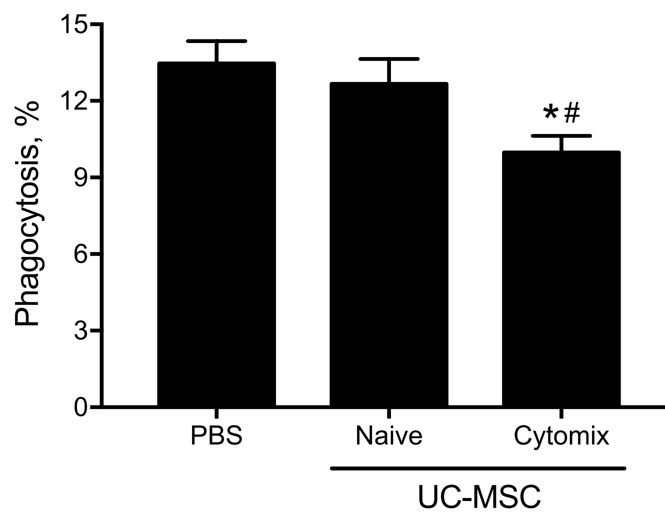

Figure 17. Phagocytosis induced by BAL of the 3 experimental groups on THP-1. The phagocytosis activity in the cytomix preactivated UC-MSC group was significantly decreased compared to PBS and naïve UC-MSCs treated animals. *p<0.05 vs PBS. #p<0.05 vs Naïve. n=9. Data as mean ± SEM.

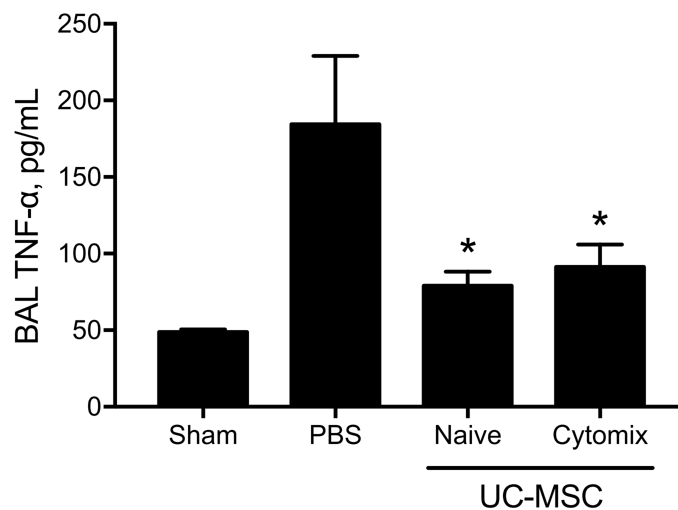

Figure 18. The levels of TNF-α were significantly reduced in the BAL of UC-MSC treated rats compared to PBS. Representative image of 7 micrometer section of lung in the different experimental groups and in healthy animals. Panel A, sham; panel B, PBS; panel C, naïve UC-MSCs; panel D, cytomix preactivated UC-MSCs.

4.3.2.4. Ex-vivo analysis: lung histology

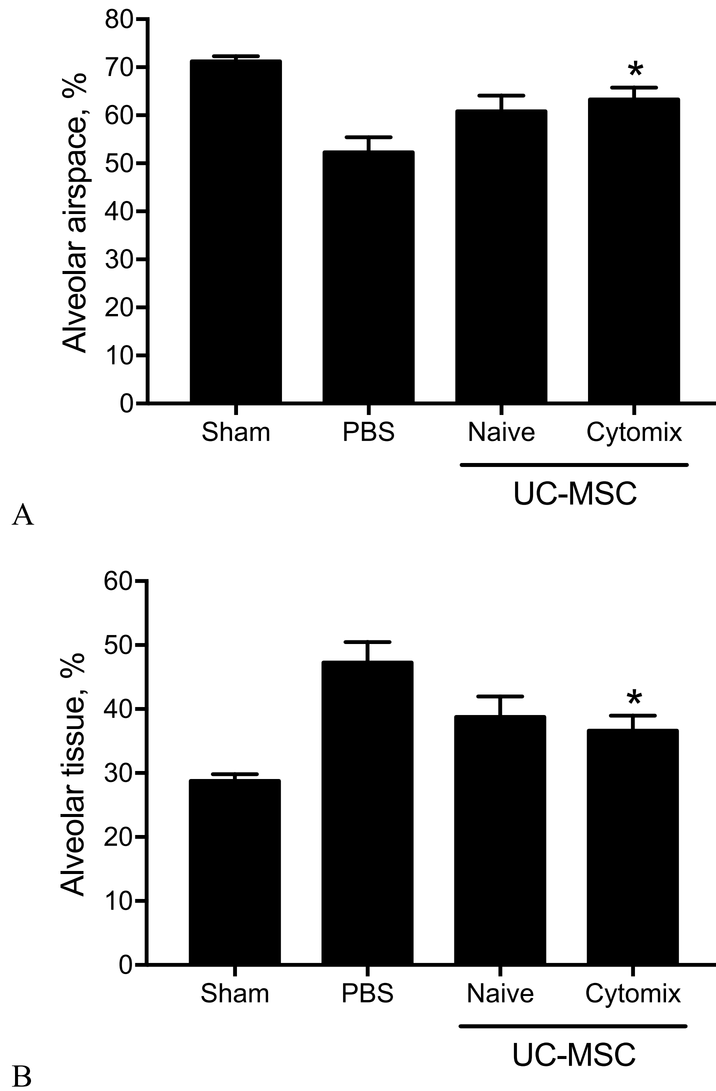

Figure 18. Preliminary lung histological analysis. The treatment with UC-MSCs primed with cytomix increased the ratio between the airspace to alveolar tissue in the lung histological analysis compared to PBS treated animals. *p<0.05 vs PBS. n=5. Data as mean ± SEM.

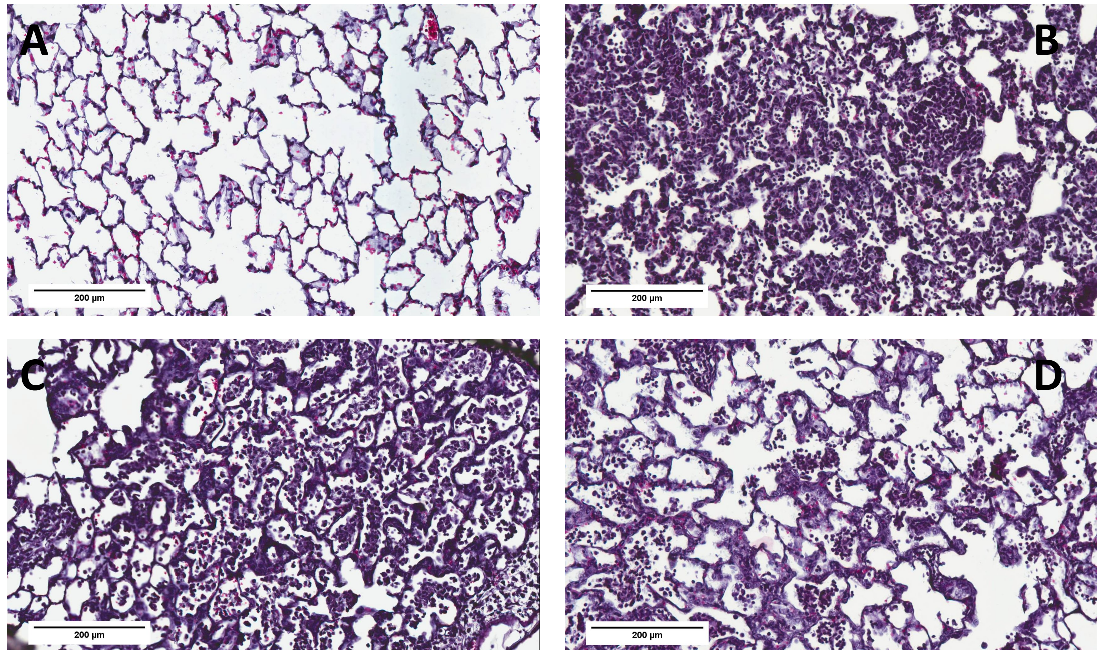

Figure 19. Representative image of 7 micrometer section of lung in the different experimental groups and in healthy animals. Panel A, sham; panel B, PBS; panel C, naïve UC-MSCs; panel D, cytomix preactivated UC-MSCs.

4.3.3. Series 2: To evaluate the lowest effective preactivated MSC doses to reduce the acute lung injury in a model of pulmonary ARDS induced by *S. aureus*.

