

**CAN COMBINED CELLULAR AND 11BETA-
HYDROXYSTEROID DEHYDROGENASE TYPE-1
GENE THERAPY ATTENUATE INFLAMMATION IN
ACUTE RESPIRATORY DISTRESS SYNDROME?**

by

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ABSTRACT

Acute Respiratory Distress Syndrome (ARDS) is an inflammatory disorder of the lungs, for which there is no effective pharmacotherapy. 11 β hydroxysteroid dehydrogenase type 1 (HSD-1) acts as a reductase to convert cortisone into cortisol, and is normally expressed by alveolar macrophages (AMs). A previous study has shown evidence of impaired alveolar HSD-1 reductase activity and relative alveolar cortisol deficiency in ARDS patients. Mesenchymal stem cells (MSCs) administration has been effective at attenuating inflammation in pre-clinical models of lung injury.

This thesis reports a series of investigations into the role of AM dysfunction in ARDS pathogenesis, and whether use of transgenic MSCs (tMSCs) expressing HSD-1 offers a potential therapy for ARDS by enhancing AM efferocytosis. It also investigates the impact of expansion on the ability of MSCs to attenuate lung injury.

Expansion of MSCs for 1 month had no effect on the ability of MSCs to promote alveolar epithelial wound healing, however the ability of MSCs to stimulate IL-10 release from macrophages was impaired. Lentiviral transfection was used to create HSD-1 tMSCs, which expressed functional HSD-1.

Development of sepsis-related ARDS is associated with decreased AM HSD-1 reductase activity and efferocytosis, which contributes to the increased alveolar neutrophil apoptosis, alveolar inflammation and mortality observed. Dysregulated AM function contributes to ARDS pathogenesis, therefore upregulation of AM efferocytosis may offer a therapeutic strategy for ARDS patients.

ARDS broncho-alveolar lavage fluid (BALF) treatment of healthy AMs *in vitro* can decrease rac1 gene expression and impair efferocytosis, thereby replicating the AM functional defect observed in ARDS patients. Following ARDS BALF treatment, AMs increase expression of pro-efferocytosis receptors CD206 and MerTK, as an unsuccessful compensatory mechanism to restore normal efferocytosis.

HSD-1 tMSC co-culture was unsuccessful at restoring AM efferocytosis both *ex vivo* and in an *in vitro* model of ARDS, indicating that HSD-1 independent mechanisms contribute to impaired AM efferocytosis in ARDS. HSD-1 tMSC therapy reduced cellular inflammation in a model of murine peritonitis, but also caused bacterial overgrowth due to immunosuppression, indicating that HSD-1 tMSC therapy is unlikely to have utility in patients with sepsis-related ARDS. However, further studies using multiple low doses of HSD-1 tMSCs in HSD-1 knockout mice in conjunction with antibiotic therapy are required to fully assess the therapeutic potential of HSD-1 tMSCs. Rho-associated protein kinase (ROCK) inhibitor treatment partially restored AM efferocytosis in an *in vitro* model of ARDS, indicating that targeting of the ROCK pathway may offer therapeutic potential in ARDS patients.

DEDICATION

To my parents, Sejal and Disha

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CHAPTER 1 : INTRODUCTION

1.1 Acute Respiratory Distress Syndrome

Acute Respiratory Distress Syndrome (ARDS) is an inflammatory disorder of the lungs which can develop in response to various insults, most commonly severe sepsis. ARDS results in acute hypoxaemic respiratory failure requiring mechanical ventilation, and is a major cause of morbidity and mortality in critically ill patients(1). Survivors of ARDS commonly have decreased health-related quality of life, and a range of persistent physical and psychological sequelae(2, 3). The mainstay of treatment currently focusses on lung-protective ventilation strategies, which has helped to reduced mortality(4-6). However, there is currently no effective pharmacotherapy available for ARDS(7, 8).

1.1.1 Diagnosis

ARDS was first described in 12 patients with acute hypoxaemia by Ashbaugh et al(9) in 1967. In 1994, the American-European Consensus Conference (AECC) definition of ARDS was published(10), which for the first time gave clinicians criteria to aid diagnosis of ARDS. However, concerns regarding the limitations and validity of the AECC definition arose over time. Therefore, the AECC definition was superseded in 2012 by the Berlin Definition of ARDS(11), developed by the ARDS definition task force (a collaboration between the European Society of Intensive Care Medicine, the American Thoracic Society and the Society of Critical Care Medicine). The Berlin definition is summarised below in table 1.1. The severity of hypoxia directly correlates with mortality, and determination of the severity helps clinicians decide the type of lung-protective ventilatory strategy required(12).

The Berlin Definition of Acute Respiratory Distress Syndrome(11)		
Timing	Within 1 week of a known clinical insult or new or worsening respiratory symptoms	
Chest imaging (radiograph or computed tomography)	Bilateral opacities: not fully explained by effusions, lobar/lung collapse, or nodules	
Origin of oedema	Respiratory failure not fully explained by cardiac failure or fluid overload. Requires objective assessment (e.g., echocardiography) to exclude hydrostatic oedema if no risk factors are present	
Oxygenation (severity of hypoxia)		
	Mild *	$26.7 \text{ kPa} < \text{PaO}_2/\text{FiO}_2 \leq 40 \text{ kPa}$ with PEEP or CPAP $\geq 5 \text{ cm H}_2\text{O}$
	Moderate	$13.3 \text{ kPa} < \text{PaO}_2/\text{FiO}_2 \leq 26.7 \text{ kPa}$ with PEEP $\geq 5 \text{ cm H}_2\text{O}$
	Severe	$\text{PaO}_2/\text{FiO}_2 < 13.3 \text{ kPa}$ with PEEP $\geq 5 \text{ cm H}_2\text{O}$
Abbreviations: CPAP, continuous positive airway pressure; FiO ₂ , fraction of inspired oxygen; PaO ₂ , partial pressure of arterial oxygen; PEEP, positive end-expiratory pressure. * This may be delivered non-invasively in the mild ARDS group		

Table 1.1: The Berlin Definition of Acute Respiratory Distress Syndrome

1.1.2 Epidemiology and Aetiology

A large multi-centre study by Reubenfield et al(1) in 2000 showed the incidence of ARDS to be 78.9 per 100,000 person-years, with an associated mortality rate of 38.5%. The incidence of ARDS and its mortality rate increased with patient age. In 2016, the international multi-centre LUNG SAFE study showed that the period prevalence of ARDS was 10.4% of all ICU admissions across 50 countries over a 4 week period(13). The LUNG SAFE study showed an inpatient mortality rate of 34.9% for those with mild ARDS, 40.3% for moderate ARDS, and 46.1% for severe ARDS(13). ARDS occurs following lung injury, however the mode of injury may be direct or indirect (8), see table 1.2. Patients may also have more than one mode of lung injury. In the LUNG SAFE study(13), 80.5% of patients had direct lung injury (59.4% from pneumonia), and 35.3% had indirect lung injury (16% from extra-pulmonary sepsis). Other, less common, causes can include acute pancreatitis,

trauma, transfusion-related lung injury, aspiration of gastric contents, burns and drug reactions(9, 13-15). This reflects the findings from other studies(16, 17), and our own experience regarding the underlying aetiology of ARDS patients in the West Midlands(18-20). There is also evidence that genetic predisposition may play a role in development of ARDS(21), along with age(3), smoking(22) and alcohol intake(23). More recent studies(24) have shown a declining mortality rate of 26% in patients recruited to the National Institutes of Health ARDS Network clinical trials between 2004-5, likely due to the introduction of lung-protective ventilation strategies including lower tidal volume ventilation, and earlier administration of antibiotics in patients with severe sepsis. The majority of deaths in ARDS patients occur in those who have developed multi-organ failure subsequent to the initial respiratory failure(25). Development of acute kidney injury can increase the mortality rate to 58% in a subset of ARDS patients(26). The development of multi-organ failure is more common in patients with underlying severe sepsis(15).

Survivors of ARDS commonly develop persistent impairments in physical function, causing reduced exercise capacity and reduced health-related quality of life(2, 27, 28). Survivors also have an increased risk of developing mental health problems, including depression and anxiety(2).

Direct Lung Injury	Indirect Lung Injury
Pneumonia Aspiration of gastric contents Smoke inhalation Drowning Pulmonary contusion	Sepsis Peritonitis Trauma Cardiac bypass Acute Pancreatitis Massive blood transfusion Post-oesophagectomy

Table 1.2: Conditions commonly leading to development of ARDS
Adapted from Parekh et al(8)

1.1.3 Pathology of ARDS

The acute phase pathogenesis of ARDS is characterised by inflammatory injury to both the alveolar epithelium and endothelium(29), with denuding of the alveolar epithelium, resulting in increased permeability. Injury to the alveolar epithelium also impairs clearance of alveolar oedema fluid in ARDS(30). Neutrophil influx may be responsible for increased alveolar epithelial permeability in ARDS: Following accumulation of activated neutrophils in the lung vasculature, migration of these cells into the alveolar space can damage the alveolar epithelium(31). Release of elastase, proteases, reactive oxygen species and matrix metalloproteinases by activated neutrophils can also damage tight junctions, leading to increased epithelial permeability(15, 31, 32). Increased apoptosis of alveolar epithelial cells also occurs, likely mediated by neutrophilic injury(33). Damage to type II alveolar epithelial cells impairs surfactant production.

Together, these events lead to the development of a proteinaceous, neutrophilic alveolar oedema(15). This oedema fluid also contains high concentrations of inflammatory cytokines(34, 35), including tumour necrosis factor-alpha (TNF- α) and interleukins (IL)-1 β , IL-6 and IL-8. Persistently elevated levels of these inflammatory cytokines are associated with greater lung injury and increased mortality(36).

Multiple inflammatory mechanisms contribute to lung endothelium damage, including release of free radicals and pro-inflammatory cytokines by neutrophils in the lung vasculature(37). These result in increased permeability of the lung vasculature, which contributes to development of the proteinaceous pulmonary oedema. The effects of inflammatory mediators (e.g. IL-1) on endothelial cells also increases leukocyte adhesion and subsequent migration into the alveolar space(38).

In some patients the inflammation may eventually resolve, leading to repair of the injured alveolar epithelium, and gradual improvement of alveolar fluid clearance associated with clinical recovery. Alveolar macrophages(39, 40), regulatory T cells(41) and lipoxins(42) are implicated in the resolution of inflammation. The action of alveolar macrophages in ARDS will be discussed further in section 1.3. A minority of patients may develop fibrosing alveolitis(43-45), which has a poor prognosis(46).

1.1.4 Treatment of ARDS

ARDS treatment is based on lung-protective ventilation strategies, including use of lower tidal volumes(4), conservative fluid management(47), decreased driving pressure(6), and prone positioning(5, 48, 49). Neuromuscular blocking agents have also been shown to improve ventilation and survival(50). Other strategies including positive end-expiratory pressure(51) and high frequency oscillatory ventilation (HFOV) (52, 53), have not previously been shown to improve outcome in ARDS patients. However, a recent study investigating use of HFOV showed that whilst this strategy increased mortality in ARDS patients with mild-moderate hypoxaemia, it may improve survival in patients with severe hypoxaemia, indicating that patient selection is critical(54). Some studies have shown that use of extra-corporeal membrane oxygenation can improve mortality in ARDS(55), whereas others have shown no change in outcome(56). There have been numerous clinical trials of potential therapeutic agents which unfortunately have not shown clinical benefit(57); including surfactant(58, 59), beta-agonists(7, 18, 60), statins(61, 62), keratinocyte growth factor (63), ketoconazole(64), lisofylline(65), prostaglandin E1(66), and nitric oxide(67). Therapeutic use of mesenchymal stem cells (MSCs) and glucocorticoids have also been investigated, these will be discussed in sections 1.2.3 and 1.4.3.

1.2 Glucocorticoids and ARDS

ARDS is triggered by a systemic inflammatory response, often due to severe sepsis. Inflammation is the appropriate physiological response to infection, and is associated with both the innate and adaptive immune responses. In this way, limited inflammation aids defence against pathogens. Normally, homeostatic mechanisms including the hypothalamic-pituitary-adrenal axis and glucocorticoid release, promote resolution of inflammation after the infective agent has been cleared. However, in certain circumstances including ARDS, an exaggerated persistent inflammatory response occurs which results in tissue injury(68).

1.2.1 Hypothalamic-pituitary-adrenal (HPA) axis

In response to various endocrine, neural and cytokine stimuli, the hypothalamus releases corticotropin-releasing hormone (CRH). CRH stimulates the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH), which is released into the bloodstream and stimulates the adrenal cortex to secrete cortisol. 90% of secreted cortisol is bound to corticosteroid-binding globulins in the blood. The remaining 10% of free cortisol is the biologically active hormone(68). The HPA axis is subject to negative feedback control; cortisol inhibits production of CRH by the hypothalamus.

1.2.2 Glucocorticoid Receptor

Cortisol mediates its anti-inflammatory actions by binding to the glucocorticoid receptor, which is found within the cytoplasm of most human cells. The cortisol-glucocorticoid receptor complex moves to the nucleus and binds to specific DNA sequences, called glucocorticoid responsive elements, in the promoter region of steroid-responsive genes(69). This enhances transcription of anti-inflammatory

genes, including mitogen-activated protein kinase phosphatase-1. The cortisol-glucocorticoid receptor complex also inhibits the action of pro-inflammatory transcription factors including nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), thereby limiting the transcription of pro-inflammatory genes(70). Glucocorticoids also increase the expression of proteins which destabilise the mRNA transcripts of pro-inflammatory genes (e.g. TNF- α) and promote their degradation, thereby preventing transcription(69).

1.2.3 Use of exogenous glucocorticoids in the treatment of ARDS

There have been multiple trials investigating the use of systemic exogenous glucocorticoid therapy in ARDS, which have had varying outcomes. In the 1980s, several trials of high-dose intravenous methylprednisolone had clear adverse outcomes for ARDS patients(71, 72). Bone et al(71) set out to determine whether early IV methylprednisolone therapy (30mg/kg every 6 hours for a total of 4 doses) in critically ill patients with sepsis could reduce the incidence of ARDS and aid reversal of ARDS. The trial recruited 304 critically ill patients with sepsis and found that IV methylprednisolone given within 48 hours of diagnosis actually impaired reversal of ARDS, and increased 14-day mortality in those patients who developed ARDS, compared to placebo. Weigelt et al(72) similarly recruited 81 critically ill patients at risk of ARDS and initiated early treatment (within 12 hours of meeting criteria) with IV methylprednisolone (30mg/kg every 6 hours for 48 hours) or placebo. Those patients treated with methylprednisolone had a significantly higher risk of developing ARDS (64% vs 33%) and infectious complications (77% vs 43%) compared to those receiving placebo. Some studies showed no significant differences with methylprednisolone therapy(73, 74).

However, in the last 2 decades, clinical trials have focussed on using a moderate dose of intravenous steroids for a longer duration. Steinberg et al(75) found that IV methylprednisolone treatment (starting at 2mg/kg then tapered down over 3 weeks) led to an increased 60-day and 180-day mortality rate in patients enrolled at least 14 days after the onset of moderate to severe ARDS, compared to placebo. However, of those ARDS patients enrolled between 7-13 days after the onset of ARDS, there was no difference in 60-day or 180-day mortality with methylprednisolone treatment compared to placebo. In this group, there was an improvement in pulmonary organ dysfunction, reduced length of intensive care unit stay, and reduced duration of mechanical ventilation. There was no difference in the number of severe infections between the treatment and placebo groups.

Meduri et al(76) showed that prolonged low dose IV methylprednisolone treatment (1mg/kg/day) for up to 28 days successfully downregulated systemic inflammation when initiated early (≤ 72 hours after diagnosis) in patients with moderate to severe ARDS. Methylprednisolone treatment reduced duration of mechanical ventilation and intensive care unit stay. There was also improved pulmonary and multi-organ dysfunction compared to placebo. However, there was no difference in mortality between methylprednisolone treated and placebo groups. There was no difference in the number of severe infections between the treatment and placebo groups.

In contrast, Brun-Buisson et al(77) showed that early corticosteroid therapy in patients with severe influenza A / H1N1 pneumonia and ARDS could actually be harmful. In this study, patients starting corticosteroids ≤ 72 hours of invasive ventilation had a higher mortality, more acquired pneumonia, and a trend towards longer duration of invasive ventilation.

The 2018 Faculty of Intensive Care Medicine / Intensive Care Society guidelines(78) on the management of ARDS used the GRADE (Grading of Recommendations, Assessment, Development and Evaluations) framework to assess four meta-analyses of corticosteroid treatment for ARDS(79-82). The guideline concluded that the evidence to support use of corticosteroids in ARDS is of low to very low quality, from clinical trials which were mostly undertaken before the widespread adoption of lung protective ventilation strategies. All these clinical trials failed to undertake long-term follow up of recruited patients. Differences in corticosteroid dose and timing between trials made direct comparisons unreliable. The quality of evidence supporting the relative risk (RR) of 0.51 (95% CI 0.24 to 1.09) for hospital mortality with corticosteroids was very low(78). Just 75% of the Cochrane risk of bias recommendations were adhered to, indicating a significant risk of bias. A post hoc power calculation for hospital mortality showed that the combined studies were underpowered at 65%, with an inadequate sample size(78). A mean reduction in length of hospital stay of 4.8 days with corticosteroid treatment was reported in one meta-analysis, however, the quality of the studies analysed was very low(79). With regards to treatment harms, a single meta-analysis(82) reported a combined analysis (including infection, neuromyopathy, diabetes, gastro-intestinal bleeding and other complications), with an RR of 0.82 (95% CI 0.5 to 1.36), however again the quality of the trials was low. This guideline recommends that the use of corticosteroids in established ARDS should be the subject of a suitably powered multicentre randomised controlled trial with long term follow up, that focuses on both potential benefits and harms(78).

1.2.4 Role of 11 β hydroxysteroid dehydrogenase in glucocorticoid metabolism

Glucocorticoid action is partly dependent on pre-receptor metabolism by the 11 β -hydroxysteroid dehydrogenase (HSD) enzyme in peripheral tissues(83). HSD has two isoforms: The type 1 isoform of HSD (HSD-1) predominantly acts as an oxo-reductase using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor to convert inactive cortisone to active cortisol(84). HSD-1 thereby locally amplifies glucocorticoid action by generating active cortisol from cortisone within specific tissues. HSD-1 is expressed widely throughout the body, especially within the liver, adipose tissue, bones, eyes, gonads, and lungs(85). Within the lung, HSD-1 is predominantly expressed within alveolar macrophages in the alveolar space and bronchial epithelial cells. It has previously been shown(86) that HSD-1 expression is induced when human monocytes differentiate into macrophages. Alveolar type I and II epithelial cells have negligible HSD-1 mRNA expression.

The catalytic domain of HSD-1 lies within the lumen of the endoplasmic reticulum (ER). Within the ER lumen, hexose-6-phosphate dehydrogenase (H6PD) reduces NADP⁺ to NADPH, using glucose-6-phosphate (G6P) as a cofactor(87). G6P levels, and consequently H6PD activity, may be dependent on extracellular glucose levels(88). The presence of H6PD results in a high NADPH / NADP⁺ ratio within the ER lumen, which drives the action of HSD-1 in a reductase direction(87, 89).

The type 2 isoform of HSD (HSD-2) acts as a dehydrogenase using NAD as a cofactor to convert active cortisol to inactive cortisone(90). HSD-2 is mainly expressed in mineralocorticoid target tissues such as the kidneys; its main function thought to be to prevent inappropriate binding of cortisol to the mineralocorticoid receptor(84).

1.2.5 Glucocorticoid metabolism in ARDS

Previous work by our group(91) revealed that in ARDS patients, lower cortisol levels in broncho-alveolar lavage fluid (BALF) are associated with greater cellular inflammation. It was found that BALF cortisol: cortisone ratios are 52% lower compared to plasma ratios in ARDS patients(91), which would suggest dysregulated alveolar steroid metabolism. It was then found that alveolar macrophages (AMs) from ARDS patients have lower HSD-1 levels early in the disease course(91); AMs from ARDS patients have 10% of normal AM HSD-1 activity (figure 1.1). Correlating with mortality data for ARDS patients, we found that HSD-1 activity was 58% higher in survivors than non-survivors(91). These results are supported by findings in animal models. Using the intra-tracheal lipopolysaccharide (IT-LPS) murine model of ARDS, we found that HSD-1 knockout (KO) mice have exaggerated and prolonged inflammation, with increased alveolar permeability & apoptotic neutrophil accumulation compared to wild type mice (figure 1.2).

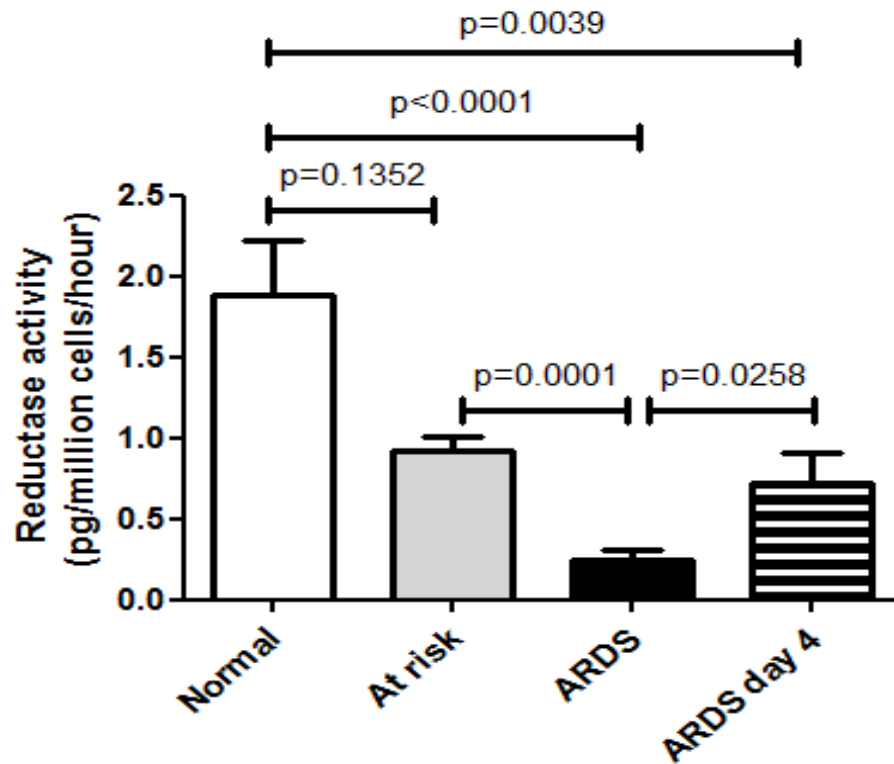


Figure 1.1: Alveolar macrophage HSD-1 reductase activity at day 0 and day 4 of ARDS compared to normal subjects and patients at risk of ARDS.

Adapted from Bassford et al(91). Alveolar macrophages (AMs) were isolated from patient BALF, before being used in HSD-1 activity assays. The data shows that AMs from patients with ARDS are deficient in HSD-1, compared with AMs from normal subjects. HSD-1 deficiency is most marked at the time when ARDS is diagnosed.

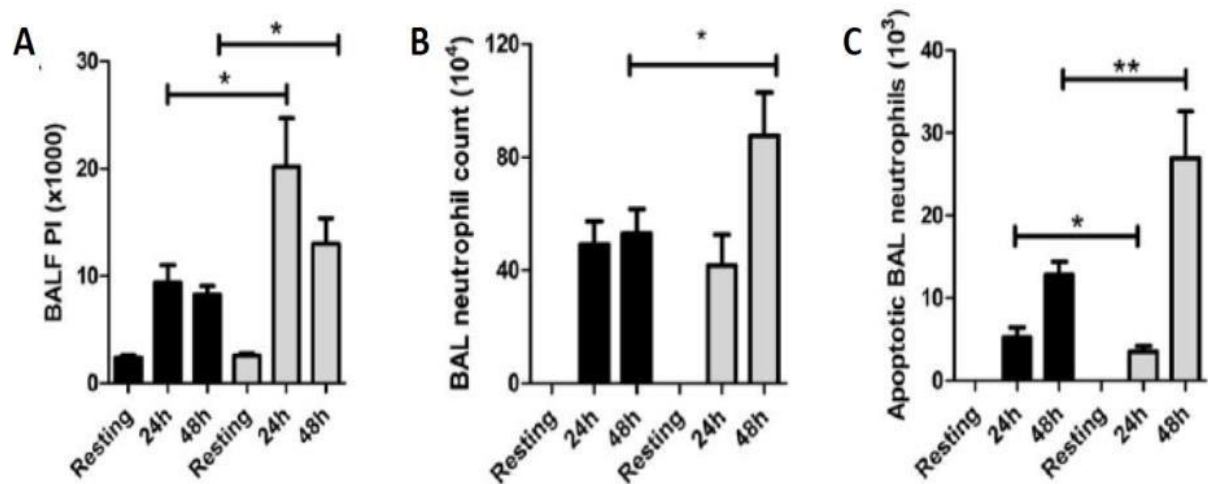


Figure 1.2: Comparison of wild type versus HSD-1 KO mice following 50 µg intra-tracheal LPS.

Wild type = black bars. HSD-1 KO = grey bars. * $p < 0.05$, ** $p < 0.01$. A) Increase in BALF permeability index is elevated & prolonged in HSD1-KO mice. B&C) Neutrophil & apoptotic neutrophil accumulation are elevated in HSD1-KO mice at 48 hours post-injury. Unpublished data kindly shared by Dr Sian Lax at the University of Birmingham.

1.3 Alveolar Macrophages in ARDS

1.3.1 Alveolar Macrophage Function and Phenotype

The unique location of alveolar macrophages (AMs) within the alveolar lumen, exposed to the outside environment, requires these cells to finely balance robust inflammatory responses to infection against resolving functions to prevent immune-mediated tissue damage. In health, AMs clear a diverse range of foreign particles (including dust, allergens and bacteria), as well as apoptotic cells, but also have reduced antigen presenting capacity compared to other tissue macrophages(92). AMs are crucial for maintaining alveolar immune homeostasis(93); it is likely that the pro-tolerogenic characteristic of AMs has evolved to prevent excessive inflammation in the face of continuous low-level stimulation(94). Resident AMs develop from progenitors at the foetal stage(95, 96), and can self-renew throughout life,(97, 98) independently from circulating monocytes. Recruited AMs are circulating monocytes which migrate into the alveolar space (often following an inflammatory stimulus) and differentiate into AMs. It was previously thought that resident and recruited AMs behaved identically, however recent work has shown that AM origin does affect function following lung injury(99, 100).

Classical 'M1' activation of macrophages occurs via exposure to inflammatory cytokines (e.g. interferon- γ , TNF- α), or by toll-like receptor (TLR) recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) or danger-associated molecular patterns (DAMPs) from necrotic cell debris (e.g. heat-shock proteins, high mobility group box-1 (HMGB1) and histones(101). Subsequent activation of pro-inflammatory transcription factors including NF- κ B results in increased phagocytosis, expression of surface markers (e.g. CD80, CD86)

and production of pro-inflammatory cytokines (e.g. IL-1, IL-6). This pro-inflammatory phenotype provides defence against infection, but can cause tissue damage if left unchecked. Alternative activation of macrophages occurs following exposure to IL-4 and IL-13, which induce an anti-inflammatory pro-repair 'M2' phenotype(101). Glucocorticoids and regulatory T cells(41) can also induce an M2 phenotype. M2 macrophages have increased efferocytosis activity (ability to phagocytose apoptotic cells), produce anti-inflammatory cytokines (e.g. IL-10), and pro-repair cytokines (e.g. Transforming Growth Factor- β [TGF- β]). M2 macrophages are a heterogeneous group and can be further classified into various subsets (M2a/b/c/d), according to the combination of activation signals required, and their specific functions(102). Table 1.3 summarises the key differences between M1 and M2 macrophages.

M1: Classically Activated	M2: Alternatively Activated
Increased phagocytosis Release of inflammatory cytokines (IL-1, IL-6, IL-12) Express surface markers CD80, CD86, CD68 Can cause tissue damage if unchecked	Increased efferocytosis Release of anti-inflammatory cytokines (IL-10, TGF- β) Express surface markers CD206, CD163, MER tyrosine kinase Promote wound healing and tissue repair

Table 1.3: Characteristics of phenotypically polarised macrophages

At rest, AMs do not fit neatly into the binary phenotypic paradigm of M1 (classically activated, pro-inflammatory) versus M2 (alternatively activated, pro-resolving), instead showing plasticity and intermediate phenotypes(93). There is a paucity of human AM phenotypic studies, especially in patients with ARDS. Much of the AM phenotyping work been undertaken in mice, however murine AMs have different surface markers to human AMs(103). Human macrophage phenotyping has mainly

been undertaken in monocyte-derived macrophages (MDMs), which function differently to AMs(93). However, when AMs are polarised *ex-vivo* with interferon- γ or interleukins (IL)-4 and IL-13, they increase expression of M1-associated pro-inflammatory genes (CD69, Toll-Like Receptors [TLR]-2/4, Chemokine ligands [CXCL]-9/10/11) or M2-associated pro-resolving genes (mannose receptor CD206, CD163, tyrosine protein kinase MER, matrix metalloproteinases [MMP]-2/7) respectively(104). Table 1.3 shows the functions and surface markers of AMs polarised in this way. Importantly, AMs show significantly plasticity in their phenotype / activation profile; once polarised to a particular phenotype, they retain the ability to return to a resting state or switch to a different phenotype following a change in stimuli(39). This means that a population of AMs in ARDS can contain functionally distinct subsets at the same time(105).

1.3.2 Role of Alveolar Macrophages in pathogenesis and resolution of ARDS

AMs initially play a role in the damaging pro-inflammatory immune response seen in early ARDS, exhibiting increased NF- κ B activation(106, 107) and M1 marker expression(105), contributing to alveolar barrier dysfunction (108) and inducing apoptosis of alveolar epithelial cells(109). In the early stages of infection-related ARDS, increased levels of monocyte chemoattractant protein (MCP)-1 in BALF and rapid influx of circulating monocytes into the alveolar space are observed(110). These recruited monocytes differentiate into AMs with a more pro-inflammatory phenotype(100). Greater monocyte influx and increased shift to M1 phenotype were associated with worse mortality(110). Murine studies suggest that AM-derived microvesicles may also mediate epithelial injury in early ARDS(111). Apoptosis and

clearance of recruited monocytes is required for resolution of inflammation in ARDS(99).

AMs are required for the resolution of inflammation in ARDS and tissue repair. After the initial acute inflammatory phase of ARDS, AMs switch to a pro-resolving M2 phenotype(105). The process of efferocytosis is essential to resolving inflammation, and is discussed further in section 1.3.3. AMs secrete anti-inflammatory lipid mediators, including lipoxins, resolvins and protectins(39). Via release of prostaglandins and TGF- β , AMs can also suppress T cell activation and promote development of regulatory T cells(112, 113); in turn regulatory T cells promote an M2 phenotype in AMs(41, 114). AMs release keratinocyte growth factor (KGF) and hepatocyte growth factor to promote wound repair(115, 116).

1.3.3 Apoptosis and Efferocytosis in ARDS

Apoptosis is a form of programmed cell death, and efferocytosis is the process by which phagocytes (mainly AMs in the airways) phagocytose apoptotic cells. Efficient efferocytosis is required to prevent the apoptotic cells undergoing secondary necrosis, which would result in cell rupture and release of pro-inflammatory cells contents (i.e. DAMPs including HMGB-1, heat shock proteins) into the surrounding tissues(117, 118). Release of these DAMPs during secondary necrosis increases mortality in sepsis models(119). DAMPs activate TLRs on leukocytes, causing inflammation and further tissue damage (120, 121). Therefore when functioning normally, the processes of apoptosis and efferocytosis allow old/damaged cells to die and be cleared without causing significant inflammation and/or auto-immunity, in contrast to necrosis(122).

The process of efferocytosis is itself anti-inflammatory. Following AM efferocytosis of apoptotic neutrophils, intracellular signalling within AMs induces an anti-inflammatory pro-resolving M2 phenotype, associated with increased production of anti-inflammatory / pro-repair cytokines (vascular endothelial growth factor [VEGF], hepatocyte growth factor [HGF], TGF- β , Prostaglandin E₂, and IL-10)(123-128). In this way, the process of efferocytosis may stimulate alveolar epithelial repair in ARDS. However, the anti-inflammatory pathways activated by efferocytosis and release of anti-inflammatory mediators may also impair the bacterial phagocytosis capabilities of macrophages(129). There is also a corresponding reduction in release of pro-inflammatory cytokines following efferocytosis (TNF- α , IL-1 β , IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor [GM-CSF]) from AMs(130). Strategies to upregulate efferocytosis could accelerate tissue repair and resolution of inflammation, helping to break the destructive cycle of inflammation in ARDS.

1.3.4 Mechanisms of Efferocytosis

When cells undergo apoptosis, phosphatidylserine (PS) is re-distributed from the inner leaflet to the outer leaflet of the cell membrane; therefore PS is exposed on the cell surface of apoptotic cells(131). Macrophages either bind PS directly via receptors (e.g. brain angiogenesis inhibitor-1, T-cell immunoglobulin and mucin domain 1 [TIM]-1/3/4) or indirectly via PS-binding bridging molecules (e.g. milk fat globule epidermal growth factor-VIII [MFG-E8], growth arrest specific-6 [Gas6] and Protein S). On macrophages, integrin $\alpha\beta 3/5$ recognises MFG-E8 and receptor tyrosine kinases Tyro3, Axl and Mer (acronym: TAM receptors) recognise Gas6 and Protein S(117). Activation of either receptor types triggers intracellular signalling resulting in Ras-related C3 botulinum toxin substrate 1 (Rac1) guanosine

triphosphate hydrolase (GTPase) activation, which causes cytoskeletal rearrangement and engulfment of the apoptotic cell(132). There are also macrophage receptors which can bind other non-PS surface ligands on apoptotic cells (e.g. mannose receptor CD206 recognises altered sugars)(117). There appears to be a large degree of functional redundancy in the number of receptors which can recognise apoptotic cells; the reason for this is not fully understood. Many of the macrophage receptors described above have not yet been studied in AMs; so far the best characterised apoptotic cell recognition receptor expressed by AMs is Mer(117, 133). Some studies have shown that Mer expression may be critical for AM efferocytosis(134-136). Mer activation induces transcription of suppressor of cytokine signalling (SOCS) proteins, which inhibit TLR-induced inflammatory signalling(137). It is likely that integrin $\alpha\beta 5$ and Mer function in conjunction, with integrin $\alpha\beta 5$ tethering the apoptotic cell to the macrophage, and Mer triggering engulfment(138). Mer signalling ultimately results in Rac1 activation, however there appear to be multiple signalling pathways involved, potentially involving activation of vav1 and phosphatidylinositol 3'-OH kinase (PI3K) pathways(138, 139).

AMs express signal inhibitory protein α (SIRP α) on their surface. In the alveolar space, surfactant proteins A and D (SPA/SPD) bind SIRP α , which results in suppression of efferocytosis(140). Binding of SIRP α to the "don't eat me" CD47 ligand found on the surface of healthy cells similarly suppresses efferocytosis(141). SIRP α activation causes phosphorylation of Src homology region 2 domain-containing phosphatase-1 (SHP-1), which results in downstream activation of the GTPase Ras homolog gene family, member A (RhoA)(141). RhoA activates Rho-associated kinase (ROCK), which then phosphorylates phosphatase and tensin homologue (PTEN)(142, 143). RhoA, ROCK and PTEN then form a complex which

opposes PI3K signalling, resulting in Rac1 inhibition and suppression of efferocytosis(144, 145). The signalling pathways for Mer and SIRP α are summarised in figure 1.3.

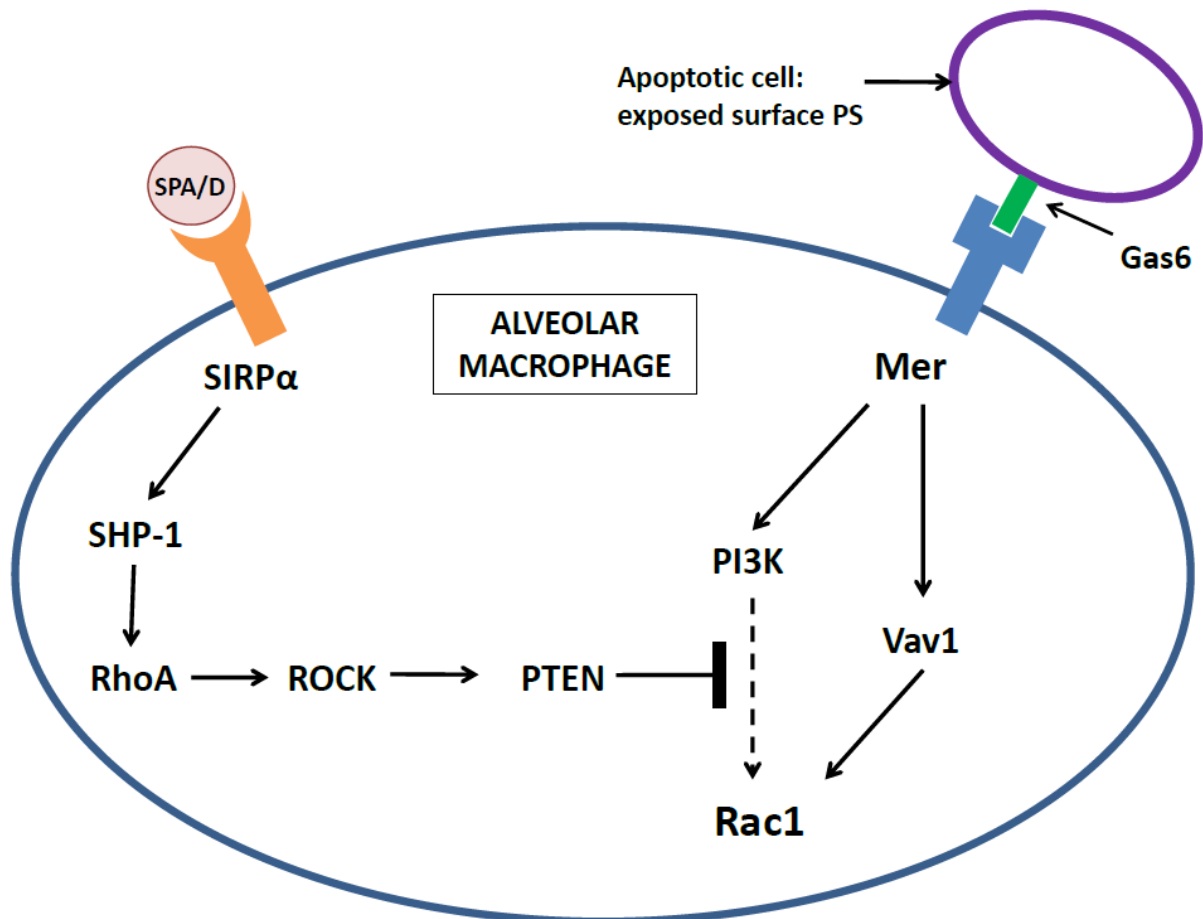


Figure 1.3: Signalling pathways for Mer and SIRP α in alveolar macrophages

Figure adapted from multiple sources(138, 139, 143, 146-148). Alveolar macrophage efferocytosis is regulated by Mer and SIRP α . Gas6 binds to PS on the surface of apoptotic cells. Activation of Mer by the PS opsonin Gas6 can trigger signalling cascades via PI3K and Vav1, which both upregulate Rac1. Activation of Rac1 results in cytoskeletal re-arrangement and efferocytosis of the apoptotic cell. Activation of SIRP α by SP-A (or SP-D) triggers a signalling cascade along the SHP1/RhoA/ROCK/PTEN pathway, which inhibits PI3K signalling, and ultimately downregulates Rac1, thereby inhibiting efferocytosis. Gas6 = growth arrest specific-6. Mer = Mer receptor tyrosine kinase. PI3K = phosphatidylinositol 3'-OH kinase. PS = phosphatidylserine. PTEN = phosphatase and tensin homologue. ROCK = Rho-associated kinase. SHP-1 = Src homology region 2 domain-containing phosphatase-1. SIRP α = signal inhibitory protein- α . SPA/D = surfactant protein A / D.

1.3.5 Factors affecting alveolar macrophage efferocytosis in ARDS

Elevated HMGB-1 levels are detected in ARDS patient BALF(91, 149, 150); HMGB-1 is a DAMP released following secondary necrosis of apoptotic cells, which causes tissue inflammation(151, 152). HMGB-1 is similarly released by cells undergoing secondary necrosis in models of severe sepsis(153). HMGB-1 has been shown to inhibit Rac1-mediated efferocytosis in macrophages(154-156). This may be via activation of the PTEN pathway(157), in a similar way to SRIP α activation as described above. HMGB-1 can also bind to PS on the surface of apoptotic neutrophils, thereby preventing their efferocytosis(158). Blocking the action of HMGB-1 has been shown to enhance MDM efferocytosis and reduce inflammation in models of ARDS(150, 159). High levels of TNF- α can also impair alveolar macrophage efferocytosis(160). It has been shown by our group and others that treatment of healthy macrophages with ARDS patient BALF can significantly reduce efferocytosis, likely due to high levels of inflammatory mediators including HMGB-1 and TNF α (91, 150).

Anti-inflammatory lipid mediators Resolvin E1 and Lipoxin both enhance neutrophil apoptosis and subsequent efferocytosis by alveolar macrophages, thus reducing lung inflammation in murine models(161, 162). Therapeutic use of these lipid mediators to enhance efferocytosis has been considered. Existing drugs such as statins(163) and azithromycin(164) have also been shown to enhance efferocytosis.

1.3.6 The ‘Neutrophil Hypothesis’

In ARDS, large numbers of neutrophils migrate to the alveolar space and undergo apoptosis(165). Persistently high neutrophil and low alveolar macrophage levels in BALF are also associated with greater mortality(165). Multiple studies have shown

that neutrophils from ARDS patients and healthy neutrophils treated with ARDS BALF have delayed apoptosis following *ex-vivo* culture for 18-24 hours (see table 1.4). This effect may be in part due to elevated alveolar inflammatory cytokines GM-CSF and IL-2 in early ARDS(166, 167). However, in these *ex-vivo* studies, any neutrophils which were apoptotic at the time of sampling will have likely undergone secondary necrosis and disintegration prior to assessment of apoptosis 18-24 hours later.

There is a paucity of studies which have assessed the proportion of alveolar neutrophils which are apoptotic at the time of ARDS diagnosis, immediately post-BAL. Matute Bello et al(168) showed that *in vivo* alveolar neutrophil apoptosis rates were higher in ARDS patients at days 1 and 3 (1.5% and 3%, n=35) compared to at-risk patients (0.5% and 1.6%, n=13), although absolute values were low and the difference was not significant. This study used cytopsin morphology as the mainstay of determining apoptosis, and only used flow cytometric assessment of apoptosis (using Annexin V and propidium iodide) on 3 ARDS patient samples. Lesur et al(166) showed that *in vivo* alveolar neutrophil apoptosis was reduced in ARDS patients (14.3%, n=9) compared to control critically ill patients (21.7%, n=10). This study used cytopsin morphology and terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assays to determine apoptosis. These are the only two studies which have assessed *in vivo* alveolar neutrophil apoptosis rates at the time of BAL, however they give opposite results, and the absolute values are not comparable. Further studies are required to clarify the degree of alveolar neutrophil apoptosis in ARDS patients.

Murine models of ARDS have shown that following influx, large numbers of neutrophils must eventually undergo apoptosis and accumulate within the alveolar

space(169). Therefore, it is likely that whilst the inflammatory alveolar environment of early ARDS may initially delay neutrophil apoptosis, these neutrophils will ultimately undergo apoptosis in the alveolar space and accumulate due to the massive influx observed in early disease. Efficient efferocytosis of these apoptotic neutrophils by alveolar macrophages is critical for the resolution of inflammation in ARDS and promotion of tissue repair (121, 132, 169, 170). The “neutrophil hypothesis” of ARDS(171) suggests that apoptotic neutrophils accumulate in ARDS due to defective AM efferocytosis and/or excessive apoptosis which overwhelms AM efferocytosis capacity. These accumulated apoptotic neutrophils then undergo secondary necrosis and trigger the prolonged exaggerated inflammation characteristic of ARDS.

Thus far, no study has assessed AM efferocytosis in ARDS patients. However, studies have shown that ARDS patients have impaired MDM efferocytosis, and that treatment of healthy macrophages with ARDS patient BALF suppresses efferocytosis(91, 150). Elevated levels of HMGB-1 have been detected in ARDS patient BALF(91, 149, 150), and HMGB-1 is known to inhibit macrophage efferocytosis(154-156). These findings suggest there may be impaired AM efferocytosis in ARDS.

Author	Source of neutrophils	Ex-vivo culture duration	Methods of quantifying apoptosis	Findings
Matute-Bello et al(168) (1997)	Circulating neutrophils from HVs	18 hours	Cytospin morphology. Annexin V and PI on flow cytometry.	ARDS BALF treatment reduced apoptosis of HV neutrophils (50%), compared to healthy volunteer BALF (76%) & saline (81%).
Matute-Bello et al(167) (2000)	Circulating neutrophils from HVs	18 hours	Annexin V and PI on flow cytometry.	HV neutrophil apoptosis was inhibited by BALF from patients with early ARDS (days 1 & 3), compared with HV BALF (61% & 59% vs 87%). Neutrophil apoptosis was not affected by BALF from patients during late ARDS (days 7 & 14) or from patients at-risk for ARDS.
Lesur et al(166) (2000)	Circulating neutrophils from HVs	10 hours	Cytospin morphology. TdT mediated dUTP nick end labelling assay	ARDS BALF treatment reduced apoptosis of HV neutrophils (26.1%), compared to HV BALF (61.3%), non-ARDS ICU patient BALF (52.1%) or saline (36.9%).
Juss et al(172) (2016)	Alveolar & circulating neutrophils from ARDS patients. Circulating neutrophils from HVs.	20 hours	Cytospin morphology. Annexin V and PI on Flow Cytometry.	Alveolar & circulating neutrophils from ARDS patients have reduced apoptosis (28.5% & 42.7%) compared to circulating neutrophils from HVs (69.2%) after 20hrs culture. Neutrophils from HVs showed reduced apoptosis when incubated with ARDS BALF compared to control BALF (70.5% vs 37.4%)
Fialkow et al(173) (2006)	Circulating neutrophils	24 hours	Morphology following Wright's Giemsa stain	Circulating neutrophils from ARDS patients showed reduced apoptosis (28%) compared to uncomplicated sepsis (57%), mechanically ventilated patients without sepsis or ARDS (53%) & HVs (69%). However, they did not differ from patients with septic shock.
Gregoire et al(150) (2018)	Circulating neutrophils from ARDS patients and HVs	24 hours	Flow Cytometry: Activated caspase-3 apoptosis kit and Annexin V / DAPI	Circulating neutrophils from ARDS patients showed reduced apoptosis (65%) compared to those from HVs (85%). ARDS BALF treatment reduced HV neutrophil apoptosis (72%) compared to saline (85%) and control BALF (80%). Exposure to ARDS BALF caused further reduction in apoptosis & necrosis of neutrophils from ARDS patients.

Table 1.4: Studies showing that ARDS patient neutrophils and ARDS BALF-treated neutrophils have delayed apoptosis following ex vivo culture

HV= Healthy volunteer. PI = Propidium Iodide. DAPI = 4',6-diamidino-2-phenylindole. All studies used BALF and saline treatments at a 50:50 ratio with culture media.

1.3.7 Relationship between HSD-1 activity, macrophage phenotype and efferocytosis

Macrophage HSD-1 deficiency is associated with increased SHP-1 expression (174) and impaired efferocytosis of apoptotic neutrophils in a model of peritonitis(175). Circulating human monocytes do not express functional HSD-1(86). However, monocyte exposure to IL-4 or IL-13 induces HSD-1 reductase activity and differentiation to M2 macrophages(86). The presence of IFN- γ (an M1 cytokine) blocks the ability of IL-4 / IL-13 to induce HSD-1 activity in monocytes (86). These findings indicate an association between M2 macrophage phenotype, increased HSD-1 reductase activity and increased efferocytic function.

As previously mentioned, HSD-1 primarily acts as a reductase *in vivo* to locally amplify the action of glucocorticoids. Glucocorticoids increase AM efferocytosis(176) by upregulating Mer expression(177) and downregulating SIRP α expression(147). Findings from our group have also shown that cortisol can stimulate AM efferocytosis, and that HSD-1 activity is required for cortisone to stimulate efferocytosis (see Figure 1.4)(91). Glucocorticoids also induce an M2c phenotype in macrophages, which is closely associated with increased Mer, CD163 and CD206 expression(178). However, whilst enhancing efferocytosis, glucocorticoids impair bacterial clearance by macrophages(179).

Our group have previously found that BALF cortisol: cortisone ratios are 52% lower compared to plasma ratios in ARDS patients(91), which would suggest dysregulated alveolar steroid metabolism. We have also shown that AMs from ARDS patients have impaired HSD-1 levels early in the disease course (Figure 1.1)(91). From these findings, it was postulated that ARDS AMs with impaired HSD-1 activity will also have impaired efferocytosis.

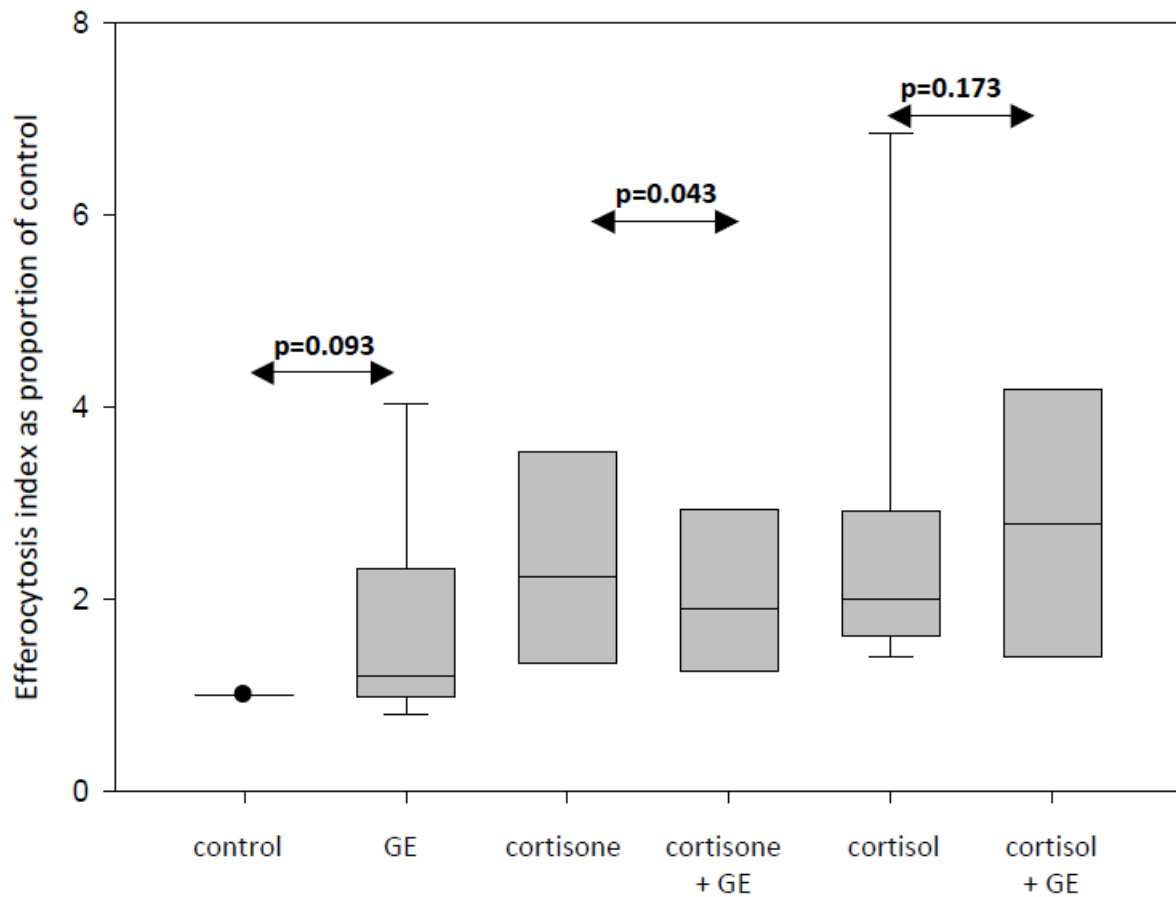


Figure 1.4: The effect of HSD-1 activity blockade on steroid influenced alveolar macrophage efferocytosis

Adapted from Bassford et al(91). GE = glycyrrhetic acid (inhibits HSD-1 activity) used at 10^{-7} M. Cortisol and cortisone both used at 10^{-7} M. Macrophages incubated with steroids for 24 hours prior to efferocytosis assay. GE has no effect on the pro-efferocytic effect of cortisol. GE reduces the pro-efferocytic effect of cortisone by preventing HSD1-mediated conversion to cortisol.

1.4 Potential Cellular Therapies for ARDS

1.4.1 Mesenchymal Stem Cells in Lung Injury

Human mesenchymal stem cells (MSCs) are multipotent cells derived from the bone marrow of adults which have the ability to differentiate into multiple different cell types including osteocytes, adipocytes, chondrocytes, myocytes and fibroblasts(180). The International Society for Cellular Therapy (ISCT) has recommended a set of criteria to define MSCs(181): 1) Cells must adhere to plastic under standard culture conditions. 2) MSCs must express CD105, CD73 and CD90, and lack expression of surface markers including CD45, CD34, CD14, CD19 and HLA-DR. 3) MSCs must have the ability to differentiate into osteogenic, adipogenic and chondrogenic cell lines (mesenchymal lineages) under appropriate culture conditions *in vitro*.

MSCs have also been isolated from adipose tissue, placenta and umbilical cord. The potential advantages of using umbilical cord MSCs include: 1) Umbilical cord MSCs are isolated at the same time-point (birth) from all donors, thereby avoiding variability associated with donor age. 2) Umbilical cord MSCs do not require an invasive procedure to obtain from the donor, and are a naturally generated 'waste product', which would otherwise be discarded. 3) Umbilical cord MSCs proliferate more rapidly *in vitro* than adult bone marrow MSCs(182). However, this thesis focusses on work performed using adult bone marrow MSCs since the majority of pre-clinical and clinical data is based on use of these cells.

There is strong pre-clinical evidence to support the therapeutic use of MSCs in ARDS. The main mechanisms by which MSCs can attenuate lung injury in ARDS(183) are: 1) Promotion of epithelial and endothelial repair, 2) Modulation of

immune function to increase bacterial clearance and aid resolution of inflammation. These mechanisms (summarised in figure 1.5) are mediated by both cell-to-cell interactions and release of paracrine factors(184-190).

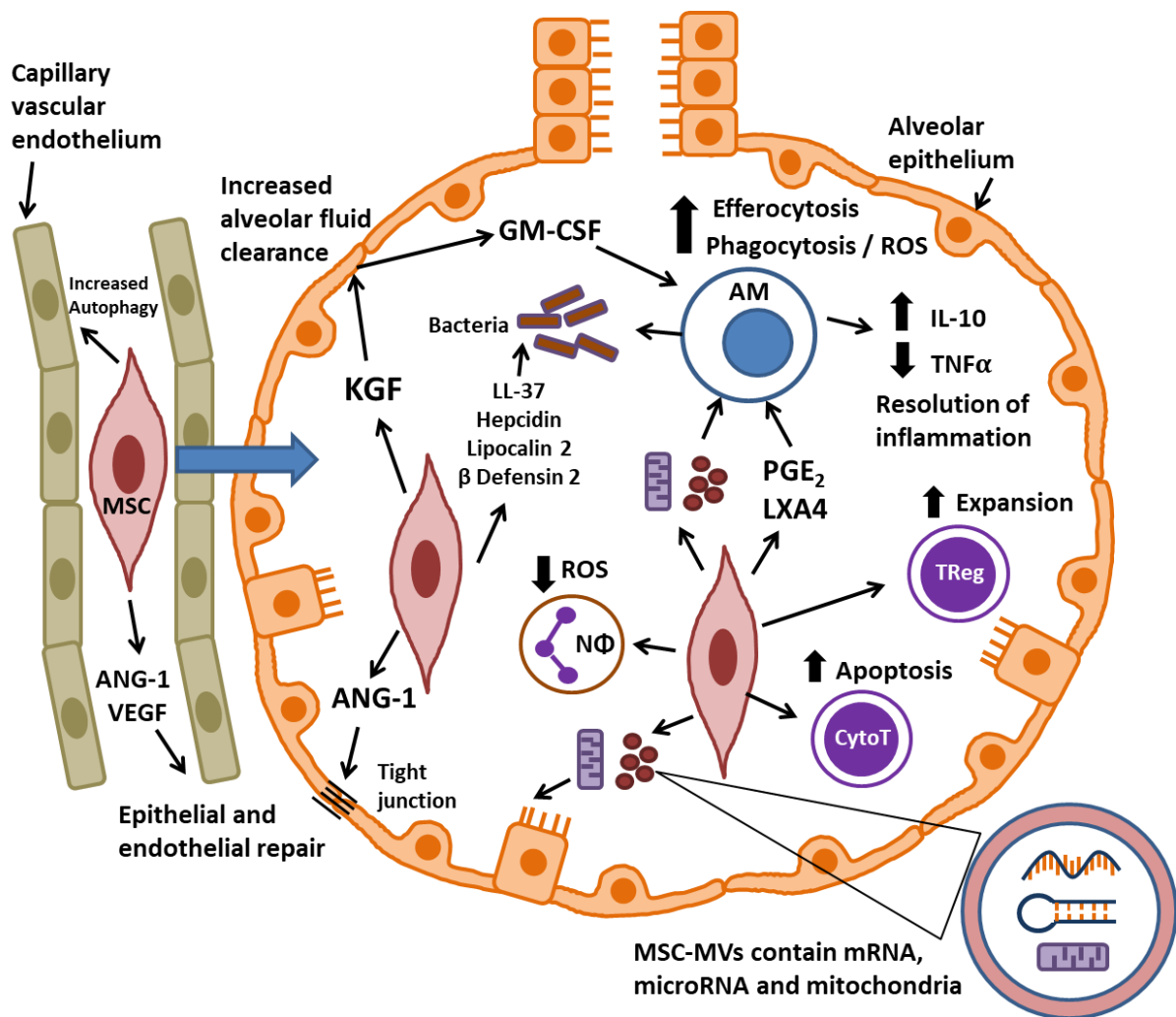


Figure 1.5: MSC mechanisms of action to attenuate lung injury in ARDS

Adapted from Laffey et al(183). Mesenchymal stem cells (MSCs) increase alveolar fluid clearance and stimulate epithelial/endothelial repair by release of paracrine factors angiopoietin-1 (ANG-1), keratinocyte growth factor (KGF) and lipoxin A4 (LXA4). KGF stimulates alveolar epithelial cells (AECs) to release granulocyte macrophage colony stimulating factor (GM-CSF), which can upregulate alveolar macrophage (AM) efferocytosis. MSCs also increase endothelial cell autophagy, thereby protecting against lung injury. Transfer of mitochondria from MSCs to type II AECs by direct contact or via microvesicles (MVs), aids epithelial integrity and increases alveolar fluid clearance. MSC-MVs can transfer protective mitochondria, microRNA and messenger RNA (mRNA) to AMs and AECs. Release of the antimicrobial peptides human cathelicidin (LL-37), lipocalin 2, β-defensin 2, and hepcidin aids bacterial clearance. MSCs modulate AM function by release of LXA4 and prostaglandin E₂ (PGE₂), and by transfer of mitochondria either via tunnelling nanotubes or MVs. AMs develop an M2 surface marker and cytokine profile (pro-resolving) but display increased bacterial phagocytosis. MSCs stimulate expansion of regulatory T cells (TRegs) and apoptosis of cytotoxic T cells (CytoT). MSCs decrease reactive oxygen species (ROS) production by neutrophils (NΦ). This immune modulation aids resolution of infection and inflammation.

MSC Promotion of Epithelial and Endothelial Repair

MSCs secrete angiopoietin-1(191) and keratinocyte growth factor(192) (KGF), which act to restore epithelial and endothelial integrity and improve alveolar fluid clearance. Angiopoietin-1 reduces epithelial protein permeability by restoring tight junctions between alveolar epithelial cells(191). MSCs can also transfer mitochondria to damaged type 2 alveolar epithelial cells by direct contact, thereby increasing intracellular adenosine triphosphate (ATP), restoring epithelial integrity, preventing apoptosis and increasing surfactant production(193, 194). MSC-derived microvesicles (MSC-MVs) also contain mitochondria and mRNA for KGF and angiopoietin-1, which can be transferred to alveolar epithelial cells and endothelial cells(195, 196). This explains the ability of MSC-MVs to restore alveolar fluid clearance in injured *ex-vivo* human lungs (197, 198). Lipoxin A4 secreted by MSCs also acts on the alveolar epithelium to increase alveolar fluid clearance in models of lung injury(199, 200). MSCs also increase autophagy of pulmonary endothelial cells thereby attenuating lung injury(201).