4.3.3.1 Assessment of lung injury: physiological measurements

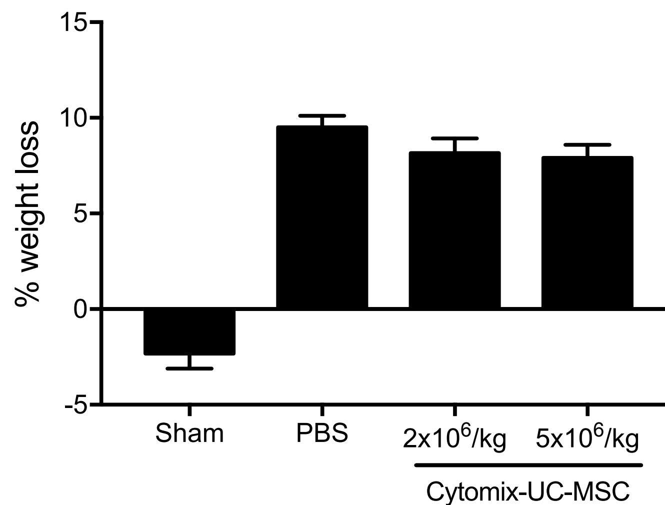

Figure 20. Animal weight loss at 48h. Weight loss was not significantly different at 48 hours after injury among groups. *p<0.05 vs PBS; n=6-10. Data as mean ± SEM.

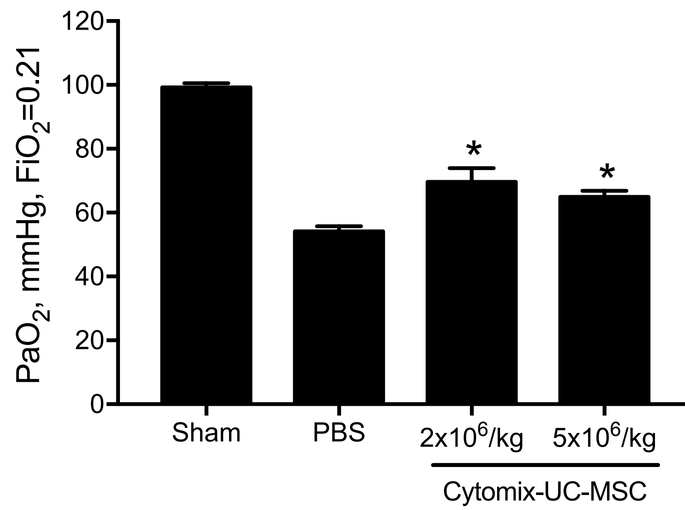

Figure 21. Animal arterial oxygenation at room air at 48h post injury. The oxygenation in the animal groups treated with cell therapy was significantly higher to the arterial PaO_2 in PBS treated animals. *$p<0.05$ vs PBS; n=6-10. Data as mean ± SEM.

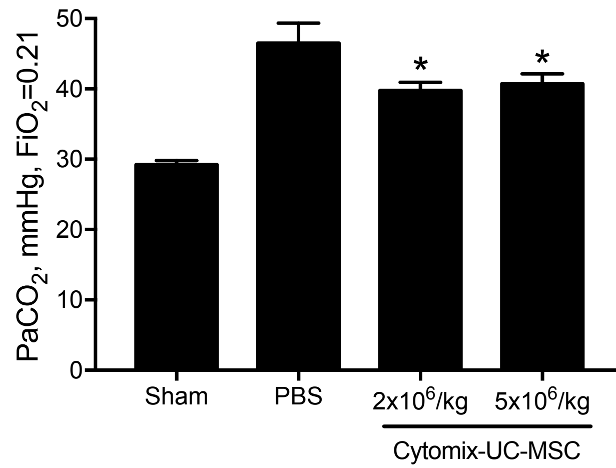

Figure 22. Animal arterial CO_2 clearance at 48h with room air. The treatment with preactivated UC-MSCs at 2 and 5 million per kg led to a better clearance of arterial carbon dioxide compared to CO_2 removal in PBS treated animals. *$p<0.05$ vs PBS; n=6-10. Data as mean ± SEM.

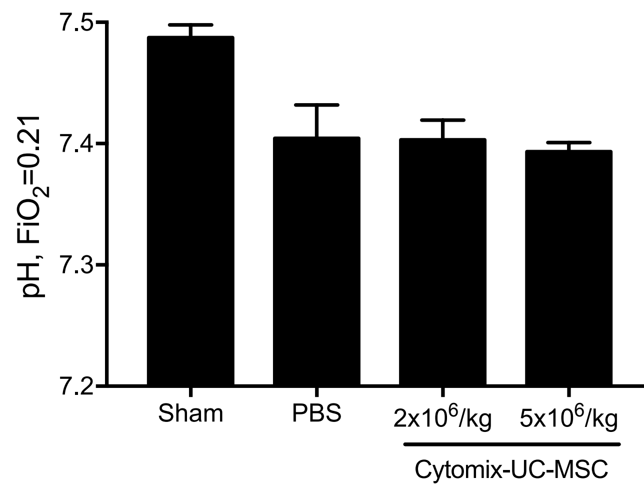

Figure 23. Animal arterial pH at 48h with room air. The arterial pH among the experimental groups did not differ at 48h after injury. n=6-10. Data as mean ± SEM.

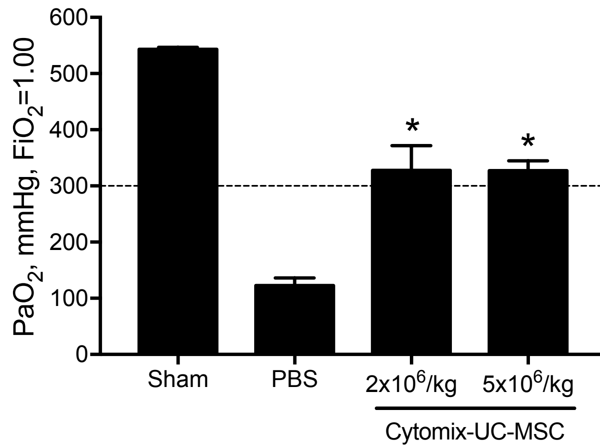

Figure 24. Animal arterial oxygenation at 48h with FiO_2=1.
Preactivated UC-MSCs at 2 and 5 million per kg prevented lung injury according to PaO_2/FiO_2 criteria compared to PBS treated animals. The dashed-line indicate threshold of oxygenation criteria for ALI in a clinical setting. *p<0.05 vs PBS; n=6-10. Data as mean ± SEM.

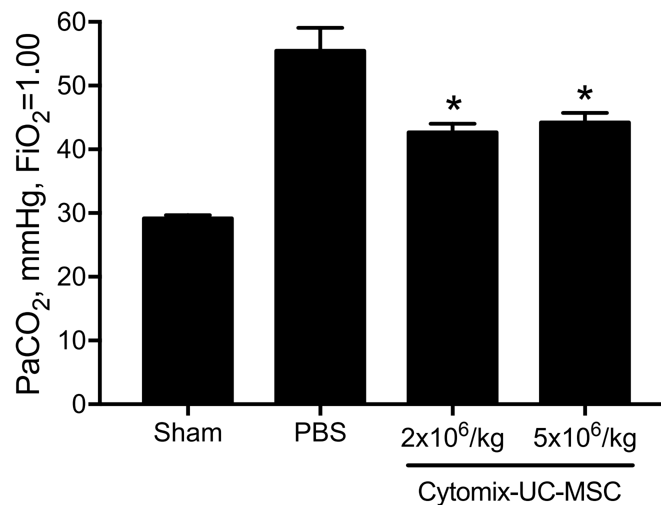

Figure 25. Animal arterial pCO_2 clearance at 48h with FiO_2=1.
Cell therapy led to a better clearance of arterial carbon dioxide,

compared to PBS treated animals. *p<0.05 vs PBS; n=6-10. Data as mean ± SEM.

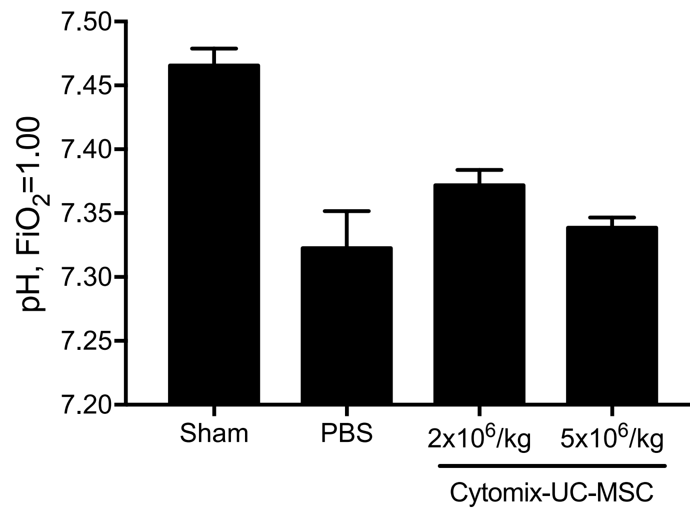

Figure 26. Animal arterial pH at 48h with FiO_2=1. The arterial pH did not differ among the experimental groups. *p<0.05 vs PBS; n=6-10. Data as mean ± SEM.