MSC Modulation of Immune Function

Models of sepsis and lung injury have shown that MSCs produce prostaglandin E2, which stimulates macrophages to secrete IL-10, an anti-inflammatory cytokine(187, 202, 203). Release of IL-1 receptor antagonist (IL-1ra) by MSCs can downregulate the inflammatory response(204). MSCs also directly aid bacterial clearance by secreting the anti-microbial peptides LL-37 (cathelicidin) (186, 205), hepcidin (206), β -defensin 2 (207) and lipocalin 2 (208). MSCs can induce apoptosis of cytotoxic T cells via the Fas ligand and cause expansion of regulatory T cells(209, 210). In the context of lung injury, MSCs can attenuate neutrophil production of reactive oxygen

species (ROS) (211). MSC release of KGF (as described above) can stimulate the alveolar epithelium to secrete GM-CSF, which can upregulate alveolar macrophage efferocytosis(212, 213). MSCs can transfer mitochondria to alveolar macrophages either via tunnelling nanotubules requiring direct contact(214), or remotely via microvesicles(215). In this way, MSC induce a modified M2 phenotype in alveolar macrophages, which have pro-resolving characteristics (increased secretion of IL-10, expression of M2 markers CD163 and CD206, reduced secretion of pro-inflammatory cytokines TNF- α and IL-8), but also increased phagocytic activity against bacteria(189, 215-217). This gives alveolar macrophages the ability to clear bacteria more effectively, whilst minimising surrounding tissue injury from release of inflammatory mediators. MSC-MVs can transfer microRNA-145 to macrophages, which increases phagocytosis(218). MSCs also enhance bacterial killing by upregulating NADPH oxidase-2 (NOX-2) dependant ROS production in macrophages(219). Release of Lipoxin A4 by MSCs also enhances macrophage phagocytosis(220).

1.4.2 MSCs in Pre-clinical Models of ARDS

No single model can replicate the complexity of human ARDS; multiple events or 'hits' are involved in disease pathogenesis(221). There are complex interactions between co-morbidities, pre-disposing factors, the immune response, and infective organisms. As previously discussed, sepsis is the underlying aetiology in the majority of ARDS cases; the resulting lung injury can be direct (e.g. pneumonia) or indirect (e.g. peritoneal sepsis)(3). Therefore, MSCs have been administered in pre-clinical

models of direct and indirect infection-related lung injury; the most important studies are summarised in tables 1.5 and 1.6.

Murine models of direct and indirect sepsis-related lung injury have shown that MSC administration can reduce mortality, lung injury, pulmonary oedema, inflammatory cytokine release, alveolar cellular infiltration, and bacterial load (where relevant)(202, 203, 222-225). Large animal (sheep) models of direct and indirect sepsis-related lung injury have also shown that either intra-bronchial or intravenous MSC administration can improve oxygenation and reduce pulmonary oedema(226, 227). Similarly, studies in porcine and human *ex-vivo* lung perfusion (EVLP) models injured with *Escherichia coli* (or *E. coli* endotoxin) have shown that administration of MSCs intra-bronchially or in perfusate can reduce endothelial permeability, pulmonary oedema, neutrophilic infiltration and bacterial load(192, 228-230). MSC-MVs have also been shown to recapitulate many of the effects of MSCs in both human EVLP and murine direct lung injury models of ARDS(195, 198, 215). However, it appears that use of MSC themselves may still be superior to the contents of their conditioned media(205).

Model	Author	Treatment	Major Findings
Mouse IP-LPS	Xu et al(203), 2007	5 x10 ⁵ murine MSCs given IV 1 hour post-injury	MSCs ↓ lung neutrophilia, lung oedema and systemic inflammatory cytokines. MSCs ↑ circulating G-CSF.
Mouse CLP	Gonzalez-Rey et al(231), 2009	1-10 x10 ⁵ human adipose MSCs given IP 4hrs post CLP	MSCs ↓ inflammatory cytokines, ↓ inflammatory cell infiltrates, ↓ bacterial load, & ↑ IL-10.
Mouse IP-LPS	Iyer et al(232), 2009	5 x10 ⁵ murine MSCs given IV immediately post-injury	MSCs ↓ circulating inflammatory cytokines TNFα, IFNγ and IL-1β. MSCs have antioxidant effects in vivo.
Mouse CLP	Németh et al(187), 2009	1x 10 ⁶ murine MSCs given IV either 24hs pre-injury or 1hr post-injury	MSCs ↑ survival, ↓ kidney / liver damage, ↓ TNFα / IL-6 / MPO. After IV injection, MSCs localise to lungs. In response to LPS, MSCs release PGE ₂ which stimulates macrophages to release IL-10. Few MSCs visible on lung histology after 24hrs.
Mouse CLP	Mei et al(224), 2010	2.5 x10 ⁵ murine MSCs given IV 6 hours post-injury	MSCs ↑ survival, ↑ bacterial clearance & phagocytosis, ↓ systemic & pulmonary inflammatory cytokines, ↓ kidney injury, ↓ lung permeability, ↓ lung cell apoptosis. Low MSC persistence after 22hrs. MSCs ↓inflammation pathway genes & ↑phagocytosis genes.
Mouse IP <i>P. aeruginosa</i>	Krasnodembskaya et al(217)	1x 10 ⁶ murine MSCs given IV 1hr post-injury	MSCs ↑ survival, ↑ bacterial clearance, ↑ monocyte phagocytosis (↑CD11b expression), ↑ M2 surface markers (CD206 / CD163) on monocytes / macrophages
Sheep IV-LPS	Rojas et al(226), 2014	4, 10 or 40 x10 ⁶ human MSCs given IB 30mins post-injury	MSCs ↑ oxygenation, ↑ CO ₂ clearance ↑ pulmonary vascular pressures, ↓ inflammation, & ↓ pulmonary oedema

Table 1.5: Studies of bone-marrow MSCs in models of indirect sepsis-related lung injury

CO₂ = Carbon Dioxide. CLP = Caecal Ligation and Puncture. IFNγ = Interferon-γ. IV = Intravenous. IP = Intra-peritoneal. MPO = Myeloperoxidase.

Model	Author	Treatment	Major Findings
Mouse IT-LPS	Gupta et al(202), 2007	7.5 x 10 ⁵ murine MSCs given IT 4hrs post injury	MSCs ↑ survival & BALF IL-10. ↓ pulmonary oedema, permeability & BALF TNFα / MIP-2
Human EVLP: IB-LPS	Lee et al(192), 2009	5 x 10 ⁶ human MSCs or CM derived from 1 x 10 ⁶ MSCs given IB 1hr post-injury	MSCs & MSC-CM ↑ alveolar fluid clearance, ↑ lung endothelial barrier integrity, and ↓ pulmonary oedema. Effects partially mediated by KGF.
Mouse I.T. live <i>E. Coli</i>	Krasnodembskaya et al (186), 2010	1 x 10 ⁶ adult human MSCs given IT 4hrs post injury	MSCs ↓ cellular infiltration, pulmonary oedema & bacterial growth (mediated by LL-37)
Mouse I.T. live <i>E. Coli</i>	Gupta et al(208), 2012	7.5 x 10 ⁵ murine MSCs given IT 4hrs post injury	MSCs ↑ survival & bacterial clearance: mediated by lipocalin 2. Also ↓ TNFα, MPO & MIP-2 in BALF
Mouse IT-LPS	Ionescu et al(189), 2012	2.5 x 10 ⁵ syngenic murine MSCs or their CM given IT 4hrs post injury	Both MSCs & MSC-CM ↓ lung injury, oedema & neutrophil influx. AMs from MSC & MSC-CM treated mice showed an M2 phenotypic shift.
Mouse IT-LPS	Islam et al(194), 2012	2.5 x 10 ⁵ human or syngenic murine MSCs given IT 4hrs post-injury	MSCs transfer mitochondria to alveolar epithelial cells via gap junctional channels & ↑ alveolar ATP.
Human EVLP: IB live <i>E. Coli</i>	Lee et al(228), 2013	5-10 x 10 ⁶ human MSCs given IB or in perfusate 1-2hrs post-injury.	MSCs ↑ alveolar fluid clearance, ↓ neutrophil influx, ↓ bacterial load, ↑ AM phagocytosis, & ↑ GM-CSF. Effects partially mediated by KGF.
Pig EVLP: IV-LPS (in perfusate)	Rojas et al,(230) 2013	1 x 10 ⁸ autologous swine MSCs given in perfusate 15 mins post-injury	MSCs ↓ pulmonary oedema, pulmonary hypertension, inflammatory infiltrates, hypoxaemia & TNFα / IL-1β.
Sheep IB live <i>P. Aeuroginosa</i>	Asmussen et al(227), 2014	5-10 x 10 ⁶ /kg cryo-preserved human MSCs IV 1hr post-injury	MSCs ↑ oxygenation and ↓ pulmonary oedema
Rat IT live <i>E. Coli</i>	Devaney et al(205), 2015	1-2 x 10 ⁷ /kg human MSCs or CM from 4 x 10 ⁶ MSCs given IV or IT 30 mins post injury	Cryopreserved MSCs ↓ lung injury, bacterial load & inflammatory cytokines. MSCs ↑ survival, AM phagocytosis and LL-37. IT & IV routes effective.
Mouse IT-LPS	Fang et al(199)	5 x 10 ⁵ human MSCs given IV 4 hours post injury	MSCs ↑ survival, ↓ pulmonary oedema & ↓ BALF TNFα / MIP-2. Effects mediated by Lipoxin A4
Mouse IT live <i>E. Coli</i>	Monsel et al(195), 2015	8 x 10 ⁵ human MSCs or MVs from 3-9 x 10 ⁶ MSCs given IT or IV 4 hours post injury	MSC-MVs ↑ survival, AM phagocytosis. ↓ cellular infiltration, inflammatory cytokines, lung permeability & bacterial load. Partially KGF mediated
Rat IT-LPS	Yang et al(233), 2016	5 x 10 ⁶ rat MSCs +/- VEGF knockdown given IV 5hrs post-injury	VEGF expression is required for MSCs to ↓ lung permeability, lung injury, inflammatory cytokines, & epithelial cell apoptosis
Mouse IT live <i>E. Coli</i>	Jackson et al(214), 2016	1 x 10 ⁶ human MSCs given IV or IN 4hrs post-injury	MSCs enhance AM phagocytosis by transferring mitochondria via tunnelling nanotubules.
Mouse IN LPS	Morrison et al(215), 2017	2.5 x 10 ⁵ murine AMs (treated ex vivo with MSC-MVs) IN 4hrs post injury	MSCs ↑ AM phagocytosis and M2 surface markers. MSC-MV treated AMs can ↓ inflammation & lung injury. Effects mediated by mitochondrial transfer.
Human EVLP: IB live <i>E. Coli</i>	Park et al(198), 2019	MVs derived from 2-4 x 10 ⁷ human MSCs given in perfusate 1hr post injury	MSC-MVs ↑ alveolar fluid clearance & AM phagocytosis [Effect enhanced by Poly (I:C) pre-treatment of MSCs]. ↓ permeability, bacterial load.

Table 1.6: Studies of bone-marrow MSCs in models of direct sepsis-related lung injury

CM = Conditioned Media. IN = Intra-nasal. IT = Intra-tracheal. IV = Intravenous. KGF = Keratinocyte growth factor. MIP-2 = Macrophage Inflammatory Protein-2. MPO = Myeloperoxidase. MV = Microvesicle VEGF = Vascular Endothelial Growth Factor. Poly (I:C) = polyinosinic:polycytidylic acid.

1.4.3 Clinical Trials of MSCs in ARDS patients

Following favourable outcomes in pre-clinical studies, therapeutic use of MSCs in clinical trials of ARDS has been considered. The advantages of potential MSC therapy include the fact that MSCs remain undifferentiated during *in vitro* expansion(183), and cryopreservation does not affect their therapeutic efficiency(205). This would allow the necessary dose of MSCs to be generated and stored prior to clinical use. Due to their low expression of major histocompatibility (MHC) antigens, allogenic MSCs can be administered without inducing an immune response or rejection(234). Following intravenous administration in models of lung injury, the majority of MSCs localise to the lungs(187, 225). This may suggest the ability of MSCs to localise to sites of injury. However, models of liver injury also show that the majority of MSCs localise to the lungs following intravenous administration, with only a minority of MSCs getting delivered to the injured liver(235). Therefore, MSC localisation to the lungs likely occurs since the alveolar capillary bed is the first major capillary bed the MSCs reach following intravenous administration. As relatively large cells, the MSCs likely become trapped in the alveolar vasculature or exit the vasculature into the alveolar space(236, 237). This characteristic favours the intravenous delivery of MSCs to target lung disease. MSCs have a strong safety record in clinical trials for multiple diseases(183). However, MSCs lose their anti-inflammatory potential with successive passage, thus significantly limiting their therapeutic potential(238). The *in vitro* expansion required to generate sufficient numbers of MSCs for clinical therapy may also cause a decrease in their anti-inflammatory capacity.

So far, only one clinical trial of bone marrow derived MSCs in ARDS patients has been completed: The Stem Cells for ARDS Treatment (START) trial led by Professor

Matthay used intravenous administration of adult human bone marrow derived MSCs in patients with ARDS(239, 240). The Phase 1 findings of this trial were that MSC doses up to 10 million per kilogram are safe and well tolerated, with no adverse effects detected(239). The Phase 2a findings were that one dose of 10 million MSCs per kilogram was safe in patients with moderate to severe ARDS. Larger trials are needed to assess efficacy. A phase 1 trial of adult human adipose-derived MSCs in ARDS patients has also shown that MSC treatment is safe and feasible(241). Details of these and other ongoing clinical trials of MSCs in ARDS are listed in table 1.7.

Study	Study Type	Treatment	Findings
Adipose-derived Mesenchymal Stem Cells in Acute Respiratory Distress Syndrome(241)	Phase 1, randomised, double-blind, placebo-controlled trial in moderate to severe ARDS	Single dose of 1×10^6 /kg human adipose-derived MSCs given IV	MSC treatment is safe and feasible. No adverse effects.
START: Stem Cells for ARDS Treatment (239)	Open-label Phase 1 dose-escalation clinical trial in moderate to severe ARDS	Single dose of 1, 5 or 10×10^6 /kg human bone marrow MSCs given IV	All doses are safe and well tolerated. No adverse effects.
START: Stem Cells for ARDS Treatment (240)	Phase 2a, randomized, double-blind, placebo-controlled study to assess safety & efficacy of MSCs in moderate to severe ARDS	Single dose of 10×10^6 /kg human bone marrow MSCs given IV	One dose of intravenous MSCs was safe in patients with moderate to severe ARDS. Larger trials are needed to assess efficacy.
MUST-ARDS: A Phase 1/2 Study to Assess MultiStem® Therapy in Acute Respiratory Distress Syndrome (NCT02611609)	Phase 1-2 clinical trial in moderate to severe ARDS.	Single dose of 3×10^8 or 9×10^8 MultiStem® cells given IV	MultiStem® therapy at both doses was well tolerated with no serious adverse effects related to administration.
REALIST: Repair of Acute Respiratory Distress Syndrome by Stromal Cell Administration (NCT03042143)	Open-label dose-escalation phase 1 trial followed by a randomised, double-blind, placebo-controlled phase 2 trial in moderate to severe ARDS	Human umbilical cord derived CD362 enriched MSCs given IV	Recruitment ongoing
Mesenchymal Stem Cells for Treatment of ARDS in Patients With Malignancies (NCT02804945)	Phase 1 single-arm open label clinical trial in patients with malignancy and moderate to severe ARDS	Single dose of 3×10^6 /kg human bone marrow MSCs given IV	Recruitment complete, results pending.
Human Umbilical Cord Mesenchymal Stem Cell Therapy in ARDS (NCT03608592)	Phase 1 single-arm open label clinical trial in patients with moderate to severe ARDS	Single dose of 1×10^6 /kg human umbilical cord MSCs given IV	Recruitment ongoing
STAT: Mesenchymal Stromal Cells For Acute Respiratory Distress Syndrome (NCT03818854)	Phase 2b, randomized, double-blind, placebo-controlled study to assess efficacy of MSCs in moderate to severe ARDS	Single dose of 10×10^6 /kg human bone marrow MSCs given IV	Not yet recruiting

Table 1.7: Selected completed and planned clinical trials of MSCs in ARDS

1.4.4 Transgenic Mesenchymal Stem Cell therapy for ARDS

MSCs have also been used as vector for gene therapy in murine models of ARDS (see table 1.8 for a summary). Two studies have shown that in murine direct lung injury models, transgenic MSCs expressing human angiopoietin-1 were more effective at reducing alveolar cellular inflammation (both total and neutrophilic), inflammatory cytokine levels, and protein permeability than non-transfected or null-transfected MSCs(242, 243). Other studies have shown that transgenic MSCs expressing IL-10, soluble IL-1 receptor-like-1 or nuclear factor (erythroid derived 2)–like 2 can also reduce lung injury, inflammatory cytokine production and protein permeability more effectively than control MSCs in murine models of direct lung injury(244-246). These studies show that synergistic anti-inflammatory action of combined cellular and gene therapy was found to be superior to use of cellular therapy alone. As discussed above, MSCs localise to the lungs following intravenous administration, making MSCs an ideal vector for delivering transgene therapy in pulmonary diseases. Therefore, use of transgenic MSCs (tMSCs) may be a strategy to compensate for the loss of intrinsic anti-inflammatory potential seen in MSCs following successive passage. There is also precedence for use of transgenic human cells in clinical trials in other diseases, including Lymphoid Leukaemia, Parkinson's Disease, and Human Immunodeficiency Virus infection, (247-251).

Model	Author	Treatment	Major Findings
Mouse IT-LPS	Mei et al(242), 2007	2.5 x 10 ⁵ transgenic murine MSCs expressing human angiopoietin-1 given IV 30mins post-injury	Both treatments ↓ inflammatory cytokines. Ang1-tMSCs ↓ lung permeability, ↓ BALF total cell & neutrophil influx more than null-transfected MSCs.
Mouse IT-LPS	Xu et al(243), 2008	1 x10 ⁵ transgenic murine MSCs expressing human angiopoietin-1 given IV 2hrs post-injury	Ang1-tMSCs ↓ endothelial permeability, BALF protein, neutrophilia, MPO and TNFα more than null-transfected MSCs
Mouse IN-LPS	Martinez-Gonzalez et al(244), 2013	1x10 ⁶ transgenic human adipose MSCs expressing soluble IL-1 receptor-like-1 (ST2) given IV 6hrs post-injury	ST2-tMSCs ↓ inflammatory cytokines, lung injury, neutrophilic infiltration and protein permeability more than control MSCs.
Mouse IT-LPS	Wang et al(245), 2018	1x10 ⁶ transgenic murine MSCs expressing IL-10 given IV 4hrs post-injury	IL10-tMSCs ↓ inflammatory cytokines, ↓ protein permeability and ↑ survival more than control MSCs.
Mouse IT-LPS	Zhang et al(246), 2018	1x10 ⁶ transgenic human amniotic MSCs expressing nuclear factor erythroid derived 2-like 2(Nrf2) given IV 4hrs post-injury	Nrf2-tMSCs ↓ inflammatory cytokines and lung injury more than control MSCs

Table 1.8: Studies of transgenic MSCs in models of direct lung injury

1.5 Summary

Despite 50 years of research in ARDS, a pharmacological therapy remains elusive. While advances in supportive management such as lung protective ventilation have improved outcomes, ARDS remains a major cause of morbidity and mortality in critically ill patients.

It has previously been shown that ARDS patients have a relative alveolar cortisol deficiency due to reduced AM HSD-1 reductase activity. It is known that increased HSD-1 reductase activity and cortisol promote macrophage efferocytosis. However, no study has yet investigated alveolar macrophage efferocytosis in ARDS. There is also a lack of studies investigating *in vivo* neutrophil apoptosis in ARDS patients, with contradictory existing data. Therefore, investigation is required into a potential pathophysiological link between impaired AM HSD-1 activity, defective AM efferocytosis and resulting accumulation of apoptotic neutrophils in the alveolar space. If not cleared, these apoptotic neutrophils would undergo secondary necrosis, stimulating prolonged exaggerated inflammation. Upregulation of HSD-1 in the alveolar space may therefore potentially offer a therapeutic strategy for ARDS.

Pre-clinical models of ARDS in animals and human *ex vivo* perfused lungs have shown that MSCs offer a potentially effective therapy due to their intrinsic anti-inflammatory and pro-repair capabilities. Studies have also shown that use of a combined cellular and gene based therapy may be superior to cellular therapy alone. As previously stated, localised upregulation of HSD-1 in the alveolar space could have therapeutic potential in ARDS. Further investigation is required into whether a combined cellular and HSD-1 gene therapy could restore AM function and attenuate inflammation in ARDS. This strategy would aim to enhance local alveolar HSD-1 activity and thereby increase alveolar cortisol levels, which should restore normal AM

function and promote apoptotic neutrophil clearance, thereby attenuating inflammation. The intrinsic anti-inflammatory effects of MSCs would act synergistically with the locally elevated alveolar cortisol levels to promote the resolution of inflammation, which would potentially be superior to use of MSCs alone. This strategy could have therapeutic potential in ARDS, and potentially avoid the deleterious effects of systemic glucocorticoid therapy.

CHAPTER 2 : HYPOTHESIS AND AIMS

2.1 Hypotheses

Alveolar macrophages in ARDS have impaired HSD-1 reductase activity and impaired efferocytosis, resulting in persistent inflammation due to secondary neutrophil necrosis, which influences clinical outcome.

Therapeutic strategies using transgenic MSC which over-express HSD-1 may restore alveolar macrophage efferocytosis and attenuate inflammation in ARDS.

2.2 Aims

- 1) To determine whether the intrinsic anti-inflammatory and pro-repair abilities of MSCs are altered with prolonged *in vitro* expansion and repeated passage.
- 2) To create transgenic MSCs which express the HSD-1 transgene by using a lentiviral vector.
- 3) To determine if ARDS is associated with impaired alveolar macrophage efferocytosis and increased neutrophil apoptosis. To then determine whether co-culture with HSD-1 transgenic MSCs can restore alveolar macrophage efferocytosis.
- 4) To determine whether alveolar macrophage phenotype and functions can be restored in an *in vitro* model of ARDS.
- 5) To determine whether HSD-1 transgenic MSC therapy can attenuate inflammation in murine models of lung injury.

CHAPTER 3 : GENERAL METHODS

3.1 Clinical Studies

The aim of the clinical studies was to determine if ARDS is associated with impaired alveolar macrophage efferocytosis and increased alveolar neutrophil apoptosis.

3.1.1 Ethical Approvals for Clinical Studies

National research ethical approval was sought for an observational clinical study entitled: “A research study to investigate whether transgenic mesenchymal stem cells expressing hydroxysteroid dehydrogenase type-1 can attenuate inflammation in Acute Respiratory Distress Syndrome” (AM-ARDS). A favourable ethical opinion was given following review by the Wales Research and Ethics Committee 1 (REC 16/WA/0169). Health Research Authority (HRA) approval for this study was given subsequently. Following REC and HRA approval, the AM-ARDS study was approved by both the University Hospitals Birmingham and Heart of England NHS Trusts. This study is registered on the National Institute for Health Research (NIHR) Portfolio, registration number 32296.

National research ethical approval was also sought for a study on human lung tissue samples entitled: “Use of Tissue from Lung Resections to Investigate Cellular Function in Human Lung Disease” (Lung Resection Study). A favourable ethical opinion was given following review by the West Midlands (Solihull) Research and Ethics Committee 1 (REC 17/WM/0272). HRA approval for this study was given subsequently. This study was then approved by both the University Hospitals Birmingham and Heart of England NHS Trusts.

3.1.2 AM-ARDS Study Patient Recruitment

Patients were enrolled into the AM-ARDS observational study from the intensive care units (ICUs) of the New Queen Elizabeth Hospital Birmingham and Birmingham Heartlands Hospital in the period from November 2016 to July 2019. These hospitals are both tertiary referral centres with a combined total of 100 ICU beds. These ICUs admit adult patients with both medical and surgical conditions. Two main groups of patients were recruited: patients with ARDS, and a control group of critically ill sepsis patients at risk of ARDS.

Potential recruits to this study were screened for on a daily basis by members of the direct clinical care teams at both ICUs. It was recognised that in this study, the patients would be unable to give informed consent due to alterations in conscious level caused by illness and therapeutic sedation. Therefore, if a patient fulfilled the study inclusion/ exclusion criteria, their next of kin would be approached by a medical or nursing member of the research team and would be requested to consider giving assent for the patient to be recruited into the study. If the patient's next of kin agreed to give assent; a signed, written agreement of assent was obtained from them.

If a potential participant had been identified through the screening process and there was no next of kin available either in person or via telephone after 24 hours of attempting to make contact, then the Intensive Care consultant doctor with responsibility for clinical care of the patient in Intensive Care was approached to act as a Nominated Consultee. Designated Consultees would always be unconnected to the study, and therefore, able to make a decision completely independent of the research team. Nominated Consultees were asked to give an opinion as to whether

the patient should be enrolled in such medical research. Following assent from the next of kin, or agreement from the Nominated Consultee; samples of blood, urine and broncho-alveolar lavage fluid (BALF) were taken from both groups of patients. If patients regained the capacity to comprehend the details of the study, retrospective consent was sought and they were asked for permission to include their data in the study.

3.1.3 AM-ARDS Study Inclusion and Exclusion Criteria

Inclusion Criteria for ARDS Patients

ARDS patients were diagnosed according to the Berlin criteria(11). Patients with a septic aetiology were selected for, since this was the population that alveolar HSD-1 deficiency was previously identified in. The inclusion criteria for ARDS patients are shown below, all these criteria would need to be met for enrolment:

- Age greater than or equal to 18 years
- Admission to an Intensive Care Unit
- Patient undergoing mechanical ventilation
- Underlying aetiology of infection/sepsis
- Patient must fulfil the Berlin diagnostic criteria for ARDS:
 - Acute onset (within 1 week of clinical insult)
 - Bilateral infiltrates on chest radiograph
 - Respiratory failure not fully explained by heart failure or fluid overload
 - $\text{PaO}_2/\text{FiO}_2$ ratio <40 kPa with PEEP or CPAP ≥ 5 cm H_2O
- ARDS diagnosed within the last 48 hours

Inclusion Criteria for Critically Ill Patients At Risk of ARDS (Control Group)

A control group of patients was identified, who were undergoing mechanical ventilation on ICU, had a septic aetiology, and were at risk of developing ARDS. The inclusion criteria for critically ill patients at-risk of ARDS patients are shown below, all these criteria would need to be met for enrolment:

- Age greater than or equal to 18 years
- Admission to an Intensive Care Unit
- Patient undergoing mechanical ventilation
- Underlying aetiology of infection/sepsis
- Patient NOT meeting Berlin criteria for ARDS
- Fulfils the Systemic Inflammatory Response Syndrome (SIRS) criteria; must have had 2 or more of the following within the last 24hrs:
 - Temperature >38 or <36 °C
 - Heart rate >90 beats per minute
 - Respiratory rate >20 breaths per minute or $\text{PaCO}_2 <4.3$ kPa (prior to mechanical ventilation)
 - White cell count >12.0 or $<4.0 \times 10^4/\mu\text{l}$

Exclusion Criteria

Under certain circumstances, it was deemed inappropriate for a patient to be enrolled to the AM-ARDS study, even if they met the inclusion criteria. These exclusion criteria mainly related to patient welfare and assent. Patients who were clinically immunosuppressed, or on long term steroid therapy prior to ICU admission,

were also excluded on the basis that these would be confounding factors which would interfere with alveolar macrophage function and HSD-1 activity. Patients with significantly abnormal clotting function were excluded since bronchoscopy could not be safely performed in this patient group. The vast majority of patients eligible for recruitment to both groups received intravenous hydrocortisone to treat for septic shock on admission to ICU. Excluding patients who received acute corticosteroid treatment would have made recruitment to this study impracticable. Therefore, a pragmatic approach was taken in which patients who had received acute corticosteroid treatment for less than 24 hours at the time of screening would be considered eligible for recruitment. The full exclusion criteria are shown below, meeting any one of these criteria would exclude a patient from being enrolled:

- Imminent treatment withdrawal
- Patient on long term steroid therapy prior to current acute illness
- Abnormal clotting function (INR > 3.0 or platelets < 20)
- Clinically relevant immunosuppression for any reason
- Personal consultee, when available, does not provide assent
- Designated consultee, if used, does not provide assent

3.1.4 AM-ARDS Study Severity Scoring

SOFA Score

The Sepsis-related Organ Failure Assessment (SOFA) Score was used to determine the severity of organ failure suffered by critically ill patients with a septic aetiology (252-254). Organ function in respiratory, neurological, cardiovascular, haematological, hepatic and renal systems are given a score between 0 - 4. Greater

total scores are associated with worse clinical outcomes. Components of the SOFA score are shown below in table 3.1:

Variables	SOFA SCORE				
	0	1	2	3	4
Respiration PaO ₂ /FiO ₂ ratio (kPa)	≥53.3	40 - 53.2	26.7 - 39.9	13.3 - 26.6*	<13.3*
Central Nervous System Glasgow Coma Scale (GCS)	15	13 - 14	10 - 12	6 - 9	<6
Cardiovascular Mean Arterial Pressure (MAP) or Inotropic requirement (µg/kg/min)	MAP >70 mmHg	MAP <70 mmHg	Dopamine ≤5 Or Any dose of Dobutamine	Dopamine >5 and ≤15 or Noradrenaline /adrenaline <0.1	Dopamine >15 or Noradrenaline /adrenaline >0.1
Coagulation Platelet count (x10 ³ /µL)	>150	100-149	50-99	20-49	<20
Liver Function Bilirubin (µmol/L)	<20	20 - 32	33 - 101	102 - 204	>204
Renal Function Creatinine (µmol/L)	<110	110-170	171-299	300-440 or 200-499 mls /day urine output	>440 or <200 mls /day urine output

Table 3.1: SOFA score components

Adapted from Vincent et al(253, 255). FiO₂: fraction of inspired oxygen. PaO₂: partial pressure of arterial oxygen. PEEP: positive end-expiratory pressure. *patient must also be mechanically ventilated.

APACHE II

The Acute Physiology and Chronic Health Evaluation II (APACHE II) system commonly utilised in ICU studies as a marker of disease severity in critically ill patients. In APACHE II, 12 physiologic variables are each assigned a score from 0 - 4 depending on the most abnormal measurement in the first 24 hours of their ICU

stay. Additional scores are given for variables including age and concurrent medical or surgical conditions. This results in a score between 0 - 71, with greater scores indicating greater disease severity and consequently increased risk of mortality (256).

Murray Lung Injury Score

The Murray Lung Injury Score (LIS) was developed prior to the Berlin and Consensus criteria for ARDS diagnosis, in order to aid identification of acute lung injury(257). LIS components include alveolar infiltrates on chest x-ray, PEEP, PaO₂/FiO₂ ratio and lung compliance (see table 3.2).

Inputs	LUNG INJURY SCORE				
	0	1	2	3	4
Chest X-Ray Quadrants with alveolar consolidation	0	1	2	3	4
PaO₂ / FiO₂ ratio (kPa)	>40	30 - 40	23 - 29	22 – 13.3	<13.3
PEEP (cm H ₂ O)	≤5	6-8	9-11	12-14	≥15
Respiratory System Compliance Score (ml / cm H ₂ O)	>150	100-149	50-99	20-49	<20

Table 3.2: Murray Lung Injury Score Components

Adapted from Murray et al(257). FiO₂: fraction of inspired oxygen. PaO₂: partial pressure of arterial oxygen. PEEP: positive end-expiratory pressure. The final score is calculated by addition of the component parts, then dividing by the number of inputs selected. Score of 1 - 2.5 indicates mild to moderate lung injury. Score >2.5 indicates severe lung injury.

3.1.5 Lung Resection Study Patient Recruitment

The Lung Resection study recruited patients with lung disease who were scheduled to have surgery to remove lung tissue as part of their clinical treatment plan (usually a lobectomy or pneumonectomy) at the Thoracic Surgery Unit in Birmingham Heartlands Hospital from September 2017 to July 2019. The vast majority of patients undergoing lung resection surgery were being treated for lung cancer. The aim of this study was to collect human lung tissue samples which were surplus to histopathological requirements following planned thoracic surgery. These lung tissue samples were then used in laboratory studies to investigate mechanisms of lung disease.

Any adult patient aged 18 years or older undergoing thoracic surgery to remove lung tissue at Birmingham Heartlands Hospital was eligible for inclusion to this study. If patients consented to be enrolled to the study, they were asked to sign a written declaration of consent. Baseline demographics including age, sex, smoking history and lung function were collected as part of the study.

3.2 Clinical Sample Collection

3.2.1 Blood collection

As part of the AM-ARDS study, 40 mls of whole blood were collected from patients on two occasions: within 24 hours of enrolment (day 1), and between 4 to 7 days after enrolment. Blood was collected by peripheral venepuncture or from pre-sited arterial lines into three 6 ml Lithium Heparin vacutainer tubes, two 6 ml serum clot activator vacutainer tubes, one 4 ml Ethylenediaminetetraacetic acid (EDTA) vacutainer tube and one 3.5ml 3.2% sodium citrate vacutainer tube (all from Becton Dickinson Ltd, Oxford, UK). Samples were then transported on ice to the laboratory for processing and analysis.

Whole blood was also taken from healthy volunteers by peripheral venepuncture into five 6 ml Lithium heparin vacutainer tubes at the University of Birmingham's Centre for Translational Inflammation Research, after obtaining consent according to University guidelines. Samples were then transported on ice to the laboratory for processing and analysis.

3.2.2 Urine Collection

As part of the AM-ARDS study, 20 mls of urine were collected from patients on two occasions: within 24 hours of enrolment (day 1), and between 4 to 7 days after enrolment. Samples were collected from a pre-sited indwelling urinary catheter already in situ as a result of their illness.

3.2.3 Bronchoscopy and collection of broncho-alveolar lavage fluid

Bronchoscopy was performed on sedated, mechanically ventilated patients using a standardised protocol as part of the AM-ARDS study. Bronchoscopy was only performed if the consultant ICU physician responsible for the patient's clinical care stated that it would be safe to proceed. Patients were ventilated using 100% inspired oxygen for 5 minutes prior to bronchoscopy. An Olympus LF-TP flexible tracheal intubation fibrescope (Olympus-Keymed, UK) was used for all bronchoscopy procedures. This scope is 5.2 mm in diameter and has a 2.6 mm suction channel. After inserting the scope through the patient's endo-tracheal tube or tracheostomy tube, the tip of the bronchoscope was wedged into a sub-segmental bronchus of the lingula or right middle lobe. Two 50 ml aliquots of sterile 0.9% saline at room temperature were instilled down the bronchoscope as a lavage, and the broncho-alveolar lavage fluid (BALF) aspirated back up into a collection chamber. At least 5 ml of BALF was always sent to the hospital microbiology department for analysis (including microscopy, culture, sensitivity and viral PCR). The remainder of the sample was immediately placed on ice and transported to the laboratory for processing. Initial bronchoscopy and collection of BALF was performed within 24 hours of enrolment to the study (described as day 1). A second BALF sample would be collected 4 to 7 days after enrolment, only if the patient was still being mechanically ventilated and the consultant ICU physician responsible for the patient's clinical care stated that it would be safe to proceed with a bronchoscopy.

3.2.4 Lung Resection Tissue Collection

Lung resections samples were immediately examined in theatre by an experienced member of the surgical team. A section of the sample distant from any tumour (if present), with no evidence of macroscopic pathology, and which was not required for histopathological purposes, was immediately immersed in sterile 0.9% saline within a sterile sealed container. Samples were transported on ice by the research team to the laboratory for processing and analysis.

3.3 Patient Sample Processing

3.3.1 Blood Sample Processing

Blood in serum clot activator tubes was allowed to clot at room temperature for 30 minutes prior to centrifugation. All blood samples from AM-ARDS study patients were centrifuged at 560g for 10 minutes at 4°C (Eppendorf AG 5810R centrifuge, Germany). The supernatant serum and plasma were aspirated and aliquoted into 1 ml cryovials. These samples were then stored at -80°C for future analysis.

Blood samples from healthy volunteers were processed to isolate neutrophils, for use in efferocytosis assays (for full details, see section 3.4.1).

3.3.2 Urine Sample Processing

Urine samples from AM-ARDS patients were centrifuged at 560g for 10 minutes at 4°C (Eppendorf AG 5810R centrifuge, Hamburg, Germany). The supernatant acellular urine was aliquoted into 20 ml tubes before being stored at -80°C for future analysis.

3.3.3 Broncho-Alveolar Lavage Fluid Processing

Broncho-Alveolar Lavage Fluid (BALF) was first filtered through sterile surgical gauze to remove mucus, into a sterile 50ml centrifuge tube (Greiner Bio-One, Kremsmünster, Austria). The total volume of filtered BALF was documented. Cell count and viability check were performed using trypan blue and a haemocytometer as described in 3.3.5. A differential cell count was then performed using cytospin and Diff-Quik labelling (Gentaur Europe, Kampenhout, Belgium) as described in 3.3.6. A

sample of filtered BALF cells were also assessed for apoptosis on flow cytometry as described in section 3.3.7. The filtered BALF was then centrifuged at 560g for 10 min at 4°C (Eppendorf AG 5810R centrifuge, Hamburg, Germany). Acellular BALF supernatant was aspirated and stored in 1ml aliquots at -80°C for future analysis. The cell pellet was re-suspended in 10 mls of RPMI 1640 media (Sigma-Aldrich, UK) containing 10% Fetal Bovine Serum (FBS; Gibco, ThermoFisher, USA) prior to alveolar macrophage isolation as described in section 3.3.7.

3.3.4. Lung Resection Sample Processing

Following arrival in the laboratory, lung resection samples were immediately washed through with 500 – 2000 ml of sterile 0.9% saline (Baxter, Newbury, UK) using a 14 gauge needle (Vasofix®, Braun, Melsungen, Germany). The needle was moved through the lung sample at multiple sites, whilst connected to an intravenous giving set, which allowed saline to run freely through the tissue. The washed through lavage fluid was collected and placed into sterile 50ml centrifuge tubes, before being centrifuged in a pre-chilled centrifuge (Eppendorf AG 5810R, Hamburg, Germany) at 4°C and 560g for 10 minutes. The supernatants were discarded and the cell pellets pooled and re-suspended in 10mls of RPMI 1640 media containing 10% FBS prior to alveolar macrophage isolation as described in section 3.3.8.

3.3.5 Cell Count and Viability

10 µl of cell solution was mixed with equal volume of 0.4% (w/v) trypan blue solution and left to incubate for 5 minutes. Cell count and viability were performed by adding 10 µl of the mixture to a haemocytometer with improved Neubauer markings and cells counted in each chamber. Non-viable cells take up the dye and appear blue whilst

viable cells exclude the dye. Total viable cell count was performed multiplying the number of viable cells on the haemocytometer grid by 2 (to account for trypan blue solution dilution) and then multiplying by 10,000 to give the number of cells per millilitre.

3.3.6 Cytospin Differential Cell Count

A differential cell count was performed using a cytopsin preparation. 100µl of cells at a concentration of 1×10^6 /ml were placed in the cytopsin apparatus and centrifuged at 300 r.p.m (revolutions per minute) for 5 minutes in a Shandon Mk II cyto-centrifuge (Thermo electron corporation, Basingstoke, UK). Slides were air dried and stained using Diff-Quik (Gentaur Europe, Kampenhout, Belgium). The differential cell count was then determined by counting 100 cells under 400x magnification and documenting the number and proportions of individual cell types. Leukocytes were identified by cell size and nuclear morphology.

3.3.7 Apoptosis Assay

An Apoptosis Detection Kit (BioLegend, UK) was used to identify apoptotic and necrotic cells. Cells were required to be in suspension prior to starting this assay. Filtered BALF cells or circulating neutrophils alone were re-suspended and transferred into 5ml polypropylene flow cytometry tubes (Falcon™, Becton Dickinson). Cultured alveolar macrophages had their media removed and were washed with Dulbecco's Phosphate Buffered Saline (PBS; Sigma-Aldrich, UK), before being incubated with 500µl of TrypLE™ express (cell-dissociation agent, ThermoFisher Scientific, UK) for 10 minutes at 37°C and 5% CO₂. Macrophages were visualised under a microscope to ensure that they had detached from the

culture well floor. 500µl of RPMI 1640 supplemented with 10% FCS was then added to each well to quench the trypsin. The macrophage suspensions were then pipetted and transferred into 5ml polypropylene flow cytometry tubes. For each cell type, 4 tubes were prepared: unlabelled, Annexin V only, 7-AAD only and dual-stained. A maximum of 100,000 cells were added to each tube.

2mls of Annexin V binding buffer (Biolegend, UK) was added to each tube prior to centrifugation at 400g for 5 minutes at 4°C (Eppendorf AG 5810R centrifuge, Germany). Supernatant was discarded and cells re-suspended in 100µl of Annexin V binding buffer and placed on ice. To the dual-stained and Annexin V only tubes, 5µl of Fluorescein isothiocyanate (FITC) conjugated Annexin V (Biolegend, UK) was added. All tubes were then vortexed for 5 seconds each, and incubated on ice in the dark for 15 minutes. During this incubation, Annexin V binds to phosphatidylserine on the surface of apoptotic cells. Tubes were then washed twice in Annexin V binding buffer as described above, and resuspended in 400µl of Annexin V binding Buffer. To the 7-AAD and dual-stained tubes, 5µl of 7-aminoactinomycin D (7-AAD, BioLegend, UK) was added. 7-AAD is a nucleic acid viability dye which can only penetrate damaged cell membranes. All tubes were then vortexed for 5 seconds each, and incubated on ice in the dark for 10 minutes. Tubes were then run on the Accuri C6 flow cytometer (BD Biosciences, USA) using the FL1 channel to detect FITC conjugated Annexin V and the FL3 channel to detect 7-AAD. Unlabelled and single colour controls were run first to set up gating and compensation. As per kit instructions, cells that were Annexin V only positive were classified as early apoptotic, whereas cells which were doubly positive for Annexin V and 7-AAD were classified as late apoptotic / necrotic.

Morphological analysis of cytopins (section 3.3.6) was used as an additional method of assessing apoptosis in neutrophils. Morphological changes of apoptosis include nuclear fragmentation, chromatin aggregation and cytoplasmic vacuolation(258).

3.3.8 Alveolar Macrophage Isolation

Re-suspended cell pellets from BALF and lung resections were treated identically from this point onwards. The cell suspension was overlaid onto 10 mls of Lymphoprep™ (StemCell Technologies, Vancouver, Canada) in a sterile 50 ml centrifuge tube. The tube was centrifuged at 4°C and 800g for 30 minutes with minimal acceleration and no brake. The interphase layer containing mononuclear cells was aspirated using a 3 ml Pasteur pipette (Alphalabs, Eastleigh, UK) into a sterile 50 ml centrifuge tube containing PBS supplemented with 10% FBS. Cells were then centrifuged at 300g for 10 minutes at 4°C to enable removal of platelets and wash off any remaining Lymphoprep™. Alveolar macrophages (AMs) were re-suspended in RPMI 1640 media supplemented with 10% FBS, 100U/mL penicillin, 100ug/mL streptomycin and 2mM L-glutamine (Sigma-Aldrich). A cell count, viability check and cytospin differential count were done (as per sections 3.3.5 and 3.3.6). AMs were then diluted in supplemented RPMI 1640 media and plated according to assay. For phagocytosis assays, AMs were plated at 5×10^4 per well in a 96-well black-walled, clear flat-bottomed culture plate (Costar®, Corning®, New York, USA). For efferocytosis and phenotyping assays, AMs were plated at 2.5×10^5 per well in a 24-well flat bottom culture plate (Costar®, Corning®, New York, USA). For HSD-1 functional assays, AMs were plated at 2×10^5 per well in a 6-well flat bottom culture plate (Costar®, Corning®, New York, USA). For RNA collection, AMs were plated at 1

$\times 10^6$ per well in a 6-well flat bottom culture plate. AMs were cultured in an incubator at 37°C with 5% CO₂ in preparation for cell function studies. AMs were adhered to the flat bottom wells of the culture plates. After 24 hours incubation, conditioned media was removed from the cultured alveolar macrophages and replaced with supplemented RPMI 1640 media. The conditioned media was centrifuged at 1000g for 5 minutes to pellet cellular debris, before the supernatant was aliquoted and stored at -80°C for future analysis.

AM activation was minimised as far as possible during isolation, by transporting biological samples on ice to the laboratory and processing within 1 hour of sample collection. The duration of AM exposure to Lymphoprep™ was minimised and AMs were immediately washed using PBS following the Lymphoprep™ stage. Use of Lymphoprep™ was necessary due to the highly neutrophilic content of ARDS BALF; a density gradient was required to isolate AMs. In order to have comparable results, all AMs therefore needed to be isolated using this technique. Preliminary studies assessed TNF α secretion by lung tissue AMs in the 24 hours following lymphoprep isolation: TNF α secretion was trivial (range 15-24 pg / million AMs, n=3) and equivalent to previous studies(259), indicating minimal activation of AMs.

3.4 Alveolar Macrophage Experiments

3.4.1 Efferocytosis Assay

A functional efferocytosis assay was used to assess the ability of alveolar macrophages to phagocytose apoptotic neutrophils(260).

Isolation and preparation of neutrophils

Neutrophils were isolated from whole blood using a Percoll® (Sigma-Aldrich, Poole, UK) discontinuous density gradient as previously described (261). Blood was taken from healthy volunteers by peripheral venepuncture into lithium heparin vacutainer tubes (Becton Dickinson Ltd, Oxford, UK) and transferred into a 50ml sterile centrifuge tube (Greiner Bio-One, Kremsmünster, Austria). 1 ml of 2% dextran (Sigma-Aldrich, UK) was added for every 6 mls of blood, then gently mixed prior to incubation for 40 minutes at room temperature; this enabled erythrocyte sedimentation.

Two stock concentrations of Percoll® were made up at 80% and 56%, both diluted in sterile 0.9% (w/v) saline. A discontinuous density gradient was prepared by first adding 5mls of 56% Percoll® in a sterile 15ml centrifuge tube (Greiner Bio-One, Kremsmünster, Austria), then under-laying this with 2.5 ml of 80% Percoll® using a 3 ml Pasteur pipette (Alphalabs, Eastleigh, UK).

After dextran had facilitated erythrocyte sedimentation, the supernatant leucocyte-rich plasma was aspirated with a 3ml Pasteur pipette and layered onto the Percoll® density gradient. The 15ml tube was then centrifuged at 220g and 20°C for 20 minutes with minimal acceleration and no brake (Eppendorf AG 5810R centrifuge,

Hamburg, Germany). This process separates leucocytes subsets into distinct layers. Diluted plasma is at the top, followed by an interphase of mononuclear cells. Below this is a layer of 56% Percoll®, then a neutrophil cellular interphase followed by the 80% Percoll® layer. The erythrocyte pellet was found at the base. The plasma and mononuclear cell layers were first removed using a 3 ml Pasteur pipette. Neutrophils were then aspirated from the 80% and 56% gradient interface using a fine bore Pasteur pipette (Alphalabs, Eastleigh, UK) and transferred to a sterile 15ml centrifuge tube. The remainder of the tube was filled with PBS, before being centrifuged at 1500g for 5 minutes at room temperature. The supernatant was then discarded and the neutrophils re-suspended in RPMI 1640 (Sigma-Aldrich, Poole, UK). Cell count, viability and cyto-spin differential count were then performed as described in 3.3.5 and 3.3.6. Neutrophil purity using this method was consistently >96%.

Neutrophils were then re-suspended in a 5µM solution of CellTracker™ Deep Red fluorescent dye (ThermoFisher Scientific, Loughborough, UK) in warmed RPMI 1640 supplemented with 10% FBS, at a concentration of 4×10^6 cells /ml. Neutrophils were incubated for 45 minutes at 37°C and 5% CO₂ to allow intracellular uptake of the fluorescent dye. This dye is taken up into the cytoplasm of viable cells and metabolised by intra-cellular esterase enzymes into a compound which has a deep red excitation/emission spectra (630/650 nm maxima). These spectral characteristics are similar to that of allophycocyanin (APC). Staining with CellTracker™ Deep Red results in fluorescent retention for 72 hours and negligible cytotoxicity. From preliminary experiments, alveolar macrophages were found to be least auto-fluorescent in the APC emission spectrum (compared to FITC and PE); therefore labelling neutrophils with CellTracker™ Deep Red would minimize any interference

from macrophage auto-fluorescence in the efferocytosis assay. After incubation, the neutrophils were centrifuged at 1500g for 5 minutes at room temperature, following which the supernatant was discarded. The cells were then re-suspended at a concentration of 2×10^6 / ml in warmed serum-free RPMI containing 100U/mL penicillin and 100ug/mL Streptomycin. Neutrophils were then incubated at 37°C and 5% CO₂ for 24 hours to allow apoptosis.

Neutrophils are known to undergo apoptosis in a time-dependant manner. It has previously been shown by our group (Dr Dhruv Parekh, University of Warwick, 2016) that maximal apoptosis, as evidenced by cell surface expression of Annexin V, occurs after 24 hours. This was confirmed by undertaking a flow cytometry assay of the time course of healthy neutrophil apoptosis using FITC Annexin V and 7AAD as previously described in section 3.3.7. Cells that were Annexin V positive were counted to calculate the percentage of total apoptosis at 20 and 24 hours. By 20 hours 78.1% neutrophils were apoptotic, and by 24 hours 92.8% of neutrophils were apoptotic. Very few cells were necrotic (<2% 7-AAD only positive) at any time point. These results correlated with the validation studies previously undertaken by our group. The flow cytometry findings were also confirmed by assessing the neutrophils under 400x magnification for morphological changes associated with apoptosis (Figure 3.1), including nuclear fragmentation and cytoplasmic vacuolation(258). Morphological analysis showed 76% apoptosis after 20 hours and 94% apoptosis after 24 hours, in keeping with the results from flow cytometry. Neutrophils were therefore incubated for 24 hours to achieve maximal apoptosis, prior to use in efferocytosis assays.

Neutrophil activation was minimised as far as possible during isolation by minimising agitation of cells. Neutrophil isolation commenced within 10 minutes of blood sample

collection. The duration of neutrophil exposure to Percoll® was minimised and neutrophils were immediately washed using PBS following the Percoll® stage. Neutrophil activation could be assessed by flow cytometric detection of a CD66b⁺ / CD11b⁺ phenotype(262). However, the main purpose of the neutrophils was to undergo apoptosis after 24 hours, then to be used in efferocytosis assays. The neutrophils were consistently found to be >90% apoptotic after 24 hours in both this study and previous studies (see above), therefore I did not test for activation post-isolation.

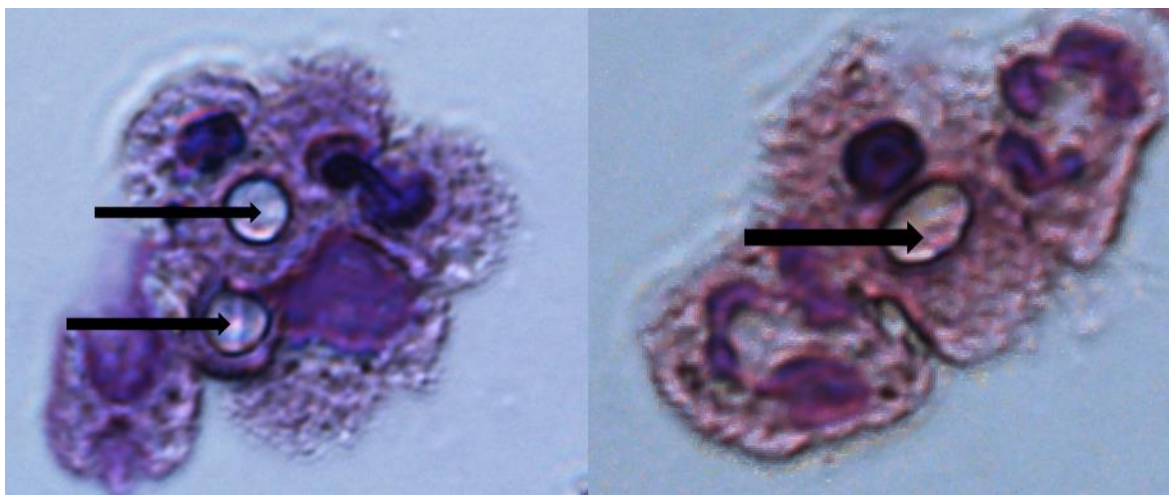


Figure 3.1: Apoptotic neutrophils showing vacuolation

Apoptotic neutrophils were labelled using Diff-Quik and viewed under 400x magnification using a bright field microscope (Zeiss AxioVert A1, Zeiss, Germany). Arrows show vacuole formation in the cytoplasm of apoptotic neutrophils. Nuclear condensation is also visible

Quantification of Efferocytosis

For efferocytosis assays, alveolar macrophages were cultured at a concentration of 2.5×10^5 per well in 24-well plates. A macrophage-only control well to which

neutrophils would not be added, was identified. For the negative control well, 5µg/ml Cytochalasin D (Sigma-Aldrich) was added to permanently inhibit actin filament polymerisation, thus inhibiting future macrophage efferocytosis. Plates were then incubated for 30 minutes in a 5% CO₂, 37°C atmosphere to allow the cytochalasin D to act, before all media was removed and wells washed with PBS. The tube containing CellTracker™ Deep Red stained apoptotic neutrophils at a concentration of 2 x10⁶ cells/mL was agitated to re-suspend the cells. The suspension was then added directly to the macrophages at 500µl per well, thereby flooding the cultured macrophages with four fold excess of apoptotic neutrophils. All wells (including the cytochalasin D treated well) received apoptotic neutrophils, except the macrophage-only control well which received 500µl of RPMI 1640 media instead. Plates were incubated for 2 hours in a 5% CO₂, 37°C atmosphere to allow efferocytosis of neutrophils. The optimal assay duration of 2 hours was determined by time course experiments undertaken using alveolar macrophages derived from the lung resections of never-smoker patients (see Figure 3.2 below). The media was then removed and wells washed twice with ice-cold PBS to remove non-adherent and non-engulfed neutrophils. 500µl of TrypLE™ express was added to each well and incubated for 10 minutes at 37°C, 5% CO₂ atmosphere. Cells were visualised under a microscope to ensure that they had detached from the culture well floor. 500µl of RPMI 1640 supplemented with 10% FCS was then added to each well to quench the trypsin. The cell suspensions were then pipetted and transferred into 5ml polypropylene flow cytometry tubes (Falcon™, Becton Dickinson). For a neutrophil-only positive control, 1 x10⁶ stained apoptotic neutrophils were also added to a separate flow cytometry tube. All cells were centrifuged at 500g for 5 minutes at 4°C (Eppendorf AG 5810R centrifuge, Hamburg, Germany). Cells were then re-

suspended in 400µl of PBS supplemented with 1% bovine serum albumin (BSA, Sigma-Aldrich) and immediately placed on ice.

Cells were agitated on a vortex (Varimix, Sciquip, Newtown, UK) immediately prior to running on the flow cytometer (Accuri C6, BD Biosciences, USA). Analysis was performed using Accuri C6 software (BD Biosciences, USA). Tubes containing only alveolar macrophages or only stained apoptotic neutrophils were used to set gates for their respective cell populations on forward and side-scatter plots. The positive control tube containing only stained apoptotic neutrophils was also used to set a positive gate on the FL-4 channel which detects the allophycocyanin (APC) fluorescent spectrum; this gate was subsequently used to identify macrophages which had engulfed CellTracker™ Deep Red stained apoptotic neutrophils. A minimum of 5,000 events (cells gated as alveolar macrophages) were counted for each experimental condition and the percentage of APC-positive macrophages was calculated. The cytochalasin D treated macrophages (negative control) were used to determine the background level of fluorescence present due to stained apoptotic neutrophils adhering to the surface of macrophages, but not being engulfed (since Cytochalasin D inhibits efferocytosis). This background fluorescence value was subtracted from the percentage of APC-positive AMs in other experimental conditions, to give a corrected net efferocytosis index truly representative of neutrophil engulfment (see Figure 3.3).

Similar flow cytometric methods of quantifying efferocytosis have been published(263-266). This flow cytometric assay has been previously validated by our group by comparing it with direct fluorescent microscopy and found to have differences in results of less than 10% (Dr Chris Bassford, PhD thesis, University of Warwick, 2011). The validation experiments were repeated, and also found the

difference between flow cytometry and fluorescent microscopy (Zeiss LSM 510, Carl Zeiss AG, Germany) to also be less than 10% (see Figure 3.4). The intra-assay coefficient of variance (CV) was also re-assessed by measuring the efferocytosis index 6 times using AMs from the same subject, then dividing the standard deviation of measurements by their mean. The intra-assay CV was calculated as 3.3%.

Steps were taken to avoid bias, including drawing gates based on single cell populations (neutrophils and AMs) prior to assessing efferocytosis. However, a limitation of this assay was that it was not possible for me to be blinded to the sample, and it was not possible to always have an independent blinded colleague to run the assay.

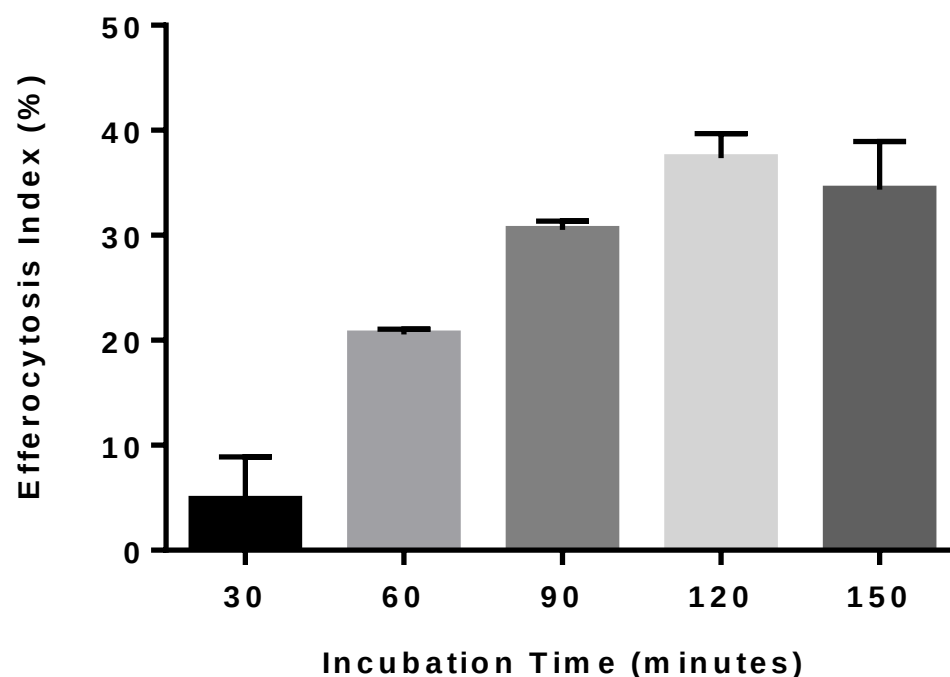
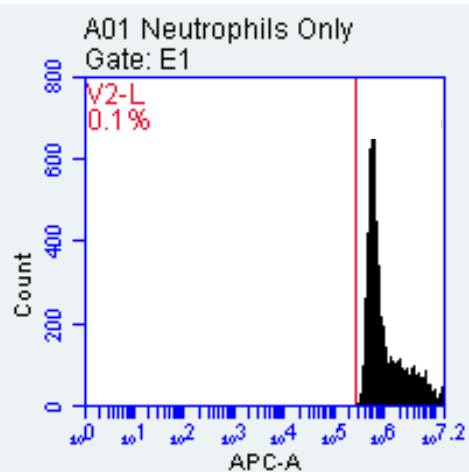
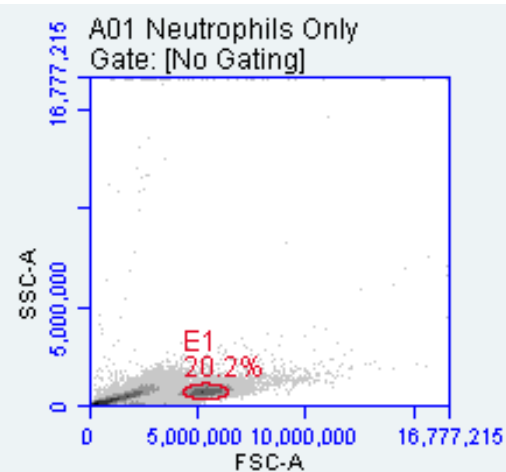
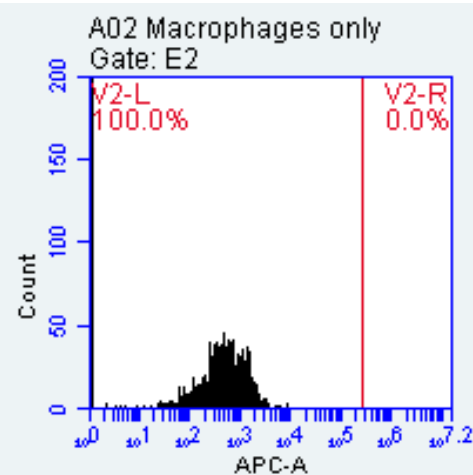
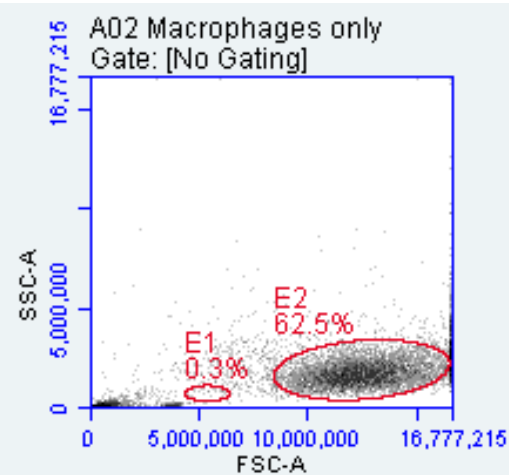


Figure 3.2: Time course of efferocytosis in alveolar macrophages.