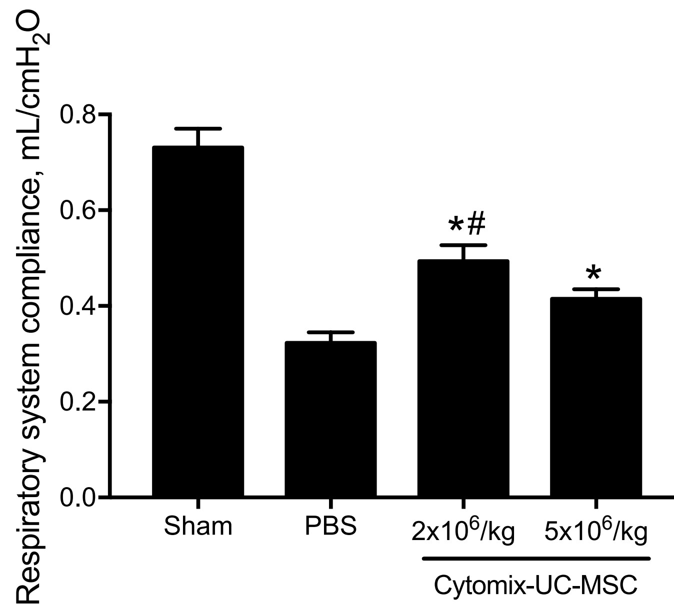

Figure 27. Respiratory mechanics at 48h post injury. The treatment with UC-MSCs primed with cytomix at low and middle dose increased the respiratory system compliance (Rs,cp) - measured in static conditions - compared to PBS treated animals. Furthermore, the administration of low dose primed UC-MSCs led to a higher Rs,cp than the one observed in animals treated with $5x10^6$/kg UC-MSCs. *$p<0.05$ vs PBS; #$p<0.05$ vs $5x10^6$/kg UC-MSCs; n=6-10. Data as mean ± SEM.

4.3.3.2. Ex-vivo analysis: wet to dry ratio

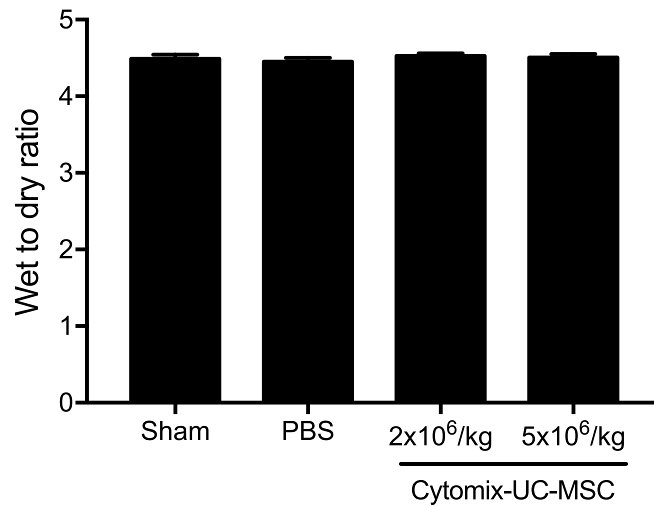

Figure 28. Lung wet to dry analysis. The wet to dry ratio did not differ among the experimental groups. n=6-10. Data as mean ± SEM.

4.3.3.3. Ex-vivo analysis: BAL

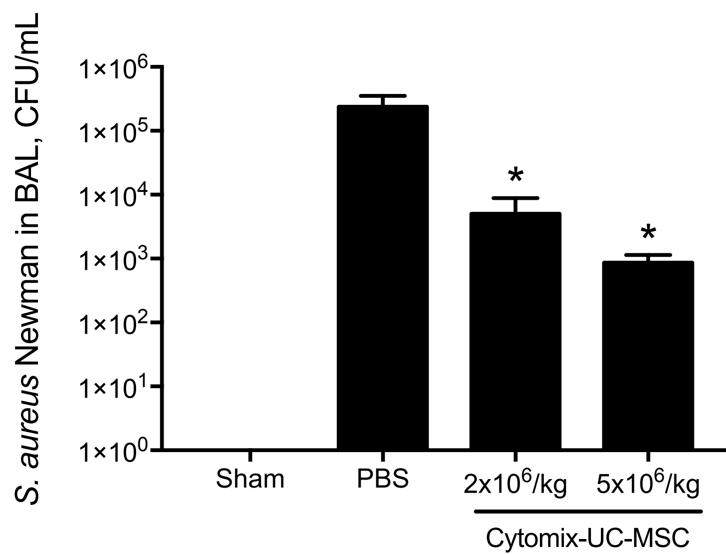

Figure 29. ***S. aureus*** **CFU count in BAL.** The treatment with preactivated UC-MSCs increased the bacterial clearance as demonstrated by significantly lower CFU count in BAL compared to the levels of CFU in PBS treated animals. *$p<0.05$ vs PBS; n=6-10. Data as mean ± SEM.

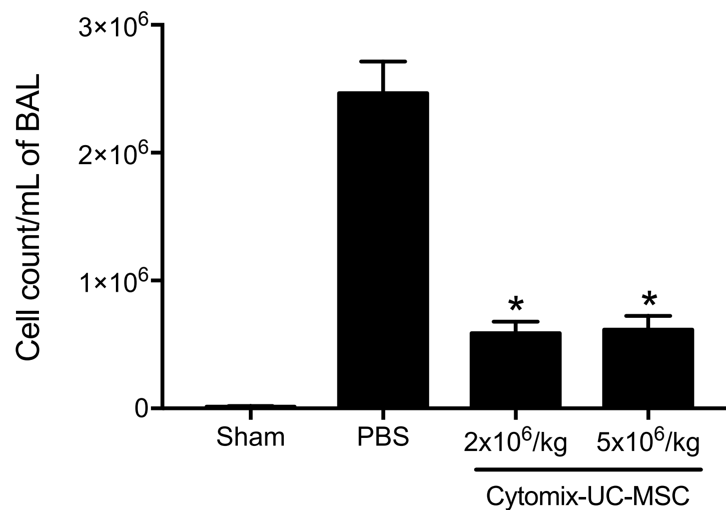

Figure 30. White blood cell count in BAL. The treatment with both doses of preactivated UC-MSCs significantly decreased the cell count in BAL compared to PBS. *$p<0.05$ vs PBS; n=6-10. Data as mean ± SEM.

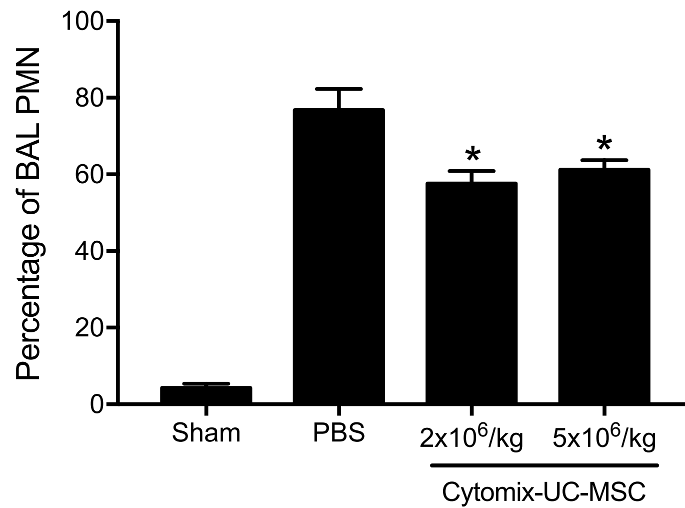

Figure 31. Differential cell count in BAL. The analysis of the differential cell count demonstrated that both doses of UC-MSCs preactivated with cytomix decreased the percentage of polymorphonuclear leukocytes in BAL compared to PBS treated animals. *p<0.05 vs PBS; n=6-10. Data as mean ± SEM.

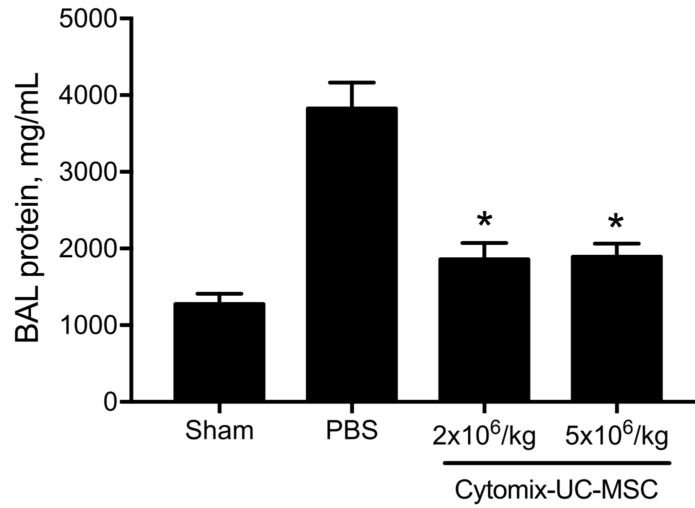

Figure 32. Quantification of BAL protein levels ex-vivo. The treatment with UC-MSCs primed with cytomix decreased the protein level in BAL compared to the animals treated with PBS. *p<0.05 vs PBS; n=6-10. Data as mean ± SEM.