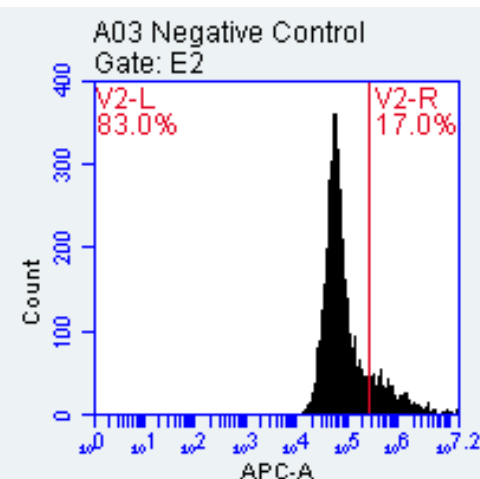
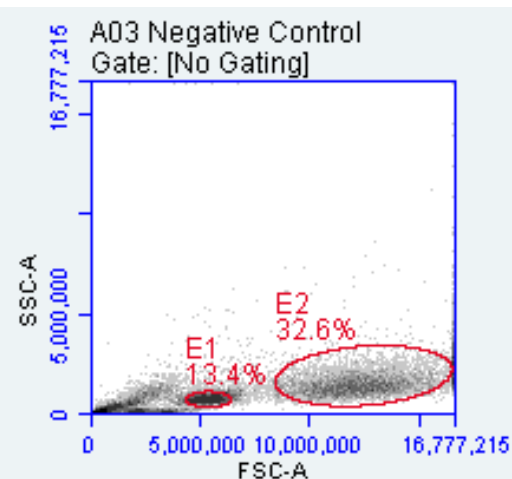
Stained apoptotic neutrophils were incubated with alveolar macrophages for different lengths of time. Alveolar macrophages were derived from lung resections of never-smoker patients, n=3.



A



B



C

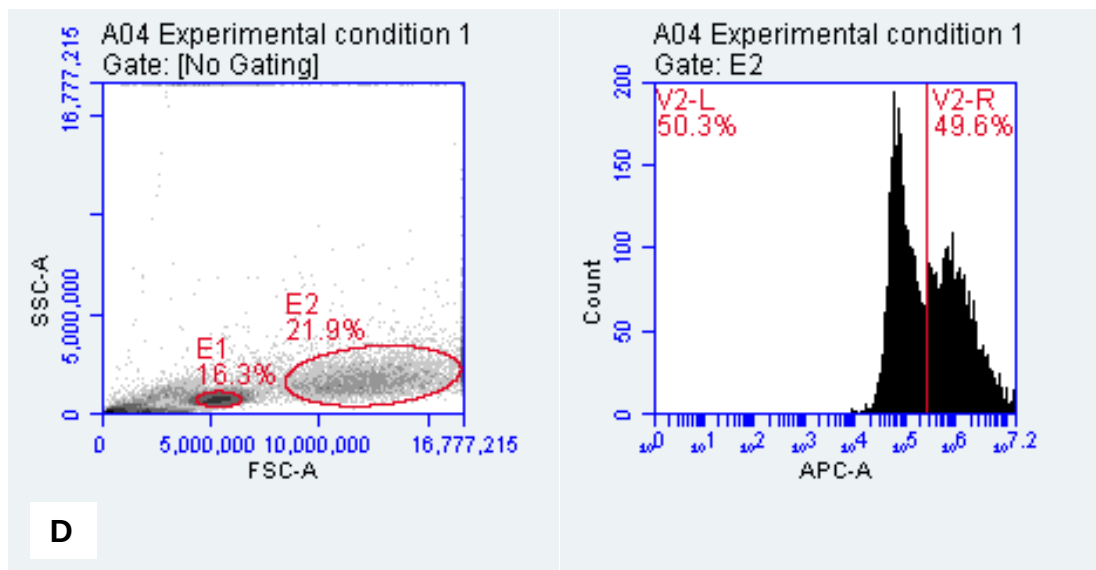


Figure 3.3: Example flow plots and histograms for efferocytosis assay

Forward vs side scatter plots and fluorescence histograms of control and test samples used to set up efferocytosis assay. Alveolar macrophages (AMs) used in this example are from the lung resection of a never smoking patient. Doublet discrimination was also performed on all samples but not shown. All fluorescence histograms are of the APC (FL4) channel, which corresponds to the emission spectra of CellTracker™ Deep Red. **A:** Stained apoptotic neutrophils were used to gate neutrophils (E1) on forward vs side scatter plot, and as a positive control used to determine the positive threshold on the APC fluorescence histogram. **B:** Tube containing AMs only used to gate macrophages on forward vs side scatter plot (E2), and used to confirm that AMs alone do not fluoresce above the positive threshold in the APC channel. **C:** Negative control (Cytochalasin D treated AMs incubated with neutrophils) in which efferocytosis has been inhibited. The APC fluorescence histogram has been gated on E2 (macrophage gate). Since efferocytosis has been inhibited, any fluorescence in the APC channel detected above the positive threshold is due to neutrophil adherence to the surface of AMs, and not engulfment. This background fluorescence value is 17%. **D:** Experimental condition (untreated AMs incubated with neutrophils). The APC fluorescence histogram has been gated on E2 (AM gate), and shows that 49.6% of AMs are APC-positive. However, the background fluorescence value of 17% from the negative control must be subtracted to determine the true efferocytosis index. The efferocytosis index for this sample would therefore be calculated as 32.6%.

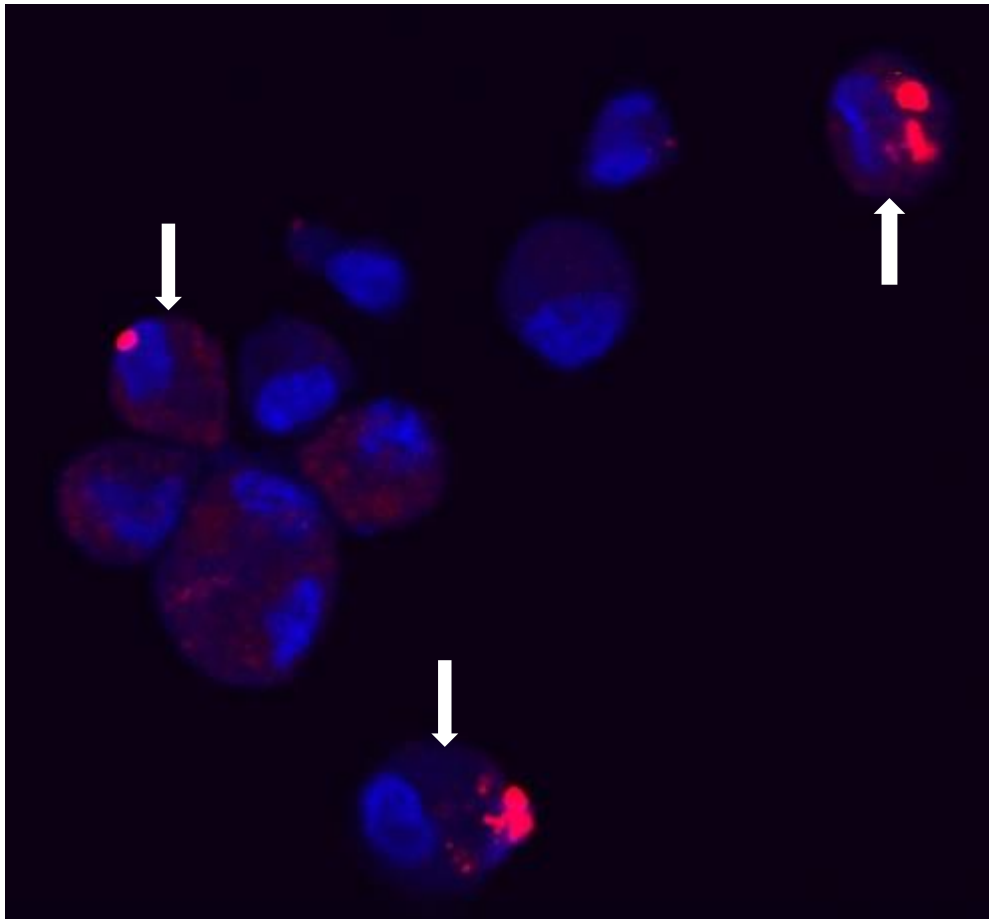


Figure 3.4: Fluorescent microscopy photograph of efferocytosis assay

This photograph shows cultured alveolar macrophages to which apoptotic neutrophils labelled with CellTracker™ Deep Red have been added. Alveolar macrophage nuclei are stained blue with DAPI. Arrows show alveolar macrophages which have engulfed apoptotic neutrophils.

3.4.2 Phagocytosis Assay

To assess macrophage phagocytosis, pHrodo™ red *E. Coli* and *S. Aureus* BioParticle® conjugates (ThermoFisher Scientific, UK) were used. These BioParticles® are killed bacteria conjugated to a pHrodo™ red dye, which increases in fluorescence as the pH of its surroundings becomes more acidic, peaking at pH 4. Therefore, in a phagocytosis assay, these BioParticles® will maximally fluoresce once they have been engulfed by a macrophage and enter a phagolysosome. The

excitation / emission maxima for pHrodo™ red dye are 560 / 585 nm. Prior to a phagocytosis assay, a vial containing 2mg of lyophilised BioParticles® was suspended in 2mls of Uptake Buffer (Hank's Balanced Salt Solution [HBSS] supplemented with 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] with pH adjusted to 7.4 using sodium hydroxide, all from Sigma-Aldrich).

Prior to this assay, alveolar macrophages were cultured with varying treatments in triplicate in 96-well black-walled clear-bottom plates at 37°C and 5% CO₂. Wells containing untreated macrophages were used for macrophage-only control and negative control. For the negative control wells, 5µg/ml Cytochalasin D (Sigma-Aldrich) was added to permanently inhibit actin filament polymerisation, thus inhibiting future macrophage phagocytosis. Plates were then incubated for 30 minutes in a 5% CO₂, 37°C atmosphere to allow the cytochalasin D to act, before all media was removed and wells washed with PBS. 50µl of RPMI 1640 supplemented with 10% FCS was then added to all wells. Vials of re-constituted pHrodo™ red BioParticles® were inverted several times and sonicated for 10 minutes, before adding 50µl of BioParticles® to all wells, except the macrophage-only wells which received 50µl of uptake buffer without BioParticles®. Plates were then incubated in the dark at 37°C and 5% CO₂ for 6 hours. Following incubation, wells were washed twice in 200µl PBS containing 1% BSA, then 100µl PBS/1% BSA was added to all wells. Plates were then read on a microplate reader (Synergy 2, Bio-Tek, USA) set at the excitation / emission spectra of pHrodo™ red dye: 560 / 585 nm. The negative control (cytochalasin D treated) macrophages were used to determine the background fluorescence present due to stained pHrodo™ red BioParticles® adhering to the outside of macrophages, but not being engulfed (since Cytochalasin D inhibits phagocytosis). This background fluorescence value was subtracted from

fluorescence values of other experimental conditions, to give corrected net fluorescence value representative of phagocytosis. For alveolar macrophages derived from BALF, the phagocytosis index of untreated macrophages was calculated and expressed as relative fluorescence units (RFU). For alveolar macrophages derived from lung resections, phagocytosis results from treated macrophages were expressed as fold change in RFU from untreated macrophages. The intra-assay CV was calculated measuring the phagocytic index 6 times using AMs from the same subject, then dividing the standard deviation of measurements by their mean. The intra-assay CV was calculated as 5.2%.

3.4.3 Alveolar Macrophage Treatments *In Vitro*

Alveolar macrophages (AMs) from the lung resections of never or long-term ex-smoker patients were plated at concentrations specified in section 3.3.8. Acellular BALF taken from 6 ARDS patients (sections 3.2.3 and 3.3.3) was pooled and mixed in a 1:1 ratio with RPMI-1640 including 10% FBS. To elicit functional changes associated with ARDS, the AMs were treated with this 50% ARDS BALF mixture. As a vehicle control treatment, AMs were also treated with a 1:1 mixture of 0.9% Saline (Baxter, UK) and RPMI-1640 including 10% FBS. Other treatments given in conjunction with 50% ARDS BALF or saline included 200nM Y-27632 dihydrochloride (Rho-associated protein kinase inhibitor, Apexbio, USA), 2 μ M SF1670 (Phosphatase and tensin homolog inhibitor, Selleckchem, USA), dimethyl sulfoxide (DMSO, vehicle control for Y-27632 and SF1670, Sigma-Aldrich), and 500ng/ml recombinant human high mobility group box-1 (HMGB-1, Abcam, UK). Other treatments not combined with 50% ARDS BALF or saline included 50ng/ml interferon- γ (Peprotech, UK), 1 μ g/ml lipopolysaccharide (Sigma-Aldrich), 40ng/ml

interleukin-4 (Peprotech, UK), and 40ng/ml interleukin-13 (Peprotech, UK). Efferocytosis, phagocytosis, HSD-1 functional assay and RNA extraction (sections 3.4.1, 3.4.2, 3.5 and 3.7.1) were performed 24 hours after treatment with 50% ARDS BALF. Phenotyping (section 3.4.4) was performed 48 hours after treatment. To assess the impact of tMSCs on restoring AM function after treatment with 50% ARDS BALF, a 6 hour Transwell co-culture of tMSCs with AMs (as described in section 3.4.5) was performed between removal of ARDS BALF and efferocytosis or phenotyping assays.

3.4.4 Phenotyping of Alveolar Macrophages

Prior to this assay, alveolar macrophages (AMs) were cultured with varying treatments in duplicate in 24-well plates at 37°C and 5% CO₂. On the day of this assay, cultured AMs had their media removed and were washed with PBS, before being incubated with 500µl of TrypLE™ express for 10 minutes at 37°C and 5% CO₂. AMs were visualised under a microscope to ensure that they had detached from the culture well floor. 500µl of RPMI 1640 supplemented with 10% FCS was then added to each well to quench the trypsin. The AM suspensions were then pipetted and transferred into 5ml polypropylene flow cytometry tubes. 2mls of FACS buffer (PBS supplemented with 1% Ethylenediaminetetraacetic acid [EDTA, Sigma-Aldrich] and 1% BSA) were added to each tube before centrifuging at 400g for 5mins (Eppendorf AG 5810R centrifuge, Germany). Supernatants were discarded and macrophages were re-suspended in 100µl of FACS buffer. Tubes containing untreated AMs were used for unstained control and isotype controls. AMs were stained with either antibody panel A or B as described in table 3.3, then vortexed and incubated on ice in the dark for 30 minutes. Two smaller panels were used instead of one large panel

to minimise the risk of spectral overlap, and to avoid using other channels in which AMs showed significant auto-fluorescence. Following incubation, tubes were washed twice with FACS buffer as described above, then re-suspended in 300µl of FACS buffer, before running immediately on a flow cytometer (Accuri C6, BD Biosciences, USA). Compensation and analysis was performed using Accuri C6 Software (BD Biosciences, USA). The AM population was gated on the forward and side scatter plot. The median fluorescence intensity (MFI) in relevant channels from isotype control AMs was subtracted from the MFIs of stained AMs, to give the net MFI for each antibody fluorophore. Results for treated AMs were expressed as a fold change in net MFI compared to untreated AMs.

Steps were taken to avoid bias, including drawing gates based on unstained and isotype control stained AMs prior to assessing phenotype. However, a limitation of this assay was that it was not possible for me to be blinded to the sample, and it was not possible to always have an independent blinded colleague to run the assay.

Panel	Antibody	Clone	Fluorophore	Manufacturer	Isotype Control	Concentration
A	Anti-human CD206	19.2	APC	BD Biosciences	Mouse IgG1 κ	1 : 20
	Anti-human CD80	L307.4	PE	BD Biosciences	Mouse IgG1 κ	1 : 20
	Anti-human CD163	GHI/61	FITC	BD Biosciences	Mouse IgG1 κ	1 : 20
B	Anti-human Mer	125518	APC	R&D Systems	Mouse IgG2B	1 : 20
	Anti-human SIRP α (CD163)	15-414	FITC	ThermoFisher	Mouse IgG1 κ	1 : 20
	Anti-human CD200R	OX108	PE	ThermoFisher	Mouse IgG2a κ	1 : 20

Table 3.3: Human antibody staining panels used on flow cytometry to assess alveolar macrophage surface marker expression

FITC: Fluorescein isothiocyanate. APC: Allophycocyanin. PE: Phycoerythrin. Mer: Mer Receptor Tyrosine Kinase. SIRP α : Signal-Regulatory Protein Alpha. All Isotype control antibodies were purchased from ThermoFisher, except for Mouse IgG1a κ , which was from BD Biosciences.

3.4.5 Transwell co-culture of HSD-1 transgenic mesenchymal stem cells with alveolar macrophages

Alveolar macrophages (AMs) from ARDS patients or lung resections were co-cultured with HSD-1 transgenic mesenchymal stem cells (tMSCs) prior to efferocytosis assays. The creation of tMSCs is described in chapter 5. AMs were cultured at 2.5×10^5 per well in RPMI 1640 containing 10% FBS in 24-well plates at 37°C and 5% CO₂. In the morning of the efferocytosis assay, wells were washed with PBS and media changed to 600µl serum-free RPMI 1640. Exogenous cortisone (Sigma-Aldrich) was added to give a final concentration of 10^{-7} M in all wells. Then, Transwell® permeable inserts containing a 0.4µm polycarbonate microporous membrane (Costar, Corning, USA) were added to each well. Then, 100µl of serum-free RPMI containing either 6.25×10^4 transgene-activated tMSCs, transgene-inactive tMSCs or no cells was added to the upper compartment. These Transwells® have previously been used for MSC and macrophage co-culture experiments(214, 215). The ratio of AMs to tMSCs was 4:1. Plates were then incubated for 6 hours at 37°C and 5% CO₂. The transwell inserts to which tMSCs had adhered to were removed, and the efferocytosis or phenotyping assay was performed as described above in sections 3.4.1 and 3.4.4. A diagram of the transwell co-culture experiments is shown in Figure 3.5.

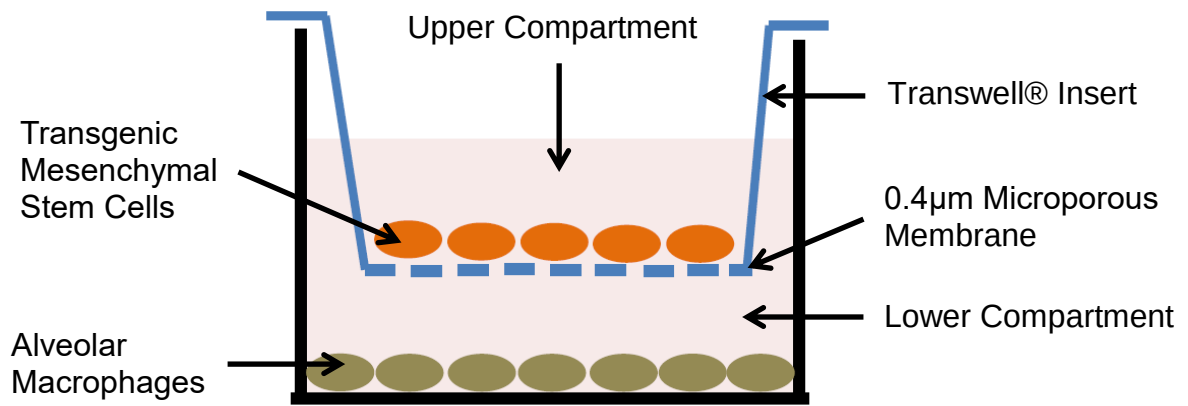


Figure 3.5: Diagram of alveolar macrophage and transgenic mesenchymal stem cell co-culture using Transwells®

3.5 11 β -Hydroxysteroid Dehydrogenase Type-1 Activity

Assay (Thin Layer Chromatography)

This assay was developed and has previously been validated by the Institute for Metabolism and Systems Research at the University of Birmingham(267, 268).

This assay was used to measure the reductase activity (conversion of cortisone to cortisol) and dehydrogenase activity (conversion of cortisol to cortisone) of HSD-1 within adherent cells. In 6-well plates, 100,000 adherent cells (alveolar macrophages or mesenchymal stem cells) were cultured per well. Duplicate wells were used for each different condition or cell type: 1 well for reductase and 1 well for dehydrogenase activity. Blank wells containing media only (no cells) were also prepared. The media used in this assay was dependant on cell type: RPMI1640 for macrophages and α MEM (Minimum Essential Media, ThermoFisher Scientific, UK) for mesenchymal stem cells.

To start the assay, culture media was removed from adherent cells in 6-well plates, and cells were washed with PBS to remove any remaining FBS (to prevent steroid contamination from serum). Then 1 ml of 'cold' (non-radioactive) cortisol or cortisone at 10^{-7} M concentration in serum-free media was added to each well, including the blank wells containing no cells. Then 3 μ l of 'hot' radioactive tritiated ^{3} H-cortisone or ^{3} H-cortisol (stock concentration 1.5 pM/ml, stock radioactivity 1.37 MBq/ml, NEN Radiochemicals, UK) were added to each well. Therefore, each well received a combination of either 'hot + cold' cortisol or 'hot + cold' cortisone. Plates were incubated at 37°C and 5% CO₂ for 6 - 12 hours. Supernatant media was transferred to glass tubes (Fisher Scientific, UK), then either analysed immediately or sealed

and frozen at -20°C for future analysis. Frozen samples were thawed at room temperature prior to analysis by thin layer chromatography.

Thin Layer Chromatography (TLC)

To start the analysis, 5ml dichloromethane (Sigma-Aldrich) was added to each tube, before rocking tubes for 5 minutes to elute steroids from aqueous to organic phase. Tubes were centrifuged at 350g for 15 minutes to separate the layers. The aqueous and interface (protein) layers were then aspirated using glass pipettes and discarded. Tubes were placed under air blowers in a heating block at 50°C for 1 hour, to evaporate the dichloromethane. Once the dichloromethane had been evaporated, steroids were re-suspended in 60µl of fresh dichloromethane. Silica plates (Sigma-Aldrich) were labelled and prepared by spotting 1 drop of 1mg/ml 'cold' mixed cortisol and cortisone (diluted in absolute ethanol) on each lane. This aided steroid separation and helped to visualize steroids under UV light at a later stage. Lanes are spaced at least 1.5 cm apart on the plate. Samples were then spotted on the silica plates using glass pipettes and capillary action. The silica plates were then placed in a glass tank containing 200mls TLC buffer (16mls ethanol mixed with 184mls chloroform), with samples at the bottom of the tank. A weighted lid was placed on top to prevent evaporation, and the plates were run in the TLC buffer at room temperature for 90 minutes. Plates were then removed, allowed to dry, and scanned for radioactivity (Bioscan 200, Washington, USA) for 10 minutes per sample. Relevant steroid peaks were identified by comparing with blank cortisol and cortisone controls. Steroid separation was also assessed manually under UV light: cortisone travels further and creates the top band, cortisol creates the lower band.

The percentage of steroid conversion was determined by adding together the radioactivity counts from the original steroid and metabolite, then calculating the percentage of the total count which was derived from the metabolite. HSD-1 activity was then calculated by multiplying percentage conversion by the original steroid concentration (10^{-7}M) and dividing by duration of steroid incubation in hours. HSD-1 activity is expressed in pM/hr/100,000 cells to allow direct comparisons. Intra-assay CV was calculated as 3.6%. Inter-assay CV was calculated from control conditions over 6 experiments. Inter-assay CV was calculated as 8.4%.

3.6 Mesenchymal Stem Cell Experiments

3.6.1 Purchase and standard culture of human bone marrow mesenchymal stem cells

Human Mesenchymal Stem Cells (MSCs) from 5 adult donors were purchased from the Institute of Regenerative Medicine at Texas A&M College of Medicine, USA. Donors were designated 8001 (24-year-old female), 8004 (22 year old male), 8013 (22-year-old male), 7083 (24 year old male) and 8011 (23 year old female). Prior to shipment, all cells had been characterised as MSCs by flow cytometric assessment of surface markers, and by both osteogenic and adipogenic differentiation. MSCs from all donors were at passage 2 (meaning the cells had been dissociated from a plastic surface on 2 successive occasions) prior to shipment. MSCs were cultured in α MEM (Gibco, ThermoFisher Scientific) containing 16% FBS, 100U/mL penicillin, 100ug/mL streptomycin and 2mM L-Glutamine, using T175 flasks (Nunc, ThermoFisher Scientific) at 37°C and 5% CO₂. MSCs from donor 8011 showed the most rapid expansion in vitro, and were therefore used in transfection experiments to create transgenic tMSCs, as described in chapter 5. MSCs from the other 4 donors were used for prolonged expansion experiments as described below.

3.6.2 Prolonged expansion of MSCs and conditioned media collection

MSCs from donors 8001, 8004, 8013 and 7083 were used for prolonged expansion experiments. MSCs were passaged every 4 days: this involved aspiration of

supernatant media, washing in 20mls PBS, then incubating with 6mls TrypLE™ express for 5 minutes at 37°C and 5% CO₂ to allow detachment of MSCs. Then 6mls of FBS-containing αMEM was added to each flask to quench the trypsin. Suspended MSCs were transferred to a 15ml falcon tube (ThermoFisher Scientific, USA) and centrifuged at 300g for 5 minutes. Supernatant media was discarded, and MSCs re-suspended in fresh αMEM before being counted as described in section 3.3.5. MSCs would then be returned to T175 flasks for standard culture in αMEM containing 16% FBS.

At alternate passages, 5x10⁵ MSCs were seeded into each of two T75 flasks (Nunc, ThermoFisher Scientific) and cultured in αMEM containing 5% FBS. One T75 flask received “cytomix”, a cocktail of pro-inflammatory mediators: 50ng/ml interferon-γ, 50ng/ml tumour necrosis factor-α, 50ng/ml interleukin-1β (all from Peprotech, UK). At the same time, 5x10⁵ MSCs were taken for direct co-cultured experiments with THP-1 macrophages, as described in section 3.6.3. The remainder of MSCs were returned to standard culture in a T175 flask containing αMEM with 16% FBS. After 24 hours culture at 37°C and 5% CO₂, conditioned media (CM) was aspirated from the T75 flasks and 6-well plates. CM was centrifuged at 500g to pellet any debris, before the supernatant was aliquoted and frozen at -20°C. Stimulated and unstimulated MSCs remaining in T75 flasks were used for RNA extraction as described in section 3.7.1. MSCs from 4 donors were expanded in this way for one month, with collections of CM and RNA at 4 equally spaced time-points / passages. After one month of *in vitro* culture, MSC bi-lineage differentiation and surface marker expression were assessed (described in sections 3.6.4 and 3.6.5). The purpose of this was to determine whether the remaining MSCs had differentiated or had

maintained a pluripotent MSC phenotype. A timeline of the MSC expansion experiments is shown in figure 3.6.

The FBS content of α MEM was reduced to 5% for stimulated and unstimulated MSC-CM collection, since the MSC-CM would later be used in wound healing assays (described in section 3.6.6), where a higher FBS concentration would cause overly rapid wound closure. Quantification of selected MSC paracrine factors within MSC-CM was performed using enzyme-linked immunosorbent assays and prostaglandin E2 parameter assays as described in sections 3.8.2 and 3.8.3.

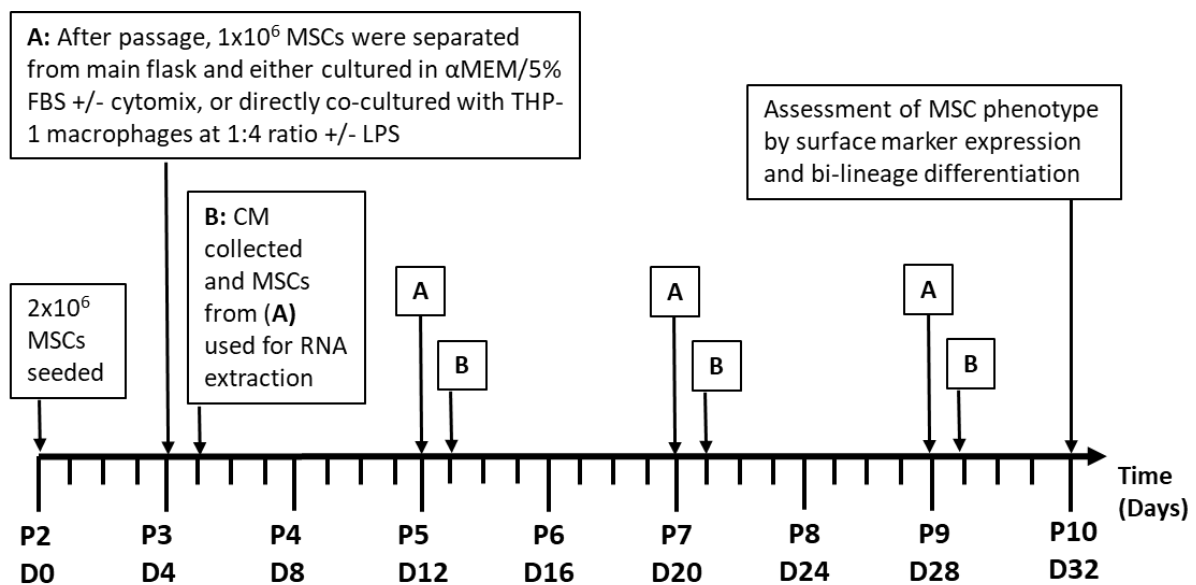


Figure 3.6: Timeline of prolonged MSC expansion experiments

MSCs not used for (A) continued to be cultured in the main flask under standard conditions (α MEM + 10% FBS). P = passage number. D = Days in culture. α MEM = α minimal essential media. FBS = foetal bovine serum. MSC = mesenchymal stem cell. CM = conditioned media. LPS = 100ng/ml Lipopolysaccharide. Cytomix = a cocktail of pro-inflammatory mediators: 50ng/ml interferon- γ , 50ng/ml tumour necrosis factor- α and 50ng/ml interleukin-1 β .

3.6.3 THP-1 macrophage direct co-culture with mesenchymal stem cells

The THP-1 monocytic cell line (Sigma-Aldrich, UK) was cultured in RPMI-1640 including 10% FBS, using T75 flasks, at 37°C and 5% CO₂. To differentiate into macrophages, 1x10⁶ THP-1 cells were cultured per well in a 6-well plate, in the presence of 100ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 hours. During this time cells would become adherent as they differentiated. Media was then changed and cells rested for a further 72 hours in RPMI 1640 including 10% FBS before being used in co-culture experiments(269). THP-1 derived macrophages were used since they would provide a consistent response to inflammatory stimulation, as opposed to alveolar macrophages whose responses varied between patients.

Following MSC passage as described in section 3.6.2, media was removed from THP1-macrophages and 2.5x10⁵ MSCs in 2mls RPMI 1640 / 10% FBS were added to wells in duplicate. This gave a 1:4 ratio of MSCs to THP-1 macrophages. Media was also changed on control wells containing THP-1 macrophages only in duplicate. One co-culture well and one macrophage-only well were treated with 100ng/ml Lipopolysaccharide (LPS, Sigma-Aldrich, UK). Cells were cultured for 24 hours at 37°C and 5% CO₂, before CM was aspirated from wells. CM was centrifuged at 500g to pellet any debris, before the supernatant was aliquoted and frozen at -20°C.