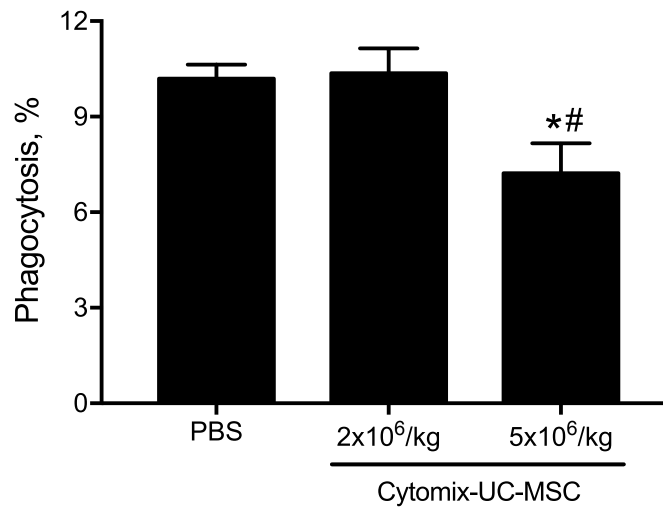

Figure 33. Phagocytosis induced by BAL of the 3 experimental groups on THP-1. The phagocytosis activity in the group of animals treated with $5x10^6$/kg UC-MSCs primed with cytomix was significantly decreased compared to PBS and $2x10^6$/kg preactivated UC-MSCs treated animals. *p<0.05 vs PBS; #p<0.05 vs $2x10^6$/kg UC-MSCs; n=8-10. Data as mean ± SEM.

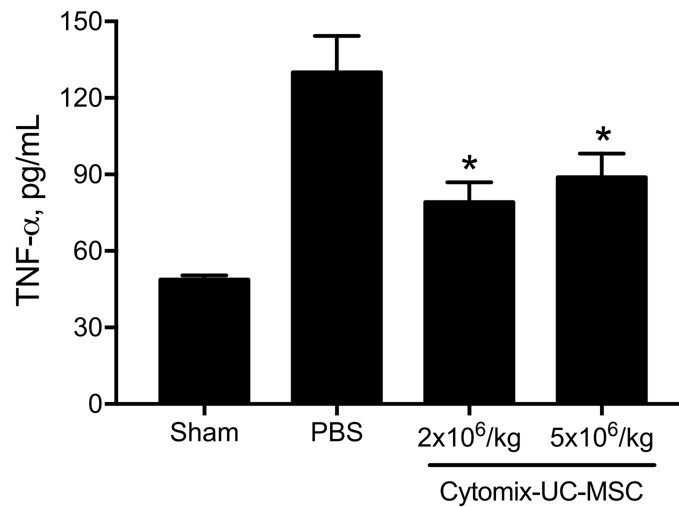

Figure 34. The levels of TNF-α were significantly reduced in the BAL of citomix primed UC-MSC treated rats compared to PBS. *p<0.05 vs PBS; n=6-10. Data as mean ± SEM.

4.3.3.4. Ex-vivo analysis: lung histology

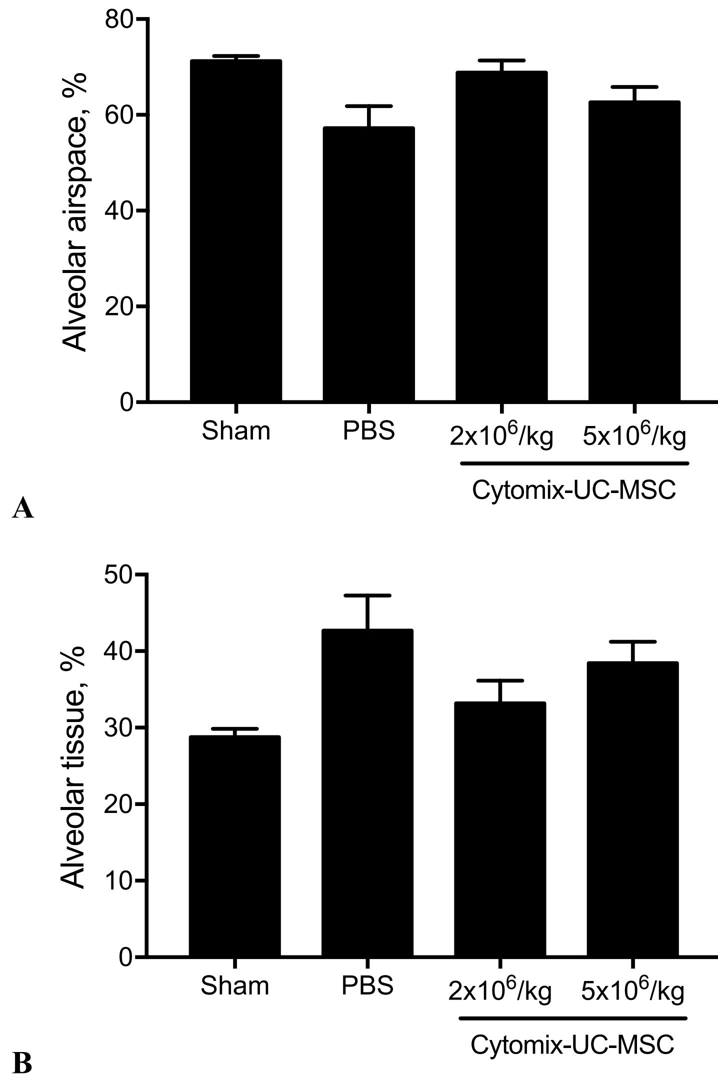

Figure 18. Preliminary lung histological analysis. The treatment with UC-MSCs primed with cytomix at 2 and $5x10^6$/kg doses demonstrated a trend to a significant increase in the ratio between the airspace to alveolar tissue in the lung histological analysis compared to PBS treated animals. n=5. Data as mean ± SEM.

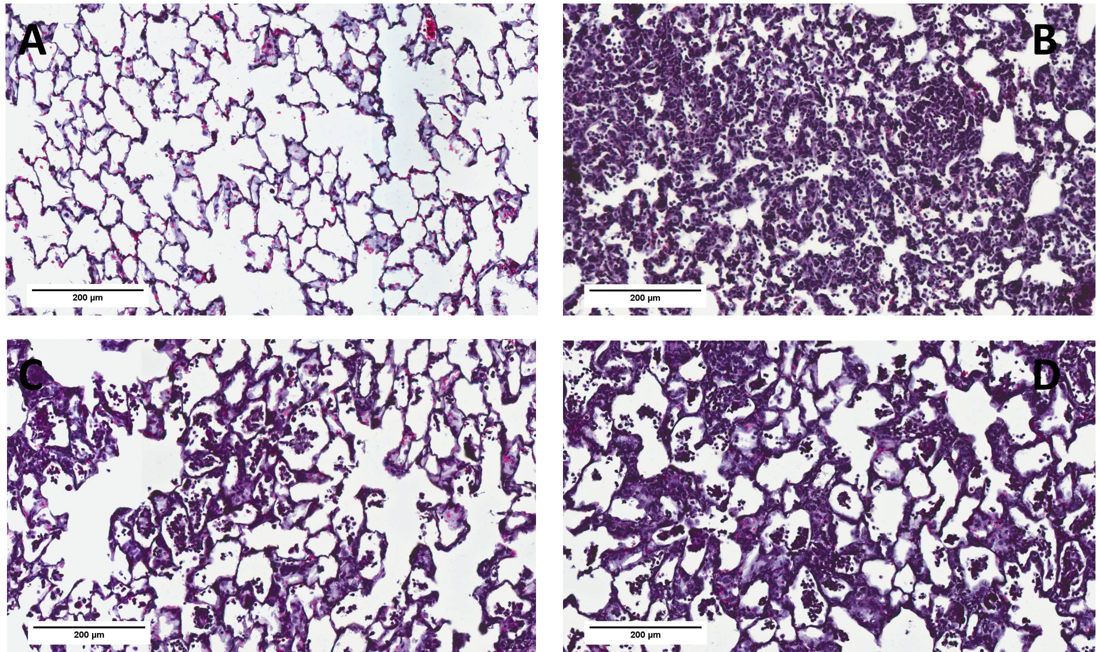

Figure 19. Representative image of 7 micrometer section of lung in the different experimental groups and in healthy animals. Panel A, sham; panel B, PBS; panel C, $2x10^6$/kg cytomix preactivated UC-MSCs; panel D, $5x10^6$/kg cytomix preactivated UC-MSCs.