3.6.4 Adipogenic and osteogenic differentiation of mesenchymal stem cells

The ability to undergo adipogenic and osteogenic differentiation was assessed in MSCs which had undergone prolonged expansion (section 3.6.2) and in transgenic MSCs (chapter 5). MSCs or transgenic MSCs were plated at a density of 6×10^4 cells/well in a 24 well tissue culture plate and incubated with α MEM including 16% FBS for 24-72 hours at 37°C and 5% CO₂, until reaching 80-90% confluency (for adipogenic differentiation) or 100% confluency (for osteogenic differentiation). MSCs were subsequently incubated with MSC adipogenic differentiation medium (StemPro, Life Technologies), MSC osteogenic differentiation medium (StemPro, Life Technologies) or α MEM including 16% FBS as a negative control. MSCs were incubated for 14 days (adipogenic differentiation) or 21 days (osteogenic differentiation) with media changed every 3 days, before staining.

For adipocyte detection, a 0.3% stock solution of Oil Red O (Sigma-Aldrich) in isopropanol was made. MSCs were washed with PBS, then 1ml 10% neutral buffered formalin added to each well for 30mins at room temperature to fix the cell monolayer. 3 parts stock solution of Oil Red O were diluted with 2 parts distilled water. Formalin was removed and the cell monolayer washed with distilled water. The water was aspirated and 1ml 60% isopropanol added to each well for 5 minutes at room temperature. The isopropanol was then aspirated and 1ml diluted Oil Red O solution added to each well for 15 minutes at room temperature. The Oil Red O solution was then aspirated and the wells washed with distilled water. Once the water became clear, PBS was added to each well and photographs were taken via a microscope (Zeiss AxioVert A1, Zeiss, Germany). Adipogenic differentiation was

evidenced by the appearance of lipid inclusion vacuoles within the cells, which take up the neutral lipid oil red O.

For osteocyte detection, HCL or NH₄OH was added to a 2% solution of Alizarin Red S (Sigma-Aldrich) until the pH reached between 4.1-4.3. The solution was then filtered and stored in the dark. Cells were washed with PBS, then 1ml 10% neutral buffered formalin added to each well for 30mins at room temperature to fix the cell monolayer. Formalin was removed and the cell monolayer washed with distilled water. The water was aspirated 1ml of 2% of Alizarin Red S solution was added to each well for 45 minutes at room temperature. The Alizarin Red S solution was then aspirated and the wells washed with distilled water 4 times. PBS was added to each well and photographs were taken via a microscope. Osteogenic differentiation was evidenced by staining of extracellular calcium deposits, which take up the Alizarin Red S Stain.

3.6.5 Flow Cytometric assessment of mesenchymal stem cell surface markers

Flow cytometric assessment of mesenchymal stem cell surface markers was assessed in MSCs which had undergone prolonged expansion (section 3.6.2) and in transgenic MSCs (chapter 5). According to the International Society of Cell Therapy(181), to be classified as MSCs, cells must express CD105, CD73 and CD90, and lack expression of surface markers including CD45, CD34, CD14, CD19 and HLA-DR. MSCs were dissociated using TrypLE express as described in section 3.7.1. MSC suspensions were then pipetted and transferred into 5ml polypropylene

flow cytometry tubes. 2mls of FACS buffer were added to each tube before centrifuging at 400g for 5mins (Eppendorf AG 5810R centrifuge, Germany). Supernatants were discarded and MSCs were re-suspended in 100µl of FACS buffer. Tubes were allocated for unstained control and isotype controls. MSCs were stained with either a positive or negative cocktail antibody panel as described in table 3.4, then vortexed and incubated on ice in the dark for 30 minutes. Following incubation, tubes were washed twice with FACS buffer as described above, then re-suspended in 300µl of FACS buffer, before running immediately on a flow cytometer (LSR Fortessa X-20, BD Biosciences, USA). Compensation and analysis was performed using FACSDiva Software (BD Biosciences, USA).

Steps were taken to avoid bias, including drawing gates based on unstained and isotype control stained MSCs prior to assessing phenotype. However, a limitation of this assay was that it was not possible for me to be blinded to the sample, and it was not possible to always have an independent blinded colleague to run the assay.

	Antibody	Clone	Fluorophore	Manufacturer	Isotype Control	Dilution
Positive Cocktail	Anti-human CD73	AD2	BV421	BD Biosciences	Mouse, IgG1k	1 : 20
	Anti-human CD105	266	BV786	BD Biosciences	Mouse, IgG1k	1 : 20
	Anti-human CD90	5E10	PE	ThermoFisher	Mouse, IgG1k	1 : 20
Negative Cocktail	Anti-human CD14	61D3	APC	ThermoFisher	Mouse, IgG1k	1 : 20
	Anti-human CD19	H1B19	APC	BD Biosciences	Mouse, IgG1k	1 : 20
	Anti-human CD34	4H11	APC	ThermoFisher	Mouse, IgG1k	1 : 20
	Anti-human CD45	HI30	APC	ThermoFisher	Mouse, IgG1k	1 : 20
	Anti-human HLA-DR	LN3	APC	ThermoFisher	Mouse, IgG2Bk	1 : 20

Table 3.4 Human antibody staining panel used on flow cytometry to assess mesenchymal stem cell surface marker expression.

To confirm a mesenchymal stem cell (MSC) phenotype, cells must show expression of CD73, CD105 and CD90. Cells must also lack expression of CD14, CD19, CD34, CD45 and HLA-DR. FITC (Fluorescein isothiocyanate) was not used in the panel since transgenic MSCs express Green Fluorescent Protein (GFP), which shares very similar excitation and emission spectra to FITC. BV: Brilliant Violet. APC: Allophycocyanin. PE: Phycoerythrin. HLA: Human Leukocyte Antigen.

3.6.6 Wound healing experiments

To assess the ability of mesenchymal stem cell conditioned media (MSC-CM) to promote wound healing, a monolayer of A549 cells (an adenocarcinomic human alveolar basal epithelial cell line) was used to mimic alveolar epithelium. Horizontal lines were drawn on the bottom of 24-well culture plates. This made it possible to consistently find the same location of a vertical wound under the microscope. In each well, 100,000 A549 cells were seeded with 500 μ l DMEM (Dulbecco's Modified Eagle Media, Sigma-Aldrich) including 10% FBS, 100U/mL penicillin, 100ug/mL streptomycin and 2mM L-Glutamine. Plates were incubated at 37°C and 5% CO₂ for 24-48 hours. Once confluent, cells were washed in PBS and media changed to serum-free DMEM to achieve a quiescent cellular state. After 2 hours further incubation, a vertical wound was made down the middle of each well using a 1000 μ l pipette tip. Each well was then washed with 1ml PBS to remove any detached cells. 500 μ l of MSC-CM or control media (α MEM including 5% FBS) was then added. The specific area where the vertical wound in each well intersected with the drawn horizontal lines was photographed using a camera and microscope (Zeiss AxioVert A1, Zeiss, Germany). These photos were recorded as 0hrs and the size of the wound quantified using Image J software (NIH, USA). If any wound was >20% greater or smaller than the mean wound area at 0hrs, then the data from that well was discounted. The plates were incubated for a further 6 hours, then a second set of photographs were taken of the wounds at the same positions, as shown in Figure 3.7. The percentage wound closure was calculated for every well. Wound closure values from control media were subtracted from conditioned media values. Intra-assay CV was calculated as 5.8%. Inter-assay CV was calculated as 9.4%.

Other studies have used a 24 hour duration for wound healing using an A549 monolayer(270). However, during preliminary experiments I found that the wound was closing up after 24 hours, therefore a shorter time course of 6 hours was chosen. The cause of the more rapid closure is not clear, but could have been due to a further mutation within the A549 cell line used within our laboratory. Contamination with another cell type is an unlikely but potential alternative explanation.

Steps were taken to avoid bias, including allocating a number to each photograph so I would be blinded to the treatment whilst calculating wound size. A limitation of this assay was that it was not possible to always have an independent blinded colleague to calculate wound size.

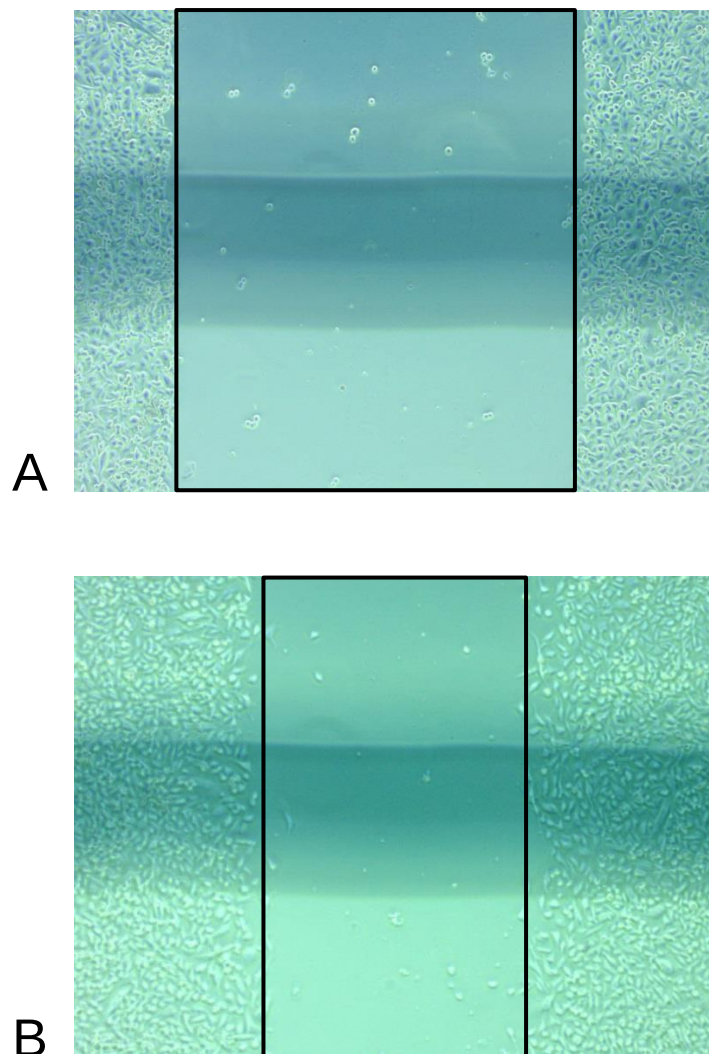


Figure 3.7: Photographs of wound healing assay using A549 cell monolayer

A: Photograph taken at time 0, immediately after wound formation. **B:** Photograph taken after 6 hours incubation with conditioned media at 37°C and 5% CO₂. Wound area (black rectangles) measured using Image J software, and percentage closure calculated.

3.7 Assessment of Gene Expression

3.7.1 RNA Extraction

RNA was isolated from Alveolar Macrophages (AMs) and Mesenchymal Stem Cells (MSCs), using the Nucleospin RNA kit (Machery-Nagel, Germany). Initially, AMs in 6-well culture plates or MSCs in T75 flasks were washed with PBS, before being incubated with TrypLE™ express for 10 minutes (AMs) or 5 minutes (MSCs) at 37°C and 5% CO₂. 1ml per well of TrypLE™ express was used for 6-well plates and 5mls for T75 flasks. Cells were visualised under a microscope to ensure that they had detached from the culture well floor. An equal volume of RPMI 1640 (AMs) or α MEM (MSCs) supplemented with 10% FCS was then added to each well to quench the trypsin. Cell suspensions were transferred to 15ml centrifuge tubes, before being centrifuged at 500g for 5 minutes. Supernatants were discarded and the pellet was re-suspended in 350 μ l RA1 buffer (Machery-Nagel) plus 3.5 μ l β -mercaptoethanol (Sigma-Aldrich). Samples were vortexed vigorously to facilitate cell lysis. The lysate was then placed into a Nucleospin® filter and centrifuged at 11,000g for 1 minute. 350 μ l of 70% ethanol was then added to the filtered lysate and mixed to adjust RNA binding conditions. Lysates were then transferred to a Nucleospin® RNA column and centrifuged at 11,000g for 30 seconds to bind RNA to the column silica membrane. To dry the membrane, 350 μ l of Membrane Desalting Buffer (Machery-Nagel) was then added to the column prior to centrifuging at 11,000g for 1 minute. A DNase reaction mixture was made by adding 10 μ l re-constituted rDNase (Machery-Nagel) to 90 μ l DNase reaction buffer (Machery-Nagel). 95 μ l of the DNase reaction mixture was then added directly to the column membrane, and incubated at room temperature for 15 minutes. The membrane was then washed with 200 μ l buffer

RAW2 (Machery-Nagel) for 30 seconds, then 600µl buffer RAW3 (Machery-Nagel) for 30 seconds, then 250µl buffer RAW3 for 2 minutes (all washes centrifuged at 11,000g). RNA was then eluted from the membrane into a nuclease-free collection tube using 60µl nuclease-free water and centrifuging at 11,000g for 1 minute. The RNA quantity was assessed immediately with the NanoDrop 2000 UV-Vis Spectrophotometer (ThermoFisher Scientific, USA) according to the manufacturer's instructions. 260/280 and 260/230 nm absorbance ratios of 1.8–2.0 indicated a pure RNA sample. RNA samples were frozen at -80°C immediately after NanoDrop quantification.

3.7.2 One-Step Reverse Transcriptase Quantitative Polymerase Chain Reaction

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is an analysis technique used to determine relative gene expression. Use of the One-Step Quantifast Probe RT PCR Kit (Qiagen, Germany) allowed RNA samples to be converted to cDNA using reverse transcriptase, then immediately run on PCR. RNA samples isolated as described in section 3.7.1 were thawed, diluted to 2.5ng/µl using ddH₂O and stored on ice. All other Quantifast kit reagents were stored on ice. For each well to be used, 2.5µl Quantifast Master Mix, 0.05µl Quantifast Reverse Transcriptase (RT) Mix, 0.4µl Taqman target primer on FAM-MGB (Applied Biosystems), 0.4µl 18S rRNA endogenous control primer on VIC-MGB (Applied Biosystems), and 1.65µl ddH₂O were mixed in a RNase-free microfuge tube (Ambion, UK). 5µl of this mixture was added to each well of a 384-well thin-wall Hard-Shell PCR plate (BioRad, USA). Then, 1µl of template RNA at 2.5 ng/µl was

added to each well in triplicate. Plates were run on a CFX384 Touch™ Real-Time PCR Detection System (BioRad, USA). Standard PCR conditions were used, with an initial 10 minutes at 50°C for the reverse transcription step. Then 5 minutes at 95°C (PCR activation step), followed by 40 cycles of: 10 seconds at 95°C (denaturation step) and 30 seconds at 60°C (combined annealing / extension step). The resulting data was then analysed using CFX Maestro software (BioRad, USA). Relative quantification of target gene mRNA was calculated relative to expression of control gene 18S rRNA. Commercially available Taqman® gene expression assays (ThermoFisher Scientific, USA) were purchased for the following genes: 18S, LL-37, VEGF, ANG-1, FGF7, PTGES2, ADIPOQ, SP7, SOX9, MYOD1, RHOA, RAC1, HSD11B1.

3.8 Biochemical Assays

3.8.1 Bicinchoninic acid Assay for protein concentrations

Protein concentration in human and murine BALF, plasma and cell lysates was measured using the Pierce™ BCA (Bicinchoninic Acid) Protein Assay Kit (ThermoFisher Scientific) which allows colorimetric detection and quantification of protein. Bovine Serum Albumin (BSA) standards were made by diluting stock BSA (at 2mg/ml) in ddH₂O to give 8 standards: 2000µg/ml, 1500µg/ml, 1000µg/ml, 750µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 25µg/ml and 0µg/ml (blank). In a 96-well microplate, 25µl of standards and diluted samples were added to appropriate wells in duplicate. The BCA Working Reagent (WR) was made by mixing BCA reagents A:B in a 50:1 ratio (ThermoFisher Scientific). 200µl of WR was then added to each well, before shaking plate for 30 seconds. Plates were then incubated at 37°C for 30mins in the dark. Plates were read at 570nm on a microplate reader (Synergy HT, BioTek, USA). Blank well mean absorbance values were subtracted from the values of standards and samples, before the BSA standard curve was plotted. Sample protein content was calculated by interpolating the standard curve and correcting for sample dilution factor. Protein permeability index was calculated as the ratio of BALF protein to plasma protein. Intra-assay CV was calculated as 7.1%. Inter-assay CV was calculated as 8.7%.

3.8.2 Enzyme Linked Immuno-Sorbent Assays (ELISAs)

Human ELISA DuoSet kits (R&D Systems) were used to measure concentrations of Angiopoetin-1, Vascular Endothelial Growth Factor (VEGF), Keratinocyte Growth

Factor (KGF), and interleukin-10 (IL-10) in mesenchymal stem cell (MSC) conditioned media. IL-10 concentrations were also measured in conditioned media from macrophage culture and MSC-Macrophage co-culture experiments. DuoSet ELISA kits contained standards, capture antibody, detection antibody and streptavidin-HRP (Horseradish Peroxidase) with dilutions varying between kits as shown in table 3.5. All DuoSet ELISAs followed the same standard protocol: Capture antibody was diluted to the working concentration in PBS and used to coat a 96-well high-binding microplate (Grenier Bio-One, Austria) with 100µl per well. Plates were then sealed and incubated overnight at 4°C. The following morning, plates were washed three times using 400µl Wash Buffer (0.05% Tween® 20 in PBS) per well. Any remaining wash buffer was removed by blotting against paper towels. Plates were then blocked by adding 300µl Reagent Diluent (1% BSA in PBS, 0.2µm filtered) to each well and incubating at room temperature for 1 hour on a plate shaker. An 8-point standard curve was made using 2-fold serial dilutions of the recombinant human target protein (e.g. VEGF) standard in Reagent Diluent. Plates were washed again as above, before adding 100µl of samples or standards in Reagent Diluent per well in duplicate. Background control samples of media (which had not been conditioned by cells) were also added to wells. Plates were incubated for 2 hours at room temperature on a shaker, before being washed as described above. 100µl of Detection Antibody diluted in Reagent Diluent was then added to each well, and plates incubated for 2 hours at room temperature on a shaker. Plates were washed again, before 100µl of a working dilution of Streptavidin-HRP was added to each well. Plates were then incubated in the dark for 20 minutes, before being washed again. Then, 100µL of Tetramethylbenzidine (TMB, Sigma-Aldrich, UK) substrate was added to each well. The plate was incubated for 20 minutes at room

temperature in the dark. 50µL of Stop Solution (1M HCl) was then added to each well. Within 10 minutes, the absorbance values of each well were determined using a microplate reader (Synergy HT, Biotek) set to 450 nm and 570nm. The readings at 570 nm were subtracted from readings at 450 nm to correct for optical imperfections in the plate. Sample concentrations were extrapolated from the appropriate standard curve, before background concentrations were subtracted. Values were then corrected for volume of media and number of cells.

The lower limit of detection, intra-assay CV and inter-assay CV were re-assessed for each ELISA kit (see table 3.6).

	Capture Antibody	Detection Antibody	Standard	Streptavidin-HRP
Angiopoetin-1	4.0 µg/ml	200 ng/ml	156 – 10,000 pg/ml	200-fold dilution
VEGF	1.0 µg/ml	100 ng/ml	31.2 – 2000 pg/ml	40-fold dilution
KGF	2.0 µg/ml	200 ng/ml	31.2 – 2000 pg/ml	200-fold dilution
IL-10	2.0 µg/ml	75 ng/ml	31.2 – 2000 pg/ml	40-fold dilution

Table 3.5: Working concentrations of reagents in DuoSet ELISA kits

	Intra-Assay CV (%)	Inter-Assay CV (%)
Angiopoetin-1	2.8	8.4
VEGF	4.2	10.4
KGF	3.2	9.8
IL-10	5.1	9.3

Table 3.6: DuoSet ELISA validation statistics

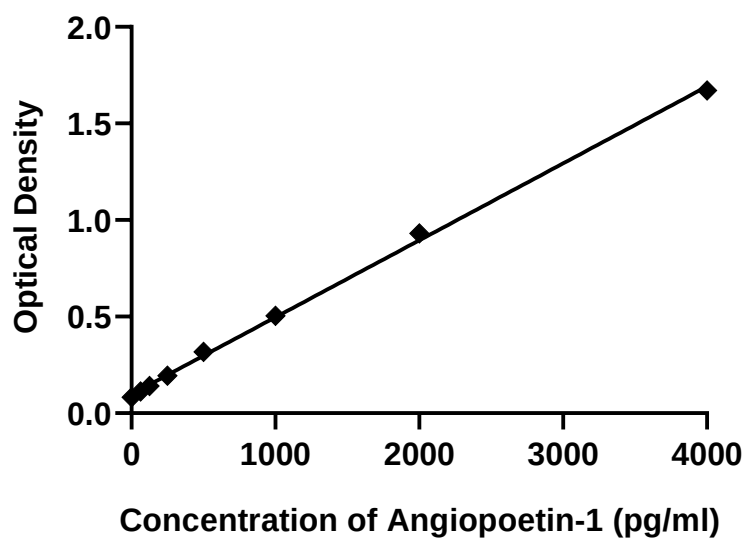


Figure 3.8: Representative standard curve for human Angiopoetin-1 ELISA

3.8.3 Prostaglandin E2 Parameter Assay

Parameter assay kits (R&D Systems, UK) used a sequential competitive enzyme immunoassay to measure concentrations of Prostaglandin E2 (PGE₂) in conditioned media from MSCs, macrophages and MSC-Macrophage co-culture experiments. All reagents described below were contained in the assay kit. Preliminary experiment determined that conditioned media samples required a 50-fold dilution for use in this assay. An 8-point standard curve (with a maximum standard of 2500pg/ml) was made using 2-fold serial dilutions of the PGE₂ standard in Calibrator Diluent RD5-56. 150µl of samples or standards in Calibrator Diluent were added in duplicate to wells in a microwell plate coated with a goat anti-mouse polyclonal antibody. 50µl of primary antibody solution was added to wells containing samples or standards. 200µl of Calibrator Diluent was added to non-specific binding wells. Plates were sealed and incubated for 1 hour at room temperature on a plate shaker. Then, 50µl of PGE₂ conjugate was added to each well. Plates were then incubated for 2 hours at room temperature on a shaker. Wells were then washed 4 times using Wash Buffer. 200µl of Substrate Solution was added to all wells, before incubating the plate in the dark at room temperature for 30 minutes. 100µl of Stop Solution was then added to each well, and plates gently tapped to allow mixing. Within 10 minutes, the absorbance values of each well were determined using a microplate reader (Synergy HT, Biotek) set to 450 nm and 570nm. The readings at 570 nm were subtracted from readings at 450 nm to correct for optical imperfections in the plate. A standard curve was created by generating a four parameter logistic curve-fit on Prism software (GraphPad, USA). Sample concentrations were extrapolated from the standard curve, before background concentrations were subtracted. Values were then corrected for volume

of media and number of cells. Lower limit of detection was calculated as 67.3 pg/ml. Intra-assay CV was calculated as 6.1%. Inter-assay CV was calculated as 11.3%.

3.8.4 Luminex Performance Assay for cytokine quantification

Human plasma and BALF inflammatory cytokines (IL-6, IL-8, TNF- α , IL-1 β , MCP-1, IL-10, IL-1ra and VEGF) were measured by a commercially available custom Magnetic Luminex® Performance Assay (R&D Systems, UK). The assay was performed as per manufacturer's instructions. Luminex® assays are calibrated against highly purified recombinant human cytokines produced at R&D Systems. This assay uses magnetic microparticles which have been pre-coated with capture antibodies specific to different analytes; this allows measurement of multiple cytokines in samples simultaneously. An 8-point standard curve was made using 3-fold serial dilutions of the standard cocktails provided with calibrator diluent. Plasma and BALF samples were also diluted in calibrator diluent. 50 μ L of microparticles were added to each well of a 96 well microplate followed by 50 μ L of standards or sample. Plates were then sealed with an opaque cover and left to incubate for 3 hours at room temperature on a microplate shaker at 800rpm. Following incubation, plates were washed 3 times with 100 μ L of wash buffer using an automated magnetic plate washer (Bioplex Pro™, Bio-rad, Hercules, USA) to ensure the magnetic beads were not washed away. 50 μ L of diluted biotin antibody cocktail was added to all wells and plates incubated as above for 1 hour. After a further wash step, 50 μ L of Streptavidin-PE was added to all wells and plates incubated as above for 30 minutes. After a further wash step, 100 μ L of wash buffer was added to all wells and plates were incubated for 2 minutes as above. The plate was then read on a Bioplex® 200 analyser (Bio-Rad, USA). The Bioplex® Manager software package

(Bio-Rad, USA) was used to determine the concentrations of analytes within each sample.

3.8.5 Liquid Chromatography Tandem Mass Spectrometry for Steroid Quantification

Cortisol and cortisone were quantified in serum and BALF at the Steroid Metabolome Core at the University of Birmingham.

The steroids were extracted from 400µL of serum or BALF via liquid/liquid extraction with 2mL of MTBE (tert-methyl butyl ether) following addition of the internal standards cortisol-d4 and cortisone-d7 (20µL of 1000ng/mL internal standard solution prepared in deuterated methanol). Samples were vortexed and left to equilibrate for 30 minutes. The MTBE layer was then removed and dried under nitrogen at 55°C, this was then reconstituted in 200µL of methanol:water 50:50 and analysed on a liquid chromatography tandem mass spectrometer, as described previously(271-273).

A Waters Xevo-XS coupled to an Acquity ultra-high performance liquid chromatography system was utilised for the experiments in positive ionisation mode. Methanol with 0.1% formic acid (FA) and water (0.1% FA) were used to elute the steroids from a HSS T3, 1.8µm, 1.2 x 50mm column held at 60°C throughout. A gradient starting at 45% methanol (0.1%FA) was held for 1 minute prior to a linear increase in methanol (0.1% FA) to 75% at 5 minutes. The column was subsequently washed at high methanol and re-equilibrated at the starting conditions prior to the next injection. 20uL of the extract was injected onto the column. Steroids were

identified due to a matching retention time and 2 identical mass transitions in comparison to a reference standard. The mass transitions for cortisol were 363.1 > 121.0 and 363.1 > 90.9; cortisol-d4 367.2 > 121.0; cortisone 360.0 > 163.0, 360.0 > 121.0; and cortisone-d7 391.0 > 121.0. Steroids were quantified relative to their deuterated internal standard through comparison to a linear calibration series ranging from 0.05 to 250ng/mL for both steroids (with inclusion of a blank). The limit of quantification for the assay was 0.3nM for cortisol and cortisone.

3.8.6 Urea Assay

Urea concentrations were measured in BALF and plasma using a colorimetric urea assay kit (Sigma-Aldrich, UK). The protocol was as per manufacturer's instructions. Plates were read at 570nm on a microplate reader (Synergy HT, BioTek, USA). Patient alveolar epithelial lining fluid (ELF) concentrations of cytokines and steroids were estimated using the urea dilution method(274). Plasma urea concentration was divided by matched BALF urea concentration to calculate the urea dilution factor. BALF concentrations of steroids and cytokines were then multiplied by the urea dilution factor to generate estimated ELF concentrations of cytokines and steroids.

3.9 Animal Studies

3.9.1 Ethical statement

All procedures were performed in compliance with UK law under the Animal [Scientific Procedures] Act 1986, with approval from the Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO). The project licence code was PAAB1C3B2 (personal licence number I6C84754C). The 3Rs principles (Reduction, Replacement and Refinement) guided the design and methodology of our animal studies.

3.9.2 Experimental animals and study design

Male wild-type (WT) C57BL/6 mice (aged 3-4 week old) were obtained from Charles River, UK and maintained at the Biomedical Services Unit (BMSU), University of Birmingham, UK. A colony of 11 β -Hydroxysteroid Dehydrogenase Type 1 Knockout (HSD-1 KO) C57BL/6 mice was also maintained at BMSU. This colony was originally a kind gift from Professor Gareth Lavery, University of Birmingham(275, 276). Caecal ligation and puncture (CLP) surgery was performed on mice aged 8-12 weeks. To ensure that mice were appropriately monitored post-procedure, CLP was performed on 4-8 mice at any one time. Mice undergoing CLP were divided into 3 intravenous treatment groups: PBS, activated HSD-1 tMSCs and inactive HSD-1 tMSCs (n=6 per group). Our experimental methods were designed to minimise the number of animals used and maximise scientific results (e.g. consistent use of the C57BL/6 strain, male gender and age). This enabled comparisons across WT and HSD-1 KO mice, and measurement of multiple endpoints in the same mouse.

3.9.3 Housing and husbandry

All mice were housed in cages with a maximum of 4 mice per cage. Mice were exposed to 12-hour light and dark cycles, with food and water given *ad libitum*. All mice were weighed before CLP surgery, each time they were assessed following surgery, and prior to euthanasia. To monitor mouse welfare, health assessment scoring sheets were filled in before, during and after CLP surgery. These assessment sheets included an established mouse severity scoring system developed previously at the University of Birmingham (table 3.7). Any mouse scoring 4 on our severity scale, at any time, was immediately humanely euthanised by Schedule 1 method, (Animal [Scientific Procedures] Act 1986) to prevent any animal from exceeding severity limits of our protocol.

Mouse behaviour	Score
Piloerect coat, with no detectable behavioural differences compared to unchallenged mice	1
Piloerect coat, intermittent huddled reflex but responds to stimulation such as cage opening	2
Piloerect coat, persistent huddled reflex with altered response to stimulation e.g. reduced activity but will still move around by themselves when handled	3
As above, but with lack of curiosity, and much reduced response to stimuli e.g. limited movements when handled	4

Table 3.7: Mouse Severity Score

3.9.4 Caecal ligation and puncture (CLP) surgery

One hour prior to CLP, mice were given intravenous treatments via tail vein of either 50µl PBS alone, activated HSD-1 tMSCs suspended in 50µl PBS, or inactive HSD-1 tMSCs suspended in 50µl PBS. A range of tMSC doses were used between 50,000 to 250,000. Analgesia was given 30 minutes prior to CLP: subcutaneous

buprenorphine at 0.1mg/kg body weight (Temgesic®, Reckitt Benckiser, Switzerland). Mice were anaesthetised with isoflurane gas (5% in oxygen delivered at 1.5L/min) for induction and maintenance anaesthesia (1-3% in oxygen delivered at 1.5L/min). All CLP surgeries were undertaken in animal surgery theatres within the BMSU, using aseptic technique, i.e. use of sterile gown, gloves, drapes, and surgical equipment. A perioperative record was kept to control for factors including time under anaesthetic and time of potentially reduced body temperature, which may account for variations in reaction. The CLP experimental design was adapted from previously published protocols(277). CLP surgery was performed on tables heated to 37.5°C. Following induction of anaesthesia, fur was shaved from the abdomen and 2% chlorhexidine (Avagard™, Australia) was then used to sterilise the skin. A midline laparotomy incision was performed followed by exposure of the caecum, ligation of the lower 30% with 2.0 Ethilon™ nylon suture (Ethicon, USA) and a single puncture of the ligated caecum with a 19G microlance needle (Becton Dickinson, UK). A small amount of faeces was then expressed by gently squeezing the ligated caecum with forceps, before being placed back into the abdominal cavity. The abdominal wall was sutured with 6.0 Vicryl® (Ethicon, USA) followed by skin suture with 4.0 Prolene® (Ethicon, USA). All mice were recovered in heat boxes and recovery incubators until euthanised. Postoperative resuscitation was with 500µl of Hartman's solution (Aquapharm 11®, Animalcare Ltd, UK) subcutaneously and a dose of buprenorphine as described before. Sham CLP surgery involved laparotomy and handling of caecum (without ligation and puncture) prior to closure. Two different time courses of CLP were used: 4 hours and 24 hours. During the 4 hour time course, animals were monitored continuously. During the 24 hour time course, mice were monitored every 2-4 hours, and given a further 500µl of subcutaneous Hartmann's solution at 6 hours

post-CLP. Mice exposed to either time course were given additional boluses of buprenorphine to maintain an appropriate level of analgesia.