4.4 Discussion

The findings that we reported in these studies provide novel insights about the potential of human mesenchymal stem cells in the treatment of pulmonary ARDS induced by gram positive bacteria.

We can summarize the main relevant results of these animal studies as follows:

1. We developed a new model of moderate-severe pulmonary ARDS in rats using a clinically relevant strain of *Staphylococcus aureus* (i.e. Newman), a gram positive coccus bacterium, which induced bilateral pneumonia;
2. In series 1, we observed that human naïve and preactivated UC-MSCs with cytomix lowered the number of bacteria and reduced the cellular white blood cell infiltrate in the bronchoalveolar lavage. However, preactivated UC-MSCs with cytomix demonstrated to be superior to naïve UC-MSCs in the prevention of acute lung injury induced by *S. aureus,* as shown by their beneficial effects on physiological variables such us respiratory system compliance, gas exchange, lung histology, lung capillary protein leak and evidence of inflammatory activation in BAL.
3. In series 2, we observed that the beneficial effect of priming with cytomix made UC-MSCs still effective even at low dose (2 million) as shown by a physiological, microbiological, immunological and histological evidence of decreased *S. aureus* induced lung injury.

S.aureus Newman is a gram positive strain known to induce a severe model of pneumonia in mice. Wardenburg et al. reported that the intranasal administration of 4 to $8x10^8$ CFU of *S.aureus* Newman to 7-week-old C57BL/6J mice was lethal in a range of 50-90% mortality (Wardenburg et al. 2007).

In our experiments aimed at establishing a model of ALI induced by gram positive bacteria, we proved that the intratracheal administration of a dose of $5x10^8$ CFU could induce a consistently moderate to severe ARDS by the development of bilateral pneumonia. The animals showed hypoxemia at 48 hours and the presence of *S.aureus* Newman was confirmed by BAL CFU count analysis. The macroscopic appearance of lung cell infiltrates was confirmed by the presence of lung cell infiltrates in BAL which was further proved at the lung histological evaluation.

S.aureus is a clinically relevant strain of gram positive bacteria and it is involved in the development of severe nosocomial pneumonia (Jones et al., 2010**;** Diekema et al., 2019 Koulenti 2017Tong et al., 2015**)**, among which ventilator associated pneumonia is still an hard challenge to fight in critically ill patients, as its still high association with high mortality rate (Melsen et al., 2013**).**

We believe that the establishment of a new model of ARDS in rats using a clinically relevant *S.aures* might be of preminent importance for researchers interested in understanding mechanisms of different therapeutics in pulmonary ARDS and to further explore the application of cell therapy.

The role of human MSCs has been characterized in the attenuation and repair of lung injury induced by ventilation (VILI) (Masterson et al., 2018; Hayes et al., 2015; Curley et al., 2013; Curley et al., 2012**).** The potential of human MSCs in the treatment of bacteria induced acute lung injury has been described in the literature, and it was almost exclusively aimed at addressing rodent models of ARDS induced by gram negative sources.

Devaney et al. reported that bone marrow (BM) MSCs could decrease the severity of ALI induced by *E. coli* in rats. The authors reported that hMSC elicited antimicrobial and immunomodulating effects that allowed to enhance the clearance of bacteria in BAL, reduce the lung cell infiltrate, improve the compliance of the respiratory system preventing the alteration of the lung architecture and then improve the oxygenation levels (Devaney et al., 2015**).** Recently, Curley and colleagues confirmed these results by the use of xeno-free cryopreserved BM-MSCs and UC-MSCs freshly thawed and administered in rats with E.coli-induced ARDS (Curley et al., 2017). The use of adipose tissue derived MSC (ASCs) has been described for the treatment of lung injury in mice induced by *P.aeruiginosa*, and their protective effects have been suggested to include the inhibition of PGE_2 production and the improved macrophages phagocytosis. (Mao et al., 2015). Furthermore, Perlee et al. confirmed the immunomodulatory effects of ASCs in a model of pneumosepsis induced in mice by *K. pneumoniae*.

To our knowledge, the role of h-MSCs in gram positive bacteria induced ALI has yet to be explored in-vivo. A single study attempted to address whether hMSC could be beneficial in models of gram

positive induced lung injury in 2016 by Qian et al. The authors reported that ASCs could exert a protective role in a model of ALI induced by S. Aureus in mice, by direct intratracheal administration of ASCs.

The authors demonstrated that ASCs could attenuate lung inflammation and elicit a direct antimicrobial effect by the LR2-MyD88-JAK2/STAT3-dependent secretion of regenerating islet-derived IIIγ (Qian et al., 2016). However, no information on lung physiological variables and on the effects of i.v. administration of hMSCs was presented.

In our experiments (series 1) we demonstrated that fresh naive UC-MSCs could increase the bacterial clearance and reduce cell infiltrates – as shown by the lower CFU and white blood cell count in BAL compared to animals treated with vehicle (i.e. PBS). However, the arterial oxygenation did not significantly improve.

In contrast, the priming of UC-MSCs with cytomix allowed to restore gas exchanges in terms of oxygenation and carbon dioxide clearance at a dose of $10x10^6$/kg. Furthermore, priming of UC-MSCs with cytomix led to a decrease of inflammatory biomarkers and the percentage of PMN leucocytes population in BAL, it decreased the capillary leakage of protein in the lung and allowed the attenuation of the lung tissue consolidation over the 48h experiment.

To our knowledge, this is the first time that the use of cytomix has been proven as a therapeutic strategy to prime hMSCs and to be effective in the treatment of an in-vivo model of bacteria induced lung injury.

Recently, Varkohoui et al. reported the potential of extracellular vescicles obtained from UC-MScs primed with IFN-γ in a rodent model of *E.coli* pneumosepsis.
IFN-γ is a proinflammatory cytokine secreted by activated lymphocytes (T cells) and natural killer cells (English et al., 2007). The authors observed that the effect of vescicles obtained from UC-MSCs primed with IFN-γ were characterize by an ameliorated capacity to attenuate ALI induced by E.coli compared to the treatment with vescicles obtained from naïve cells, by enhancing the endothelial nitric oxide synthase production in the lungs (Varkhoui et al., 2019).
Jerkic and colleagues proposed a strategy to enhance the immunomodulatory effecs of UC-MSCs in a model of *E.coli* induced pneumosepsis by the overexpression of IL-10, which was made possible by the use of a recombinant adenovirus. The authors reported that IL-10 overexpressing UC-MSCs were superior to naive UC-MSCs in terms of efficacy in *E.coli* pneumosepsis (Jerkic et al., 2019).
The use of therapeutic strategies aimed at priming hMSCs to treat ALI suggest that the development of a immunostimulating microenvironment might be the missing key about a promising potential of cell therapy in clinical trials (see Chapter 1).
Furthermore, this hint has been recently delivered by Ismal D. and colleagues, who reported how the microenviroment is crucial to how hMSCs can be beneficial or detrimental to attenuate lung injury, as reported in in preclinical in-vitro and in-vivo experiments mimiking ALI (Islam et al., 2019**.**).

As last, in our series 2 we demonstrated that the effect of UC-MSCs priming with cytomix persist even at low dose of cells, in our case 2 million cells per kg.
In both low and middle dose of preactivated UC-MSCs arterial oxygenation could be restored (i.e. PaO_2/FiO_2>300 mmHg) at 48 h after the injury.
Devaney et al. Examined the effect of different doses of BM-MSCs in a model of *E.coli* induced ALI. The authors showed the best dose of cells – that were not preactivated – was at $10x10^6$/kg and that 2 and $5x10^6$/kg BM-MSCs could not restore any physiological parameter or prevent the alveolar protein leakage the experiments (Devaney et al., 2015).
Our finding is novel and extremely appealing as it suggests the idea that the use of a priming strategy in hMSCs that can mimick a positive immunomodulatory misroenvironment might be effective avoiding the use of large number of cells to treat acute lung injury.

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Chapter 5.

"Sepsis Therapies – insights from population health to cellular therapies and genomic medicine."

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Seymour CW, et al. Time to Treatment and Mortality during Mandated Emergency Care for Sepsis. *N Engl J Med (1)*
Reviewed by Emanuele Rezoagli

Sepsis, a syndrome of patho-physiologic abnormalities due to a disordered immune response to microbial infection (2), is a key public health concern (3), and is implicated in 50% of hospital deaths (4). International guidelines for management of sepsis emphasize the importance of the early antibiotic therapy and crystalloid fluid resuscitation (5). However, the efficacy of early treatment to improve outcome remains controversial due to the low-moderate quality of the available evidence.

In a large retrospective study from New York State, Seymour and colleagues explored the association between timing of treatment and risk-adjusted mortality in septic patients (1). All hospitals included in the study were mandated to develop and adhere to evidence-informed protocols for early identification and treatment of severe sepsis or septic shock (6). The sepsis ‘protocol’ had 2 components: The ‘3-hour bundle’ required: blood cultures (prior to antibiotics); serum lactate measurement; and broad-spectrum antibiotic administration. The ‘6-hour bundle’ required: a fluid bolus of 30 ml/kg in patients with hypotension or lactate ≥4.0 mmol/L; vasopressor therapy for refractory hypotension; and lactate re-measurement. Exclusion criteria were protocol initiation after 6 hours of hospital arrival (focus on community-acquired sepsis), the 3-hr bundle completed over 12 hours, contra-indications to the protocol and centers with <50 cases of sepsis.

Analysis of data from 49,331 patients demonstrated that faster completion of the 3-hour bundle and each individual component were associated with lower mortality. Each hour of time to completion of the 3-hour bundle was associated with a 4% hourly increase in mortality (OR 1.04; CI 1.02 – 1.05; P<0.001). Patients who had the bundle completed during hours 3-12 had a 14% higher odds of in-hospital death (OR 1.14, CI 1.07 – 1.021, P<0.001). In contrast, time to completion of the fluid bolus administration was not associated with hospital mortality.

The authors ranked the hospitals in deciles from the lowest to greatest likelihood of completing the 3-hour bundle. A 1.5 fold variation in 3 hourly completion rates for the bundle occurred across the hospitals in the highest decile compared to the hospitals in the lowest decile (94.3% versus 64.1%, respectively).

Liu and colleagues recently reported a similar association between the time to antibiotic treatment and hospital mortality in septic inpatients from Northern California (7).

Limitations of the study include its retrospective design (i.e. findings 'associative', not demonstrating a cause-effect relationship), lack of data regarding antibiotic regimen effectiveness, and potential for timing inaccuracies. In addition, strategies targeting rapid antibiotic administration must be balanced to avoid antibiotic overuse and increased resistance (8).

In conclusion, given that randomized clinical trial data regarding timing of antibiotic therapy is unlikely to be forthcoming given equipoise issues, this population-level analysis provides

significant support for the contention that faster sepsis recognition and treatment may improve outcome.

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Tsoyi H, et al. Carbon Monoxide Improves Efficacy of Mesenchymal Stromal Cells During Sepsis by Production of Specialized Proresolving Lipid Mediators. *Crit Care Med (9)*
Reviewed by Bairbre A. McNicholas

Mesenchymal stromal cells (MSCs) demonstrate benefits in multiple sepsis models, including bacterial pneumonia (10, 11), abdominal sepsis (12-14) and early clinical trials suggest promise (15). MSCs may reprogram the innate immune 'hyper-inflammatory' response, enhance immune cell function, and both directly (16, 17) and indirectly (10, 12-14) enhancing antimicrobial killing. MSC reparative effects may be mediated by specialized pro-resolving lipid mediators (SPM) which modulate immune cell function.

The potential for, and mechanisms underlying, preconditioning-mediated enhancement of MSC function, remain unclear. Tsoyi and colleagues (9) hypothesized that ex vivo preconditioning of MSCs with carbon monoxide (CO), which is endogenously produced during heme degradation, and directly improves survival in preclinical sepsis models (18, 19), would enhance MSC efficacy in a murine cecal ligation and puncture (CLP) model.

CO pre-conditioning enhanced the effects of MSCs, allowing them to remain effective in attenuating sepsis when administered later (6hr) after onset of sepsis. This effect was lost when MSC deficient in hemo-oxygenase gene were pre-treated with CO indicating the role of this pathway in mediating the effects of CO. Markers of organ dysfunction were lower while neutrophil phagocytosis and macrophage efferocytosis were enhanced by CO pre-conditioning. In cell culture, the investigators induced specific subtypes of SPM's by changing the substrate available to cells - with improved survival only noted when Docosahexaenoic acid (DHA) was used which produce

SPM. HPLC analysis of culture media found the SPM subtype Resolvins increased. Resolvins alone increased neutrophil phagocytosis, whilst MSC treated with Resolvin D1 and D2 further enhanced phagocytosis to a degree analogous to CO-pretreatment of MSC. Pharmacologically inhibiting SPM production resulted in a loss of the effect of CO pre-conditioning MSCs on neutrophil phagocytosis. Silencing of 5-lipoxygenase and 12-15- lipoxygenase mRNA genes reduced production of resolvins, abolishing enhanced activity of CO-pretreated MSCs.

There are some limitations to be considered. This is a mouse model of sepsis in which standard treatments such as hemodynamic resuscitation or antibiotics were not used. The paracrine versus cell mediated effect of CO-enhanced MSC requires further resolution, as not all effects of CO-MSC treatment were replicated by Resolvin treatment. The effect of CO preconditioning on MSC longevity and safety remains to be elucidated.

In summary, CO preconditioning enhances MSC efficacy in a preclinical sepsis model by enhancing lipoxin A4 production, providing an elegant proof-of-concept for strategies to enhance MSC function. Furthermore, these findings elucidate the role of resolvins in mediating MSC effects, and suggest that resolvins may be a useful therapeutic target in sepsis.

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Davenport EE, et al. **Genomic landscape** of the **individual host response** and **outcomes in sepsis**: **a prospective cohort study**. **Lancet Respir Med (20)**

Reviewed by Peter Moran

Patient heterogeneity and a lack of specific biomarkers have limited advancements in the development of immunomodulatory treatments for sepsis (21, 22). Genetic associations with response to infectious diseases have previously been recognized, but the functional significance of these genetic associations have not been well established (23-25).

Davenport et al. use expression quantitative trait loci (eQTL - genetic variants that influence gene expression) from peripheral blood leukocytes to map associations between genetic variants and differences in gene expression in patients with sepsis due to community-acquired pneumonia, firstly in a discovery (265 patients) cohort and then a validation (106 patients) cohort. This enabled investigators to both define genes modulated by genetic variation and to identify specific functional regulatory polymorphisms.

Gene transcriptional analysis to characterize the inter-individual variability in leukocyte transcriptome in the discovery cohort, and two groupings of transcriptional sepsis response signatures (SRS1 and SRS2) were defined. Two human endotoxin tolerance data sets were also analyzed to define an endotoxin tolerance gene. 3,080 genes were differentially expressed between the two groups with 2,260 downregulated in SRS1. Key mediators of endotoxin tolerance were also differentially expressed between SRS groups. For the validation component, just seven genes that predicted SRS group membership were used to assign patients to SRS1 or SRS2. The SRS1 group was characterized by an immunosuppressed phenotype, with T-cell exhaustion, endotoxin tolerance, and HLA-II downregulation. Interestingly, clinical variables did not predict SRS grouping. Patients

with SRS1 phenotype had an increased 28 day mortality in both the discovery (27% vs 17%, p=0.037) and in the validation (65% vs 41%, p=0.003) cohorts.

Study limitations include its relatively small sample size. Samples were taken following ICU admission – which could result in a lead time bias. Data regarding end-of-life practices and timing of antimicrobials are absent Only a sepsis cohort was investigated - a critically ill non-sepsis comparator group may have provided additional insights.

This study and others (23, 25) highlight the degree of heterogeneity in the host sepsis response, the contribution of variable gene expression to this heterogeneity, and the potential impact on outcome. While application to clinical practice will take time, this approach could facilitate identification of septic patient subsets who may benefit from individualized novel targeted therapies. Critical care trials could then potentially focus on testing the efficacy of therapies that address specific defects in the host immune response in these patient subsets, heralding the advent of personalized medicine for sepsis.

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Chapter 6. Summary, conclusions and future perspectives

6.1 Summary and conclusions

Pneumonia and sepsis are the two major causes of ARDS. Despite advances in the management of mechanical ventilation, use of adjunctive therapies and development of novel antibiotic treatment, ARDS mortality is estimated as high as 40% (Bellani, Laffey et al. 2016).

The presence of an over exaggerated immune response play a major role in the pathophysiology of both ARDS and sepsis. In ARDS, the inflammatory response causes tissue damage. Promising therapeutic strategies to face up the challenge against ARDS is immune modulators for enhancing host defence responses. This research has mainly focused on the modulation of the systemic immune inflammatory response initially using primed MSCs with cytomix. MSCs have already made it to clinical trials for the treatment of ARDS. However clinical studies are still aimed at proving safety, tolerability and feasibility for cell therapy in ARDS, with recent findings suggesting that optimization is mandatory in terms of viability of cells before administration (Matthay et al. 2019).

Two major issues problems with stem cells are:

1. MSCs and host biological variability that can lead to variable responses;
2. MSCs mechanism of action is not fully understood.