3.9.5 Sample collection and processing

On completion of the CLP time course (4 or 24 hours post-CLP), mice were deeply anaesthetised with 5% isoflurane in oxygen delivered at 1.5L/min. Cardiac puncture was performed and death confirmed. Cardiac blood was aliquoted for processing later. Immediately post-mortem, peritoneal lavage fluid (PLF) was collected by instilling 2 mls of PBS including 1% EDTA into the lower quadrants of the abdomen, gently compressing the abdomen several times, then aspirating back from the lower quadrants. The chest cavity and neck were then opened, and a small incision was made in the upper trachea. A fine bore polythene tube was inserted into the trachea until the tip was slightly above the carina. Broncho-alveolar lavage fluid (BALF) was collected by lavaging the lungs twice with 600µl of PBS including 1% EDTA via the tracheal tube. The liver and caecum were examined for colour change and signs of necrosis. All samples were immediately transferred on ice to the laboratory for processing and analysis.

Cardiac blood was centrifuged at 13,500 r.p.m. in a micro-centrifuge (Stuart SCF2, Bibby Scientific Ltd, UK) for 10 minutes. The serum layer was aspirated and stored at -40°C for future analysis. Prior to centrifugation, 20µl of neat PLF and BALF from each mouse was taken for bacterial culture, as discussion in section 3.9.7. PLF and BALF were centrifuged at 400g for 10 minutes; supernatants were then aspirated and stored at -40°C for future analysis. The cell pellets were re-suspended in 1ml PBS including 2% BSA and 10% murine serum (Sigma-Aldrich), and incubated on

ice for 15 minutes to allow blocking of non-specific Fc receptor binding, prior to antibody labelling as discussed in section 3.9.6. During the incubation period, a cell count and cytospin of BALF and PLF cells were performed as described in sections 3.3.5 and 3.3.6.

3.9.6 Flow cytometric cell identification

After blocking, BALF and PLF cells from section 3.9.5 were diluted to 1×10^6 /ml in FACS Buffer (PBS containing 1% BSA and 1% EDTA) and 100 μ l added to flow cytometry tubes. Pooled cells were used for unlabelled control. Cells were then incubated with anti-mouse antibodies (as detailed in table 3.7) for 20 minutes on ice in the dark. Compensation beads (UltraComp eBeads, Invitrogen) were used for single colour controls. Following incubation, 2mls of FACS buffer was added to all tubes before centrifuging at 400g for 5 minutes at 4°C. The supernatant was then discarded, and cells re-suspended in 2mls of Annexin V binding buffer before centrifuging again as above. To assess apoptosis, cells were then incubated with BV421-Annexin V (BD Biosciences) at a 1:20 dilution for 15 minutes on ice in the dark. All cells were washed again in 2mls of Annexin V binding buffer as described above, then re-suspended in 300 μ l of Annexin V binding buffer. To assess viability, SYTOX Green nucleic acid stain (Invitrogen) was added at a 1:1500 dilution immediately prior to analysis on flow cytometer (LSR Fortessa X-20, BD Biosciences, UK). Colour compensation and analysis were performed using FACSDiva software (BD Biosciences, UK). Cell identification by surface marker expression is summarised in Table 3.8. Example flow plots and gating strategy are shown in Figure 3.9.

Antibody	Clone	Fluorophore	Manufacturer	Isotype Control	Dilution
Anti-mouse F4/80	T45-2342	PE-CF594	BD Biosciences	Rat, IgG2Ak	1 : 100
Anti-mouse CD11c	HL3	PE	BD Biosciences	Hamster, IgG1 λ 1	1 : 100
Anti-mouse CD11b	M1/70	APC	BD Biosciences	Rat, IgG2Bk	1 : 100
Anti-mouse Ly6G (Gr1)	1A8	BV786	BD Biosciences	Rat, IgG2Ak	1 : 100

Table 3.8: Murine antibody staining panel used on flow cytometry to identify cells.

APC: Allophycocyanin. PE: Phycoerythrin. BV: Brilliant Violet. All Isotype control antibodies were purchased from BD Biosciences.

Cell Type	F4/80	CD11b	CD11c	GR1
Alveolar Macrophages	++	-	++	-
Peritoneal Macrophages	++	+	-	-
Infiltrating Monocytes	+	++	++	-
Neutrophils	-	++	-	++

Table 3.9: Cell surface marker strategy to identify murine cells

The expression of each antigen on different cell types is quantified by a symbol: moderate expression (+), high expression (++), and low / no expression (-).

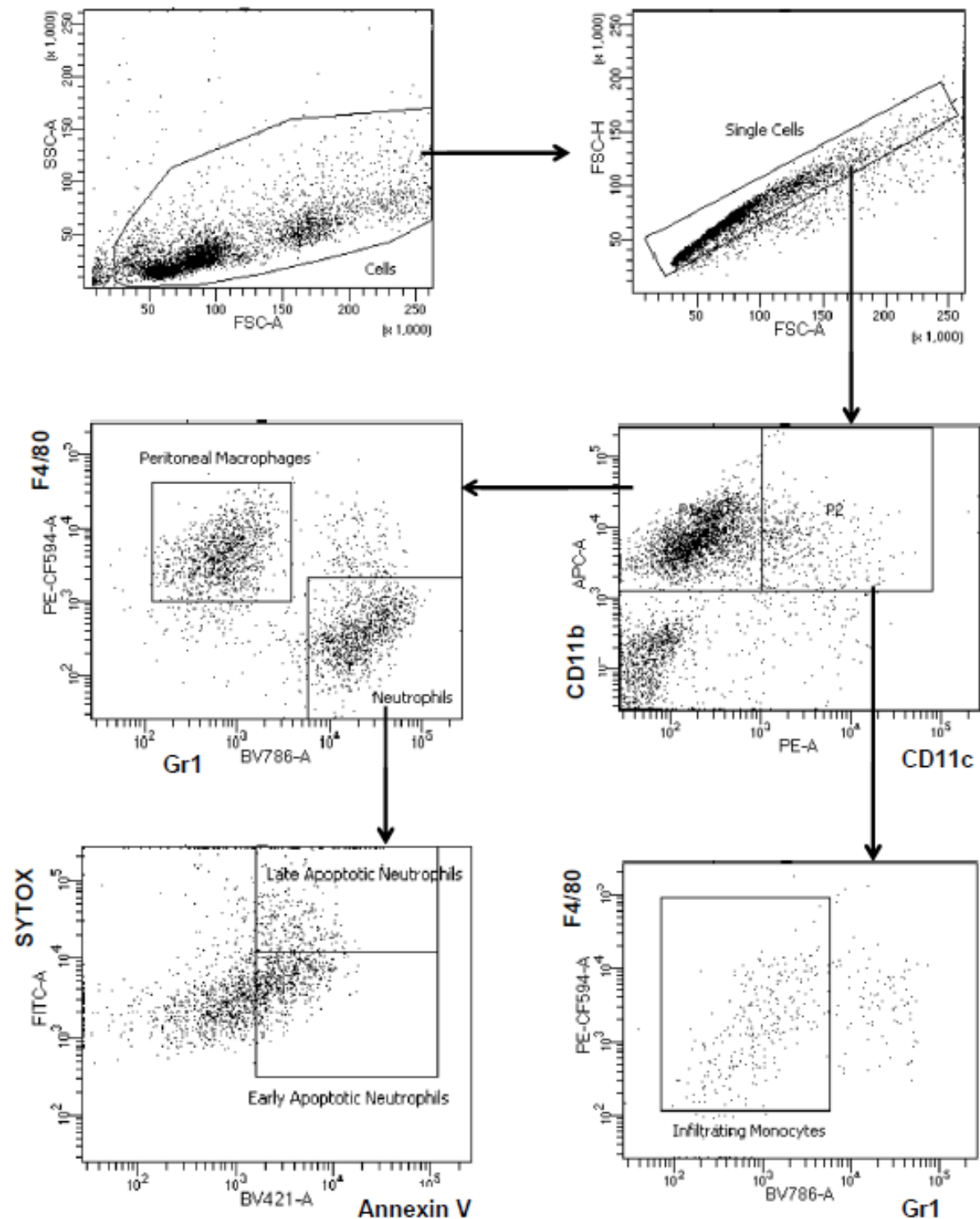


Figure 3.9: Flow cytometry gating strategy for murine cell identification in peritoneal lavage fluid

3.9.7 Bacterial culture

Agar plates were prepared by first adding ten 1.68g tablets of Luria-Bertani Lennox broth with Agar (LB Lennox, Sigma-Aldrich) to 483mls of water and autoclaving at 121°C for 20 minutes to sterilise. After cooling slightly, the broth was poured into petri dishes, where it cooled further and solidified. Marker pen was used to divide the petri dishes into 8 equal segments. For each mouse, 20µl of neat PLF and BALF were diluted in 180µl of sterile PBS in the first well of a column in a 96-well cell culture plate (CoStar®, Corning®) to give a 10-fold dilution. Eight serial 10-fold dilutions (20µl of the preceding well transferred to the next well containing 180µl PBS) were then performed, with wells mixed thoroughly at each step. The final dilution factor was 10^8 . 20µl of BALF and PLF from each dilution were plated into the corresponding segment of LB Agar plates, labelled with dilution factors then incubated at 37°C and 5% CO₂. After 24 hours, Colony Forming Units (CFU) were counted in the most diluted segment which showed bacterial growth. The CFU/ml was calculated from the dilution factor and initial total volumes of neat BALF and PLF.

3.9.8 Protein concentrations

Total protein concentrations in sera, BALF and PLF were measured using the Pierce™ BCA Protein Assay Kit as described in section 3.8.1. Protein permeability index was calculated as a ratio fluid protein to plasma protein. This assay has previously been validated by our group.

3.9.9 Cytokine assays

Murine serum, PLF and BALF inflammatory cytokines (MCP-1, macrophage inflammatory protein [MIP]-1 α , MIP-1 β , MIP-2, KC [murine homolog of chemokine C-X-C motif ligand 1], IL-1 β , IL-6, IL-10, TNF α and VEGF) were measured by a Luminex[®] screening assay (R&D systems), as described in section 3.8.4. These assays were performed as per the manufacturer's protocols with no deviations and have previously been validated by our group.

CHAPTER 4 : IMPACT OF PROLONGED *IN VITRO* EXPANSION ON MESENCHYMAL STEM CELLS

4.1 INTRODUCTION

Human bone marrow mesenchymal stem cells (MSCs) are pluripotent cells which have the ability to differentiate into several different cell types. Numerous pre-clinical studies have shown that administration of MSCs can attenuate inflammation and promote repair in models of lung injury. MSCs have been shown to be efficacious in small and large animal models of lung injury(222, 226, 227, 278), and in both animal and human injured *ex-vivo* lung perfusion models(192, 228-230). Phase 1/2 clinical trials in patients with ARDS have shown that MSC administration is safe, feasible and well-tolerated, with no adverse effects(239-241). However, further phase 2/3 trials are required to determine MSC efficacy in these patients.

The 2 main mechanisms by which MSC attenuate lung injury are: 1) Promotion of epithelial and endothelial repair, 2) Modulation of immune function to increase bacterial clearance and aid resolution of inflammation(183). These mechanisms can be initiated by either cell-to-cell contact or release of paracrine factors. MSCs are known to secrete pro-repair factors including vascular endothelial growth factor (VEGF)(233), angiopoietin-1 (Ang-1)(191), transforming growth factor- β (TGF- β), lipoxin A4(199) and keratinocyte growth factor (KGF)(192). MSCs are also known to secrete prostaglandin E₂ (PGE₂), which stimulates macrophages to release the anti-inflammatory cytokine interleukin-10(187, 202). MSCs can also upregulate macrophage phagocytosis by paracrine action: either via release of lipoxin A4 or transfer of mitochondria via microvesicles(215, 220). These actions also result in upregulation of M2 surface markers on macrophages(189, 216, 217). The effect of MSCs on macrophage efferocytosis (the ability to phagocytose apoptotic neutrophils) is not currently known. However, studies have shown that MSCs may lose their

intrinsic anti-inflammatory capabilities with repeated *in vitro* passage(238). In the START phase 2 clinical trial, 10 million MSCs / kg were administered intravenously in ARDS patients; meaning a 70kg patient would receive 7×10^8 MSCs(240). To generate doses of this magnitude for use as clinical therapy, significant *in vitro* expansion of MSCs would be required. However, the expansion required to generate sufficient MSCs may also decrease their anti-inflammatory functions, thereby limiting their therapeutic potential. It currently remains unclear what effect prolonged *in vitro* expansion has on the MSC secretome.

The aim of the work in this chapter was to determine the effect of prolonged expansion on MSC modulation of macrophage function and secretion of pro-repair paracrine factors. These functions were assessed in the absence and presence of pro-inflammatory cytokines and lipopolysaccharide (LPS), in order to mimic the inflammatory environment of sepsis-related ARDS.

4.2 METHODS

Detailed methods are provided in section 3.6.

4.2.1 Mesenchymal Stem Cell Culture and Prolonged Expansion

Human bone-marrow derived Mesenchymal Stem Cells (MSCs) from 4 young adult donors were purchased from the Institute of Regenerative Medicine at Texas A&M College of Medicine, USA. Donors were designated 8001 (24-year-old female), 8004 (22 year old male), 8013 (22-year-old male), and 7083 (24 year old male). MSCs from all donors were at passage 2 prior to shipment. MSCs were only available from a limited number of donors, and MSCs at passage 1 or 2 were only available from 4 young adult donors at the time of purchase. MSCs were cultured in α MEM (Gibco, ThermoFisher Scientific) containing 16% FBS, 100U/mL penicillin, 100ug/mL streptomycin and 2mM L-Glutamine, using T175 flasks (Nunc, ThermoFisher Scientific) at 37°C and 5% CO₂. MSCs were passaged every 4 days.

At alternate passages, 5×10^5 MSCs were seeded into each of two T75 flasks (Nunc, ThermoFisher Scientific) and cultured in α MEM containing 5% FBS. One T75 flask received “cytomix”, a cocktail of pro-inflammatory mediators: 50ng/ml interferon- γ , 50ng/ml tumour necrosis factor- α , 50ng/ml interleukin-1 β (all from Peprotech, UK). At the same time, 5×10^5 MSCs were taken for direct co-cultured experiments with THP-1 macrophages, as described in section 4.2.4. The remainder of MSCs were returned to standard culture in a T175 flask containing α MEM with 16% FBS. After 24 hours culture at 37°C and 5% CO₂, conditioned media (CM) was aspirated from the T75 flasks and 6-well plates. CM was centrifuged at 500g to pellet any debris, before the

supernatant was aliquoted and frozen at -20°C. MSCs from 4 donors were expanded in this way for one month, with collections of CM at 4 equally spaced time-points / passages. After one month of *in vitro* culture, MSC bi-lineage differentiation and surface marker expression were assessed (described in sections 3.6.4 and 3.6.5). The purpose of this was to determine whether the remaining MSCs had differentiated or had maintained a pluripotent MSC phenotype.

The FBS content of α MEM was reduced to 5% for stimulated and unstimulated MSC-CM collection, since the MSC-CM would later be used in wound healing assays (described in section 3.6.6), where a higher FBS concentration would cause overly rapid wound closure. Quantification of selected MSC paracrine factors within MSC-CM was performed using enzyme-linked immunosorbent assays (ELISAs) for Angiopoietin-1, Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor- β 1 (TGF- β 1), Keratinocyte Growth Factor (KGF), Interleukin-10 (IL-10) and Prostaglandin E₂ (PGE₂) parameter assays as described in sections 3.8.2 and 3.8.3. MSC-CM was also used to treat primary human AMs from lung tissue (isolation described in section 3.3.4) or THP-1 derived macrophages (described below) prior to phagocytosis, efferocytosis or phenotyping assays (see sections 3.4.1, 3.4.2 and 3.4.4)

4.2.2 THP-1 macrophage direct co-culture with mesenchymal stem cells

The THP-1 monocytic cell line (Sigma-Aldrich, UK) was cultured in RPMI-1640 including 10% FBS, using T75 flasks, at 37°C and 5% CO₂. To differentiate into macrophages, 1×10^6 THP-1 cells were cultured per well in a 6-well plate, in the

presence of 100ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 hours. Media was then changed and cells rested for a further 72 hours in RPMI 1640 including 10% FBS before being used in co-culture experiments(269).

Following MSC passage as described in section 3.6.2, media was removed from THP1-macrophages and 2.5×10^5 MSCs in 2mls RPMI 1640 / 10% FBS were added to wells in duplicate. This gave a 1:4 ratio of MSCs to THP-1 macrophages. Media was also changed on control wells containing THP-1 macrophages only in duplicate. One co-culture well and one macrophage-only well were treated with 100ng/ml Lipopolysaccharide (LPS, Sigma-Aldrich). Cells were cultured for 24 hours at 37°C and 5% CO₂, before CM was aspirated. CM was centrifuged at 500g to pellet any debris, before the supernatant was aliquoted and frozen at -20°C. ELISAs were used to quantify IL-10 and PGE₂ concentrations in CM from direct co-culture experiments.

4.2.3 Statistical Analysis

Data were analysed using Prism 8 software (GraphPad, USA). Normality of data was assessed using the D'Agostino & Pearson test. Differences between three or more paired non-parametric data sets were assessed using the Friedman one-way analysis of variance (ANOVA) and Dunn's multiple comparison tests. The influence of cytomix treatment versus no treatment on MSC paracrine factor release at successive passages was assessed using two-way repeated measures ANOVA. Two-tailed p-values of ≤ 0.05 were considered as significant. Results from parametric data are shown as mean and standard deviation. Results from non-parametric data are shown as median and interquartile range. Since MSCs were only available from a limited number of human donors (n=4), the results may be under-powered.

4.3 RESULTS

4.3.1 Effect of prolonged expansion on MSC secretion of paracrine pro-repair factors.

Untreated MSCs from all 4 donors showed an increase in VEGF secretion with prolonged expansion (Figure 4.1A, Friedman $Q = 8.10$, $p=0.033$); VEGF secretion was significantly increased at P9 compared to P3 (figure 4.1A, rank sum differences = 10.0, $p=0.037$). Cytomix-treated MSCs from all 4 donors showed an increase in VEGF secretion with prolonged expansion, however this increase approached but did not reach statistical significance (figure 4.1B, Friedman $Q = 8.10$, $p=0.054$). VEGF secretion was lower in cytomix-treated MSCs compared to untreated MSCs at all passages (figure 4.1C, 2-way repeated measures ANOVA, $F(1,6)$, $p=0.033$).

Untreated MSCs from all 4 donors showed a decrease in Angiopoietin-1 (Ang-1) secretion with prolonged expansion (figure 4.2A, Friedman $Q = 8.4$, $p=0.036$); Ang-1 secretion was significantly decreased at P5 compared to P3 (figure 4.2A, rank sum differences = 10.0, $p=0.037$). Cytomix-treated MSCs from all 4 donors showed an increase in Ang-1 secretion with prolonged expansion (figure 4.2B, Friedman $Q = 7.8$, $p=0.036$); Ang-1 secretion was significantly increased at P9 compared to P5 (figure 4.2B, rank sum differences = 10.0, $p=0.037$). Ang-1 secretion was not significantly different between cytomix-treated and untreated MSCs (figure 4.2C, 2-way repeated measures ANOVA, $F(1,6)$, $p=0.091$).

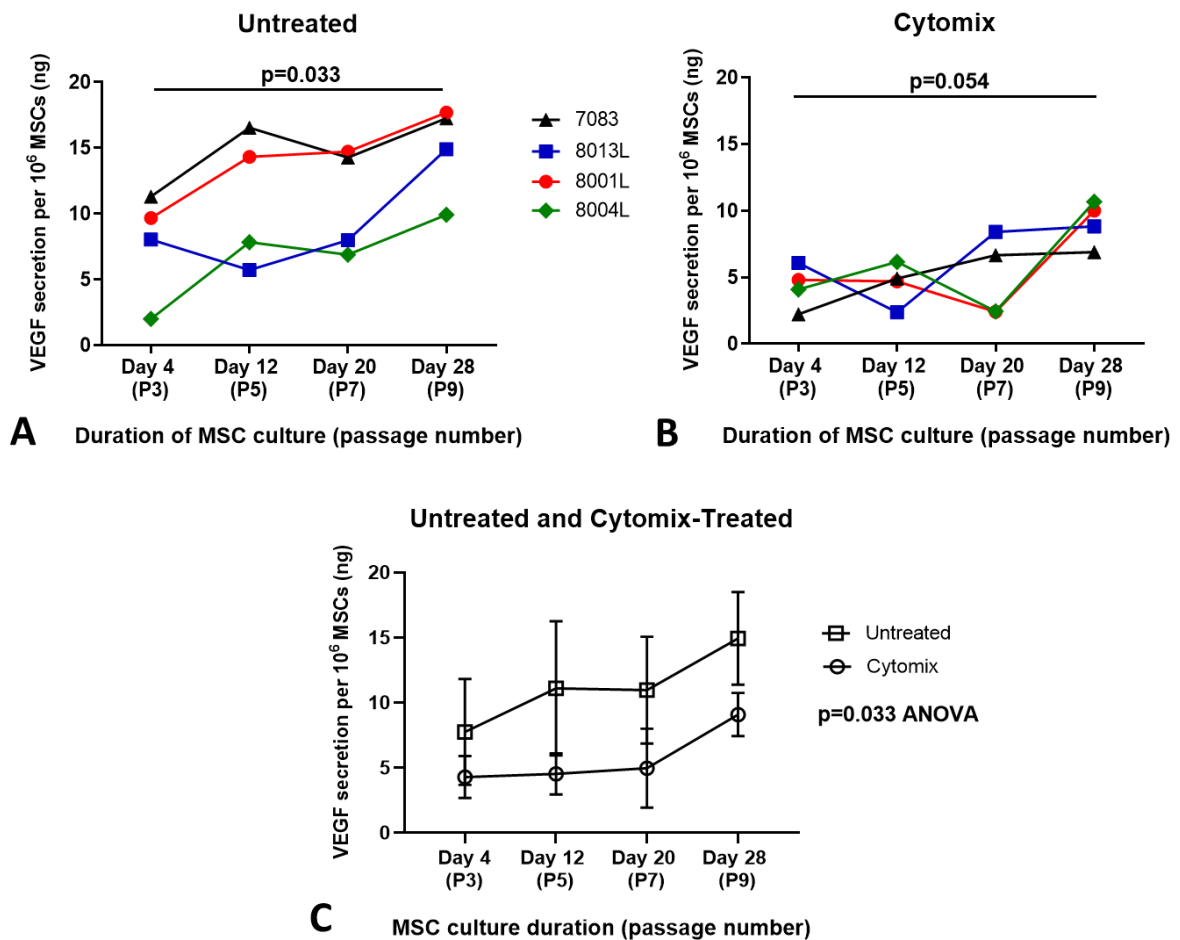


Figure 4.1: Effect of prolonged expansion on VEGF secretion by untreated and cytomix-treated MSCs.

All data shown are for secretion over 24 hours. VEGF concentrations from non-conditioned control media (α MEM + 5% FBS) have already been subtracted from all data points. Values averaged from 3 replicates, $n=4$ for all groups. **A:** Untreated MSCs from all 4 donors showed an increase in VEGF secretion with prolonged expansion (Friedman $Q = 8.10$, $p=0.033$). VEGF secretion was increased at P9 compared to P3 (rank sum differences -10.0, $p=0.037$). **B:** Cytomix treated MSCs from all 4 donors showed no significant change in VEGF secretion with prolonged expansion (Friedman $Q = 8.10$, $p=0.054$). **C:** VEGF secretion by untreated versus cytomix-treated MSCs at different passages. Values shown as mean and standard deviation. Untreated MSCs secrete higher amounts of VEGF than cytomix-treated MSCs (2-way repeated measures ANOVA, $F(1,6)$, $p=0.033$).

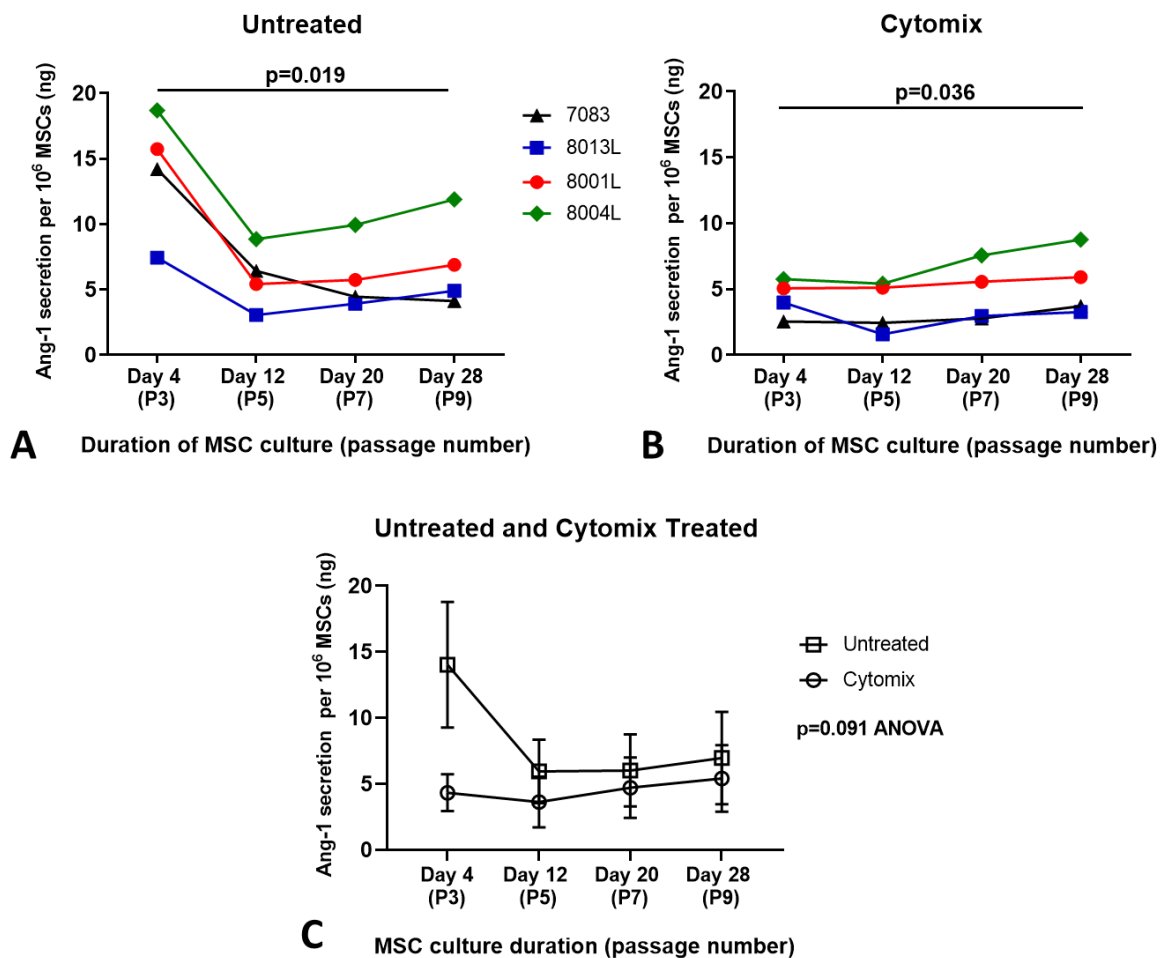


Figure 4.2: Effect of prolonged expansion on Angiopoietin-1 secretion by unstimulated and stimulated MSCs

All data shown are for secretion over 24 hours. Ang-1 = Angiopoietin-1. Ang-1 concentrations from non-conditioned control media (α MEM + 5% FBS) have already been subtracted from all data points. Values averaged from 3 replicates, $n=4$ for all groups. **A:** Untreated MSCs from all 4 donors showed a decrease in Ang-1 secretion with prolonged expansion (Friedman $Q = 8.4$, $p=0.036$). Ang-1 secretion was significantly decreased at P5 compared to P3 (rank sum differences 10.0, $p=0.037$). **B:** Cytomix-treated MSCs from all 4 donors showed an increase in Ang-1 secretion with prolonged expansion (Friedman $Q = 7.8$, $p=0.036$); Ang-1 secretion was significantly increased at P9 compared to P5 (rank sum differences 10.0, $p=0.037$). **C:** Angiopoietin-1 secretion by untreated versus cytomix-treated MSCs at different passages. Values shown as mean and standard deviation. No significant difference in Angiopoietin-1 secretion between untreated and cytomix-treated MSCs (2-way repeated measures ANOVA, $F(1,6)$, $p=0.091$).

Untreated MSCs from all 4 donors showed a decrease in TGF- β 1 secretion with prolonged expansion; this decrease approached but did not reach statistical significance (figure 4.3A, Friedman Q = 7.5, p=0.052). Cytomix-treated MSCs from all 4 donors showed no significant change in TGF- β 1 secretion with prolonged expansion (figure 4.3B, Friedman Q = 0.9, p=0.900). There was also no significant difference in TGF- β 1 secretion between untreated and cytomix-treated MSCs (figure 4.3C, 2-way repeated measures ANOVA, F(1,6), p=0.893).

Only untreated MSCs from 2 donors (8001L and 8004L) were found to secrete detectable levels of KGF (see figure 4.4); n numbers were too low to perform statistical analysis. There was no detectable KGF secreted by cytomix-treated MSCs from any donor. There was also no detectable secretion of lipoxin A4, LL-37 (human cathelicidin) or IL-1ra by untreated or cytomix-treated MSCs from all 4 donors.