If the mechanism of how MSCs elicit all these beneficial effects was fully known, variance might be reduced. It must be remembered that MSCs are biological entities from human donors. Variances in results could a be based on donor immunological history.

The most common sources of MSC for therapeutic use are bone marrow, adipose tissue and umbilical cord blood. MSCs will demonstrate variance in immunomodulation, paracrine release profiles and differentiation tendencies depending on source and classification. A comprehensive understanding of the different classifications of MSCs in immunomodulation is limited. BM-MSCs and UC-MSCs are the most commonly used MSC class in current clinical trials. Autologous stem cells are stem cells from self. The likelihood of immune response is low to minimal and tumor generation also reduced. Also no donor is required. Making autologous stem cells an ideal therapeutic option. However autologous MSCs have limitations based on the status of the patient as attaining autologous MSCs is based on the health of the patient.

This project aimed at improving umbilical cord stem cells and using these improved cells in a gram positive model of ARDS. Umbilical cord stem cells are an ideal source for cell therapy due to their accessibility and pain-free collection. Investigators suggest that the microenvironment where stem cells have “to play the battle” is determinant in directing the type of stem cell response (Islam et al. 2019). Cells were primed with cytokines to mimic an inflammatory milieu. By priming the cells, they are more prepared for the inflammatory, injurious environment in a disease state.

From in-vitro experiments we observed how UC-MSCs primed with cytomix could release anti-inflammatory biomarkers and how they could induce a change of the expression of specific cytokines from epithelial according to the difference in terms of microenvironment (e.g. with or without an injury).

The results of this project were promising showing for the first time that preactivated UC-MSCs could exert a beneficial effect to attenuate acute lung injury in a model of pulmonary ARDS induced by S. aureus. In contrast naïve UC-MSCs demonstrated incompletely a role in the decrease of ALI, without a solid evidence of improvement in terms of physiologic variables, expression of inflammatory biomarkers and attenuation of protein edema.

Furthemore, we herein observed that cytomix priming allowed a significant decrease (2 million/kg) of the cell dose to administer to the animals, without losing the positive beneficial effects demonstrated at higher cell doses (i.e. 10 million/kg).

One of the major setbacks or limitations of this project is that the cells need to primed for 24 hrs before administration. In a clinical hospital setting this is not realistic. To overcome this, cells could be primed in large batches and cryopreserved and be ready for administration into patients. Quality control measures must be in place before infusion. For therapeutic strategies, the cells must be efficient and reproducible when delivered to the patient. It must be defined what the originating source is, the extraction procedure, as well as cell culture density and the medium used as well as any supplements. If this work were to be continued the next step would be to repeat the assays using preactivated cryopreserved cells to aim the achievement of optimizing the cell treatment ready to be used at the bedside.

6.2 Future perspectives:

Different areas of interest are open to future investigation in the treatment of ARDS with cell therapy.

As we just stated, a way to optimize the therapeutic effect of the cell treatment and make the cell therapy more available and applicable at bedside would be the preactivation of hMSCs before cryopreservation. This might enhance the probability of achieving a clinical effect, that should be balanced with, on the other side, with the reduction of cell viability is compared to the use of fresh cells in culture.

The use of conditioned media for gram positive bacteria have reported preliminary but promising results in vitro, and this might exploit the paracrine effects of stem cells avoiding their use with the limitations related to the use of MSCs, including the need of trained personnel, a dedicated cell facility nearby the hospital, the short cell life as soon as after the thawing process (Krasnodembaskaya et al., 2010)

Another potential application of MSCs includes the use of microvescicles of exosomes that are released into cell media that showed positive effects in various models of disease, and that at the moment are specifically used in preclinical models or ex-vivo lung perfusion (Park et al., 2019) models of lung inury induced by gram negative bacterial.

A further application of hMSC would be the so called population enrichment, that is to say to define the patient population based on

clinical and laborator criteria that might present a higher or lower probability to cell treatment (Shankar-Hari et al, 2019).

Recently, the potential of alveolar mesenchymal stem cells and organoids obtained from human pluripotent MSC has been introduced as a advanced promising area of research in the developmental biology and that might potentially have a future application in the field of regenerative medicine (Yamamoto et al., 2017; Miller et al., 2019).

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Publications, presentations and awards

Original articles

1. "Patterns of use of adjunctive therapies in patients with early moderate- severe Acute Respiratory Distress syndrome: Insights from the LUNG SAFE Study" Duggal A[1], Rezoagli E[1], Pham T, McNicholas B, Fan E, Bellani G, Rubenfeld G, Pesenti A, Laffey J.G., On behalf of the LUNG SAFE Investigators and the ESICM Trials Group. Chest, 2019, under review [1]= contributed equally

2. "Patterns of hyperoxemia and inspired oxygen use and in patients with early Acute Respiratory Distress Syndrome: Insights from the LUNG SAFE study" Madotto F, Rezoagli E, Pham T, Schmidt M, McNicholas B, Protti A, Panwar P, Bellani G, Fan E, van Haren F, Brochard L, Laffey J.G., On behalf of the LUNG SAFE Investigators and the ESICM Trials Group, Crit Care Med, 2019, under review

3. "Extracorporeal Chloride Removal by Electrodialysis (CRe-ED): a novel approach to correct acidemia." Zanella A, Caironi P, Castagna L, Rezoagli E, Salerno D, Scotti E, Scaravilli V, Dead SA, Langer T, Mauri T, Ferrari M, Dondossola D, Chiodi M, Zadek F, Magni F, Gatti S, Gattinoni L, Pesenti A. American Journal of Respiratory and Critical Care Medicine, 2019, doi: 10.1164/rccm.201903-0538OC

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16. "Cardiac output measurements based on the pulse wave transit time and thoracic impedance exhibit limited agreement with thermodilution method during orthotopic liver transplantation." Magliocca A[1], Rezoagli E[1], Anderson TA, Ichinose I, Chitilian HV. Anesth Analg. 2018;126(1):85-92. [1]= contributed equally

17. "Hypoxia treatment reverses neurodegenerative disease in a mouse model of Leigh syndrome." Ferrari M, Jain IH, Goldberger O, Rezoagli E, Thoonen R, Chen KH, Sosnovik DE, Scherrer-Crosbie M, Mootha VK, Zapol WM. Proc Natl Acad Sci U S A. 2017;114(21):E4241-E4250.

18. “Endothelial dysfunction inhibits the ability of haptoglobin to prevent hemoglobin-induced hypertension.” Graw JA, Yu B, Rezoagli E, Warren HS, Buys ES, Bloch DB, Zapol WM. Am J Physiol Heart Circ Physiol. 2017;312(6):H1120-H1127.

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20. “Neurological assessment with validated tools in general ICU: multicenter, randomized, before and after, pragmatic study to evaluate the effectiveness of an e-learning platform for continuous medical education.” Mistraletti G, Umbrello M, Anania S, Andrighi E, DI Carlo A, Martinetti F, Barello S, Sabbatini G, Formenti P, Maraffi T, Marrazzo F, Palo A, Bellani G, Russo R, Francesconi S, Valdambrini F, Cigada M, Riccardi F, Moja EA, Iapichino G; SedaICU Investigators. Minerva Anestesiol. 2017;83(2):145-154.

21. “Pulmonary and systemic vascular resistance after cardiopulmonary bypass: role of hemolysis.” Rezoagli E, Ichinose F, Strelow S, Roy N, Shelton K, Matsumine R, Chen L, Bittner EA, Bloch DB, Zapol WM, Berra L. J Cardiothorac Vasc Anesth. 2017;31(2):505-515.

Reviews and commentary

1. “How I set up positive end-expiratory pressure – Based on physiology!” Rezoagli E, Bellani G. Crit Care, 2019, under review

2. “CO_2 Oscillation During Cardiopulmonary Resuscitation: The Role of Respiratory System Compliance.” Rezoagli E, Magliocca A, Ristagno G, Bellani G. Am J Respir Crit Care Med. 2019;199(10):1290-1291.

3. “Recommended Reading from the Galway University Hospitals, Department of Anaesthesia and Intensive Care Medicine Fellows.” Rezoagli E, McNicholas B, Moran P,

Laffey JG. Am J Respir Crit Care Med. 2018. doi: 10.1164/rccm.201804-0782RR.

4. "Sepsis: Therapeutic Potential of Immunosuppression versus Immunostimulation. Rezoagli E, Masterson CH, McCarthy SD, Laffey JG. Am J Respir Cell Mol Biol. 2019;60(1):128-130.

5. "Identification of biological phenotypes in Acute Respiratory Distress Syndrome: from biomarkers to clinical outcome." Rezoagli E, Magliocca A, Scalia Catenacci S, Bellani G, Fumagalli R. Am J Respir Crit Care Med. 2018;197(9):1209-1211.

6. "Definition ad epidemiology of ARDS." Rezoagli E, Funagalli R, Bellani G. Ann Transl Med. 2017 Jul;5(14):282.

Book Chapters

1. "The safety and efficiency of addressing ARDS using stem cell therapies in clinical trials". Rezoagli E, Murphy EJ, Laffey JG, O'Toole D. Chapter 12. Stem cell-based therapy for lung disease. Janette Kay Burgess and Irene Heijink (Eds). 2019. In press.

2. "La pressure support ventilation". Rezoagli E, Foti G, Bellani G, Villa F. Chapter 4.4. Ventilazione artificiale meccanica. Invasiva e non invasive. 3a ed. Torri-Calderini. Delfino Editore. 2020. *In press.*

3. "Is Carbon Dioxide Harmful or Helpful in ARDS?" Masterson C, Horie S, Rezoagli E, Laffey JG. Section 4: ARDS. Chapter 17. Evidence-Based Practice of Critical Care 3/e. Deutschman CS and Neligan P. Elsevier Inc. 2019

4. "Anemia ed emorragia acuta." Dentali F, Magliocca A, Rezoagli E. Manuale di emergenze medico-chirurgiche. Milone-Di Minno. Edra S.p.a. 2019

Invited lectures

1. "Simulation cases on mechanical ventilation" Instructor, Smart Course, Mechanical Ventilation, 2nd Edition, 2019, Krakow, Poland

2. "Workshop on mechanical ventilation", Instructor, Workshop Proxima, "Societa Italiana di Analgesia, Anestesia e Rianimazione e Terapia Intensiva" SIAARTI, 2017, Rimini, Italia

3. Moderator and Faculty at the Hi-Impact Critical Care Conference 2.0, 2018, Galway, Ireland

4. "Acute kidney Injury, ARDS, and Hemolysis", Hi-Impact Critical Care Conference 2.0, 2018, Galway, Ireland

5. "When do I start ExtraCorporeal CO_2 removal?" Hi-Impact Critical Care Conference 2.0, 2018, Galway, Ireland

6. "Patient-ventilator interaction" Workshop on mechanical ventilation, Hi-Impact Critical Care Conference 2.0, 2018, Galway, Ireland

Oral presentation

1. *European Society of Intensive Care Medicine, LIVES 2019, Berlin*
   "Cytomix Preactivated UC-MSCs as a Treatment of Acute Lung Injury in a New Model of Rodent Staphylococcus Aureus–Induced Pulmonary Acute Respiratory Distress Syndrome" Rezoagli E, O'Toole D, Murphy EJ, Laffey JG.

2. *European Society of Intensive Care Medicine, LIVES 2018, Paris*
   "Impact of Renal Replacement Therapy in patients with Acute Respiratory Distress Syndrome: Insights from the LUNG SAFE study." McNicholas B, Rezoagli E, Tai Pham, Madotto F, Fanelli V, Guiard E, Griffin M, Bellani G, Ranieri M, Laffey JG.

Poster presentation

1. *Irish Plant Scientists' Association Meeting 2019, Teagasc | Agriculture and Food Development Authority*
   "A novel extraction procedure for 1,3 1,6 linked beta-glucans from Basidiomycetes."
   Murphy EJ, Masterson CH, Rezoagli E, O'Toole D, Laffey JG, Major I, Stack G, Rowan N.

2. *European Society of Intensive Care Medicine, LIVES 2019, Berlin*
   "CO_2 oscillation during Cardiopulmonary Resuscitation: mechanical versus manual chest compression in a porcine model of cardiac arrest." Magliocca A, Rezoagli E, Bellani G, Ristagno G.

3. *American Thoracic Society International Conference in Dallas, 2019, Dallas, Texas, USA*
   "Immunomodulation Properties of a Novel β-glucan Extract from the Mushroom *Lentinus Edodes* in an in-vitro Lung Injury Model." Murphy EJ, Masterson CH, Rezoagli E, O'Toole D, Laffey JG, Major I, Stack G, Rowan N

4. *Athlone Institute of Technology Research Day 2019, May 2nd, 2019, Douglas Hyde Theater, Athlone, Ireland*
   "The Effects of Two Anti-cancer Drugs in a Cervical Cancer Cell Line
   (A Preliminary Study)." Murphy EJ, Masterson K, Rezoagli E, Major I.

5. *30° SMART Simposio Mostra Anestesia e Rianimazione, 2019, Milan, Italy*
   "CRILI: Cardiopulmonary Resuscitation-induced Lung Injury -a lung CT analysis in a porcine model" Magliocca A, Rezoagli E, Bellani G, Ristagno G.

6. *30° SMART Simposio Mostra Anestesia e Rianimazione, 2019, Milan, Italy*
   "Lung Recruitment in Obese Patients with Acute Respiratory Distress Syndrome" Fumagalli J, Santiago RRS, Teggia Droghi M, Zhang C, Pirrone M, Rezoagli E, Marrazzo F, Florio G,

Morais CCA, Amato MBP, Kacmarek RM, Berra L; on behalf of the MGH Lung Rescue Team Study Group.

7. *American Society of Nephrology, 2018, San Diego, USA*
   "Association of AKI on outcomes in patients with ARDS-Secondary analysis of LUNG-SAFE patient cohort." McNicholas B, Rezoagli E, Guiard E, Bellani G, Griffin M, Tai Pham, Laffey JG.

8. *European Society of Intensive Care Medicine, LIVES 2018, Paris*
   "Impact of gender in patients with Acute Respiratory Distress Syndrome: Insights from the LUNG SAFE study." Rezoagli E, McNicholas B, Masterson C, Pham T, Madotto F, Bellani G, Laffey JG.

9. *European Society of Intensive Care Medicine, LIVES 2018, Paris*
   "Impact of major comorbidities in patients with Acute Respiratory Distress Syndrome: Insights from the LUNG SAFE study." Rezoagli E, Madotto F, McNicholas B, Pham T, Bellani G, Laffey JG.

10. *European Society of Intensive Care Medicine, LIVES 2018, Paris*
    "Extracorporeal chloride removal to treat acidemia: in vitro evaluation of three techniques". Rezoagli E, De Falco S, Fumagalli J, Busana M, Rigoli A, Protti I, Tomaselli A, Battistin M, Castagna L, Giani M, Abd El Aziz El Sayed Deab S, Pesenti A, Zanella A.

11. *29° SMART Simposio Mostra Anestesia e Rianimazione, 9-11 May 2018, Milan, Italy*
    "Extracorporeal techniques for chloride removal to treat severe acidemia: in vitro study". De Falco S, Busana M, Fumagalli J, Rigoli A, Protti I, Guzzardella A, Tomaselli A, Biancolilli O, Rezoagli E, Zanella A, Pesenti A.

Awards and Positions

1. AIT *Visiting Research Fellow*, 3-year honoraroy position, Athlone Institute of Technology (AIT), Athlone, Ireland

2. Regional Representative (Lombardy) in the group Proxima Network for young Consultants in Anesthesia and Intensive Care Medicine for the "Societa Italiana di Analgesia, Anestesia e Rianimazione e Terapia Intensiva: (SIAARTI), AA 2018-2019, AA 2019-2020

3. ESICM NEXT Fellowship Programme – Best Practices in Advanced Haemodynamic Monitoring, supported by Edwards, and promoted by the European Society of Intensive Care Medicine, Rotterdam, (The Netherlands), 22-26 July 2019.

4. ESICM NEXT Fellowship Programme – Infections in ICU, supported by Bayer, and promoted by the European Society of Intensive Care Medicine, London (United Kingdom), 12-16 November 2018.

5. ESICM NEXT Fellowship Programme - ARDS Management, supported by Medtronic, and promoted by the European Society of Intensive Care Medicine, Rotterdam (The Netherlands), 26-30 March 2018.

6. Winner team of the First Edition of "SIAARTI Academy" of Italian Society of Anesthesia Analgesia Resuscitation and Intensive Care, 22-26 May, 2017, Lampedusa, Italy – Theoric and Practical course on emergency medicine and on the management of catastrophic medical events

Funding awards

1. Award “Giovani Talenti 2019”, #2 position for the Section “Medical Sciences and Neuroscience” at the University Milan-Bicocca, Monza, Italy, 2019 (2000 euro)

2. ESICM Young Investigator Award 2018 with the project titled: “Role of the exhaled breath condensate as non-invasive monitoring of the lung inflammation during ARDS: a prospective cohort study” (20000 euro)

3. National Research Grant 2017 awarded by the “Societa Italiana di Anestesia Analgesia Rianimazione e Terapia Intensiva – SIAARTI”, MSD Italia S.r.l., on behalf of Merck Sharp & Dohme Corporation, entitled: “Exhaled nitric oxide as marker of acute lung injury in mechanically ventilated adult patients with acute respiratory distress syndrome (ARDS)” (15000 euro)