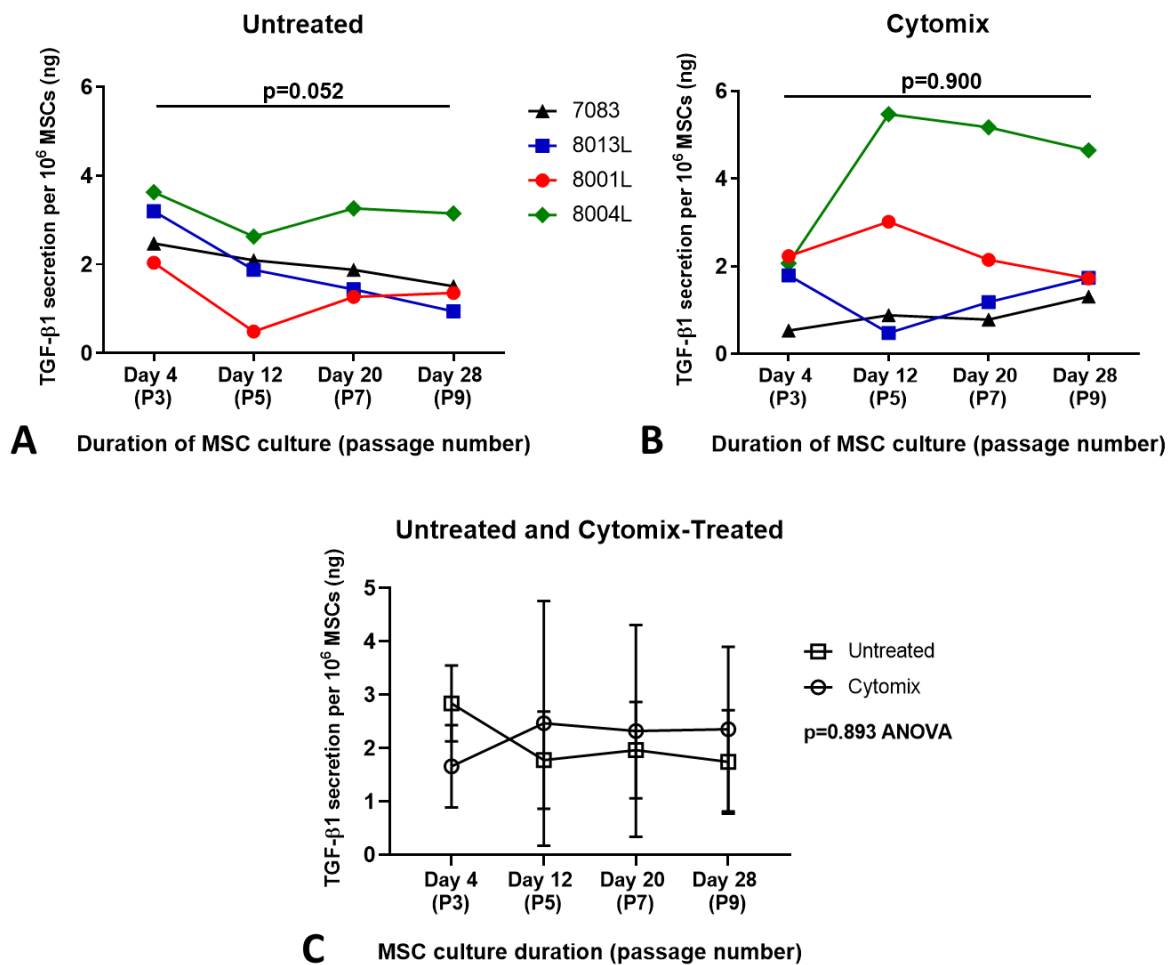


Figure 4.3: Effect of prolonged expansion on TGF- β 1 secretion by unstimulated and stimulated MSCs

All data shown are for secretion over 24 hours. TGF- β 1 concentrations from non-conditioned control media (α MEM + 5% FBS) have already been subtracted from all data points. Values averaged from 3 replicates, $n=4$ for all groups. **A:** Untreated MSCs from all 4 donors showed a decrease in TGF- β 1 secretion with prolonged expansion; this decrease approached but did not reach statistical significance (Friedman $Q = 7.5$, $p=0.052$). **B:** Cytomix-treated MSCs from all 4 donors showed no significant change in TGF- β 1 secretion with prolonged expansion (Friedman $Q = 0.9$, $p=0.900$). **C:** TGF- β 1 secretion by untreated versus cytomix-treated MSCs at different passages. Values shown as mean and standard deviation. No significant difference in TGF- β 1 secretion between untreated and cytomix-treated MSCs (2-way repeated measures ANOVA, $F(1,6)$, $p=0.893$).

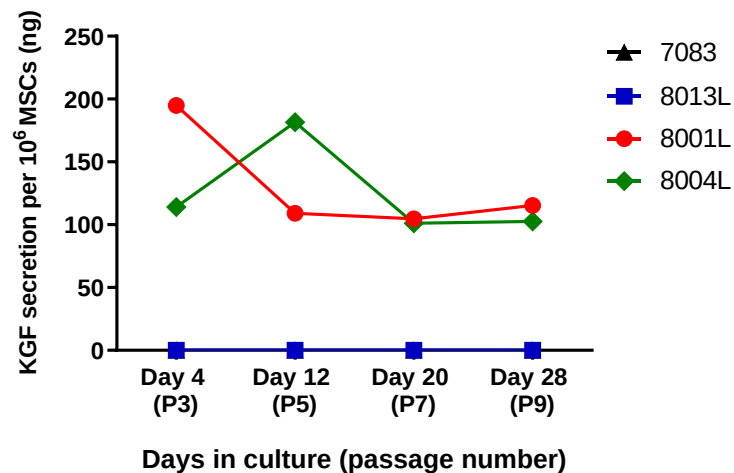


Figure 4.4: Effect of prolonged expansion on KGF secretion by untreated MSCs.

All data shown are for secretion over 24 hours. KGF concentrations from non-conditioned control media (α MEM + 5% FBS) have already been subtracted from all data points. Values averaged from 3 replicates. There was no detectable KGF secretion by untreated MSCs from donors 7083 and 8013L, as a result n-numbers were too low to perform statistical analysis. There was also no detectable KGF secretion by cytomix-treated MSCs.

4.3.2. Effect of prolonged expansion on the ability of MSC conditioned media to promote wound healing

Prolonged MSC expansion decreases the ability of conditioned media (CM) from untreated MSCs to promote wound healing (figure 4.5A, Friedman $Q = 9.9$, $p=0.0062$). Prolonged MSC expansion had no significant impact on the ability of CM from cytomix-treated MSCs to promote wound healing (figure 4.5B, Friedman $Q = 2.7$, $p=0.0508$). There was no significant difference between the ability of CM from untreated or cytomix-treated MSCs to promote wound healing (figure 4.5C, 2-way repeated measures ANOVA, $F(1,6)$, $p=0.171$).

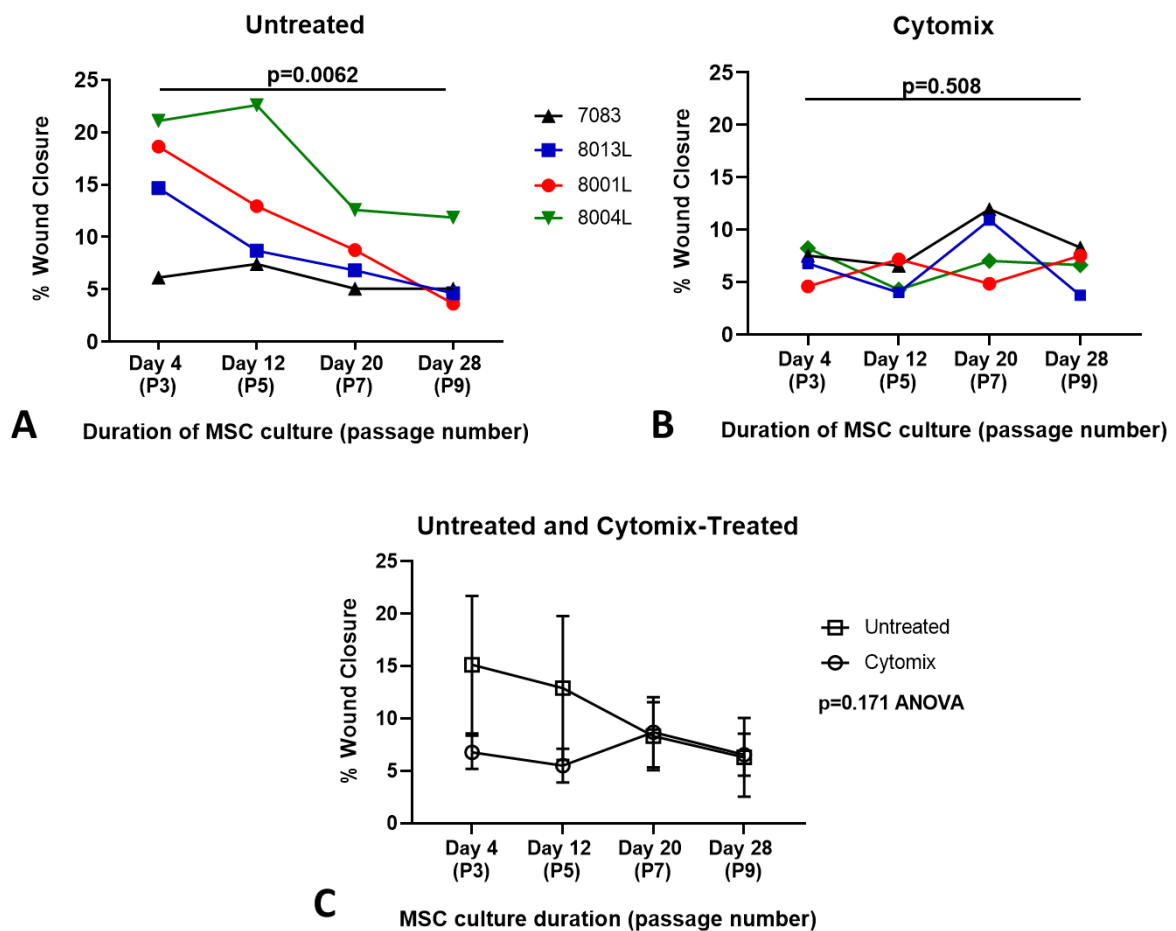


Figure 4.5: Effect of prolonged expansion on the ability of MSC-CM to promote wound healing.

All MSC-CM was collected over a 24hr period. Wound closure values from non-conditioned control media (α MEM + 5% FBS) have already been subtracted from all data points. Values averaged from 5 replicates, $n=4$ for all groups. **A:** Prolonged MSC expansion decreases the ability of conditioned media (CM) from untreated MSCs to promote wound healing (Friedman $Q = 9.9$, $p=0.0062$). **B:** Prolonged MSC expansion had no significant impact on the ability of CM from cytomix-treated MSCs to promote wound healing (Friedman $Q = 2.7$, $p=0.0508$). **C:** Wound closure mediated by CM from untreated versus cytomix-treated MSCs at different passages. Values shown as mean and standard deviation. No significant difference in wound closure between untreated and cytomix-treated MSC-CM (2-way repeated measures ANOVA, $F(1,6)$, $p=0.171$).

4.3.3. Effect of prolonged expansion on ability of MSCs to modulate macrophage function

As described in section 4.2.4, at odd-numbered passages 2.5×10^5 MSCs were directly co-cultured with 1×10^6 THP-1 derived macrophages, either in the presence of absence of LPS. After 24hrs direct co-culture, CM was collected. The P5 co-culture dataset is missing because insufficient THP-1 macrophages were available to perform the experiments at the day 12 (P5) MSC expansion time-point.

Prolonged MSC expansion significantly increased PGE₂ secretion following untreated MSC-macrophage co-culture (figure 4.6A, Friedman Q = 12.0, $p < 0.0001$); PGE₂ secretion was significantly elevated at P9 compared to P3 (figure 4.6A, rank sum differences = 12.0, $p = 0.0061$). Prolonged MSC expansion had no significant effect on PGE₂ secretion following LPS-treated MSC-macrophage co-culture (figure 4.6B, Friedman Q = 4.5, $p = 0.125$). THP-1 macrophages cultured alone in the presence or absence of LPS did not secrete PGE₂. Untreated MSCs cultured alone also did not secrete PGE₂. However, LPS-treated MSCs from all donors did secrete detectable PGE₂ when cultured alone. LPS-treated MSCs showed an increase in PGE₂ secretion with prolonged expansion (figure 4.6C, Friedman Q = 11.1, $p = 0.0009$); PGE₂ secretion was significantly increased at P9 compared to P3 (figure 4.6C, rank sum differences = 11.0, $p = 0.016$).

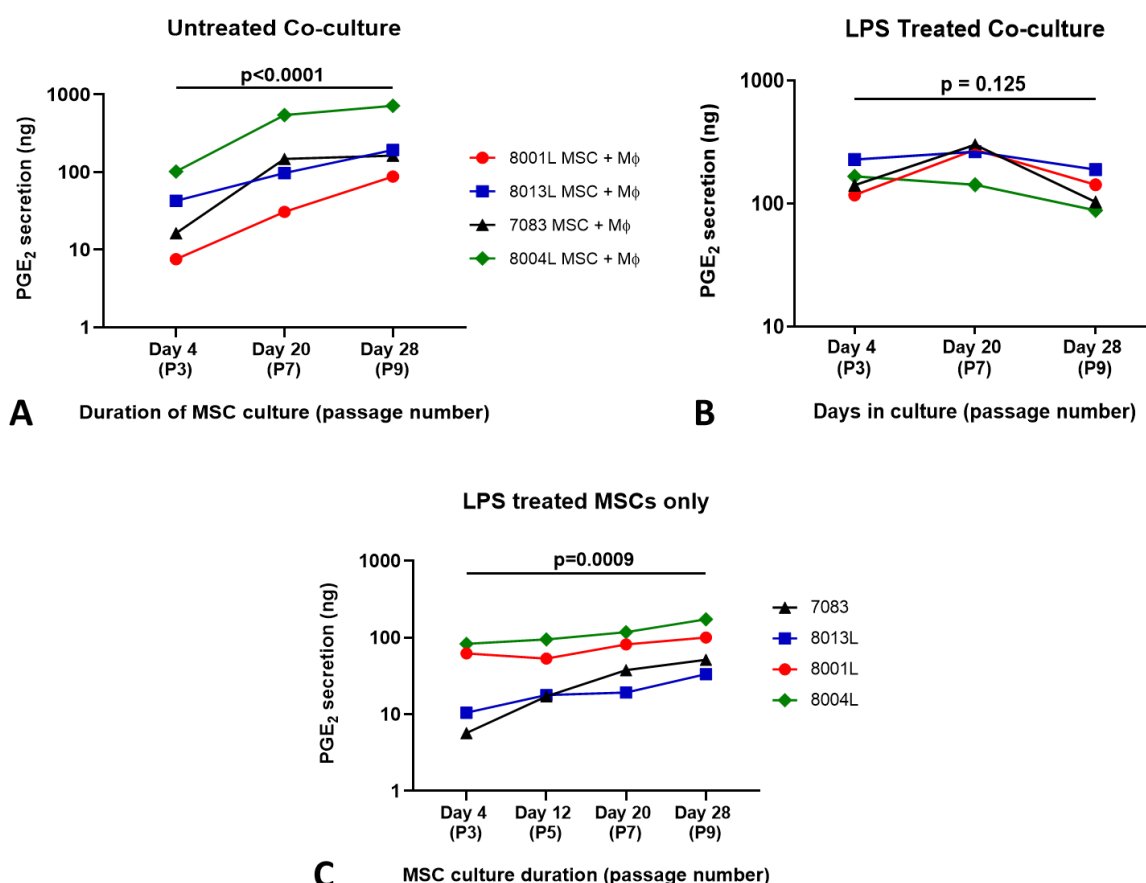


Figure 4.6: Effect of prolonged MSC expansion on PGE₂ secretion following MSC-THP1 Macrophage direct co-culture.

All data shown are for secretion over 24 hours. Non-conditioned control media (α MEM + 5% FBS) contained no detectable PGE₂. Values averaged from 3 replicates, n=4 for all groups. Log scale used for all graphs. **A:** Prolonged MSC expansion significantly increased PGE₂ secretion following untreated MSC-macrophage co-culture (Friedman Q = 12.0, p<0.0001). PGE₂ secretion was significantly elevated at P9 compared to P3 (rank sum differences, p=0.0061). **B:** Prolonged MSC expansion had no significant effect on PGE₂ secretion following LPS-treated MSC-macrophage co-culture (Friedman Q = 4.5, p=0.125). **C:** LPS-treated MSCs showed an increase in PGE₂ secretion with prolonged expansion (Friedman Q = 11.1, p=0.0009); PGE₂ secretion was significantly increased at P9 compared to P3 (rank sum differences 11.0, p=0.016).

Prolonged MSC expansion significantly increased IL-10 secretion following untreated MSC-macrophage co-culture (figure 4.7A, Friedman $Q = 7.8$, $p=0.036$); IL-10 secretion was significantly higher at P9 compared to P3 (figure 4.7A, rank sum difference = 10.0, $p=0.037$). Prolonged MSC expansion significantly reduced IL-10 secretion following LPS-treated MSC-macrophage co-culture (figure 4.7B, Friedman $Q = 8.0$, $p=0.0046$) IL-10 secretion was significantly lower at P9 compared to P3 (figure 4.7B, rank sum difference = -8.0, $p=0.014$). THP-1 macrophages or MSCs cultured alone did not secrete detectable amounts of IL-10; this was unaffected by the presence or absence of LPS.

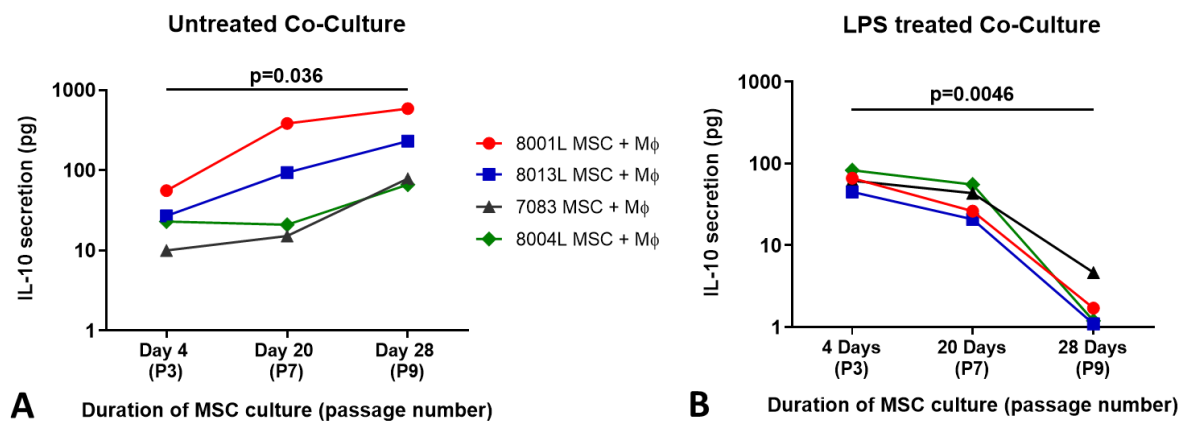


Figure 4.7: Effect of prolonged MSC expansion on IL-10 secretion following MSC-THP1 Macrophage direct co-culture

All data shown are for secretion over 24 hours. Non-conditioned control media (α MEM + 5% FBS) contained no detectable IL-10. Values averaged from 3 replicates, $n=4$ for all groups. All graphs shown with log scale. **A:** Prolonged MSC expansion significantly increased IL-10 secretion following untreated MSC-macrophage co-culture (Friedman $Q = 7.8$, $p=0.036$). IL-10 secretion was significantly higher at P9 compared to P3 (rank sum differences = 10.0, $p=0.037$). **B:** Prolonged MSC expansion significantly reduced IL-10 secretion following LPS-treated MSC-macrophage co-culture (Friedman $Q = 8.0$, $p=0.0046$) IL-10 secretion was significantly lower at P9 compared to P3 (rank sum differences = -8.0, $p=0.014$).

In a separate set of experiments, lung resection-derived primary alveolar macrophages (AMs) were incubated with untreated or cytomix-treated MSC-CM for 24 hours prior to performing efferocytosis and phagocytosis assays. Incubation of primary AMs with untreated MSC-CM significantly reduced the efferocytosis index (figure 4.8, Friedman Q = 6.5, $p=0.042$); P3 untreated MSC-CM significantly reduced AM efferocytosis compared to control media (figure 4.8, rank sum differences = 7.3, $p=0.040$). However, incubation of primary AMs with cytomix-treated MSC-CM had no significant effect on the efferocytosis index (figure 4.8, Friedman Q = 0.5, $p=0.931$).

Incubation of primary AMs with untreated MSC-CM (figure 4.9, Friedman Q = 2.0, $p=0.431$) or cytomix-treated MSC-CM (figure 4.9, Friedman Q = 0.0, $p=0.999$) had no significant effect on the phagocytic index.

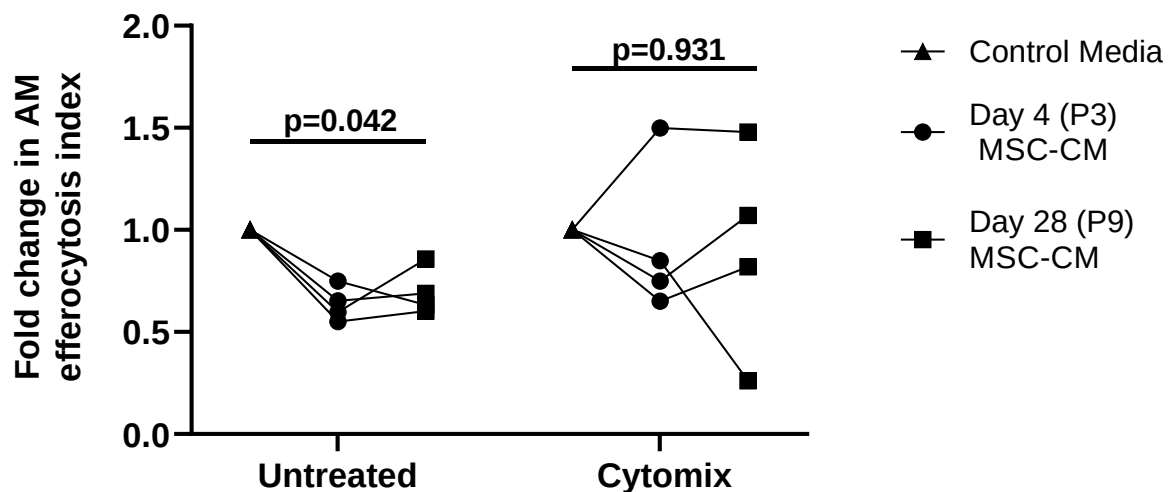


Figure 4.8: Effect of prolonged MSC expansion on the ability of MSC-CM to influence alveolar macrophage efferocytosis.

Incubation of primary AMs with untreated MSC-CM significantly reduced the efferocytosis index (Friedman Q = 6.5, $p=0.042$); P3 untreated MSC-CM significantly reduced AM efferocytosis compared to control media (rank sum differences = 7.3, $p=0.040$). However, incubation of primary AMs with cytomix-treated MSC-CM had no significant effect on the efferocytosis index (Friedman Q = 0.5, $p=0.931$).

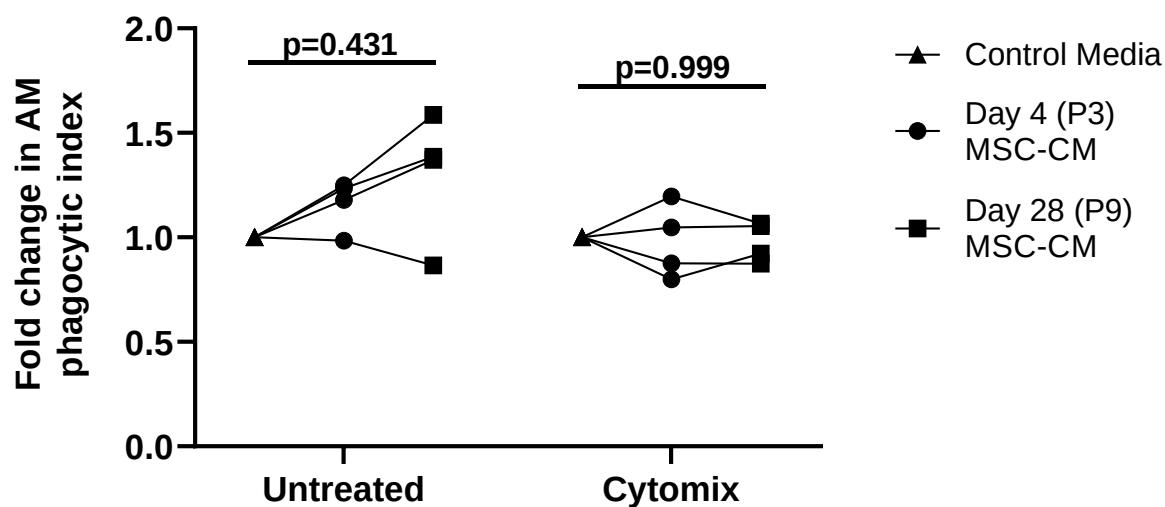


Figure 4.9: Effect of prolonged MSC expansion on the ability of MSC conditioned media to influence alveolar macrophage phagocytosis of *S. Aureus* bioparticles.

Incubation of primary AMs with untreated MSC-CM has no significant effect on the phagocytic index (Friedman $Q = 2.0$, $p=0.431$). Incubation of primary AMs with cytomix-treated MSC-CM had no significant effect on the efferocytosis index (Friedman $Q = 0.0$, $p=0.999$).

4.3.4. MSCs maintain a stem cell phenotype following 1 month of prolonged *in vitro* expansion

After 32 days of *in vitro* culture (passage 10), all MSCs underwent phenotypic assessment with flow cytometric analysis of surface markers (table 4.1) and bi-lineage differentiation. Flow cytometry revealed that MSCs from all 4 donors expressed positive markers CD90, CD105 and CD73. MSCs from all 4 donors also lacked expression of negative markers CD14, CD19, CD34, CD45 and HLA-DR. Bi-lineage differentiation experiments showed that MSCs from all 4 donors were able to differentiate down osteogenic and adipogenic lines. Representative images of P10 8001 MSCs following osteogenic and adipogenic differentiation are shown in figure

4.10. These results indicate that all MSCs retained a stem cell phenotype following 32 days of *in vitro* culture, including 10 passages.

		7083	8013	8001	8004
Positive Markers	CD90	99.0%	98.4%	98.1%	97.9%
	CD73	96.4%	95.3%	97.6%	96.8%
	CD105	97.1%	95.9%	98.4%	99.3%
Negative Marker Cocktail	CD14	0.6%	0.3%	0.2%	0.4%
	CD19				
	CD34				
	CD45				
	HLA-DR				

Table 4.1: Assessment of MSC surface markers to confirm maintenance of stem cell phenotype

All MSCs underwent phenotypic assessment after 32 days *in vitro* culture (P10).

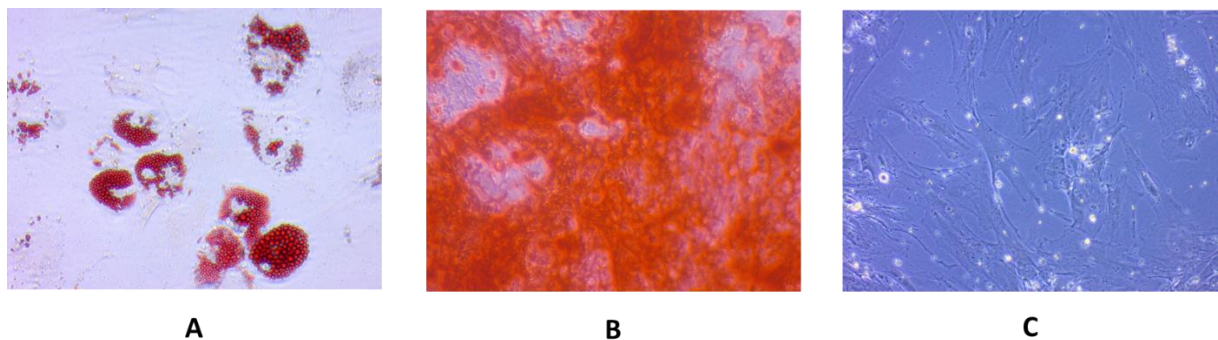


Figure 4.10: Representative bi-lineage differentiation of 8001 MSCs following 32 days *in vitro* expansion (passage 10)

A: Adipogenic differentiation media followed by Oil Red O staining, 100x magnification **B:** Osteogenic differentiation media followed by Alizarin Red S staining, 100x magnification **C:** Control MSC culture media (α MEM + 16% FBS, should not cause differentiation) followed by dual staining, 100x magnification

4.4. DISCUSSION

Changes in the MSC pro-repair secretome following prolonged expansion are more diverse than initially expected. Firstly, there appears to be a large degree of variability in the amount of pro-repair factors secreted by MSC from different donors. For example, in figure 4.1A unstimulated MSCs from 7083 produce double the amount of VEGF compared to 8001, at all passages. The impact of cytomix on pro-repair factor secretion by MSCs is also variable. MSC secretion of VEGF appears to be blunted by the presence of cytomix at all passages, however cytomix has no impact on TGF- β 1 secretion. Prolonged expansion of cytomix-treated MSCs causes a small but significant increase in Ang-1 secretion. Choi et al(238) previously found that VEGF secretion by MSCs decreases with prolonged expansion, in contrast to the findings presented here. However, the MSCs used by Choi et al were derived from an older population of patients with amyotrophic lateral sclerosis, which may account for the differences seen since the MSCs used in my study were derived from young (22-24 year old) healthy volunteers. The benefits of using MSCs from multiple donors allowed me to show that changes in secreted factors with prolonged expansion were a general feature of the MSC cell type, and were not just specific to MSCs from a single individual.

This discussion will mainly focus on assessing the content of MSC-CM derived from cytomix-treated MSCs, as this is most relevant to the inflammatory environment of ARDS. VEGF, Ang-1 and TGF- β 1 are all associated with epithelial and endothelial repair in the context of ARDS. Prolonged expansion causes an increase in Ang-1 secretion, but no significant effect on VEGF and TGF- β 1 secretion by cytomix-treated MSCs. These results are supported by the finding that prolonged expansion has no

significant effect on the ability of CM from cytomix-treated MSCs to promote wound healing. In terms of further work, the impact of prolonged expansion on the ability of MSC-CM to promote endothelial cell proliferation needs to be assessed. The prolonged MSC expansion experiments need to be repeated, with MSCs cultured in serum-free media (instead of 5% FBS) prior to CM collection. This would allow isolation of microvesicles, and to determine whether prolonged expansion has an effect on their production. Unfortunately, FBS contains microvesicles, and it would not be possible to distinguish which microvesicles were produced by MSCs in the existing CM samples.

Cytomix treated MSCs did not secrete detectable amounts of KGF. On review of the literature, previous studies have shown that MSCs treated with KGF siRNA lose their ability to restore fluid transport across human type 2 alveolar epithelial cells in a transwell co-culture model(192). Human ex-vivo lung perfusion (EVLP) models have shown that MSCs and MSC-CM could restore alveolar fluid clearance and raise alveolar fluid KGF levels in an endotoxin-injured lung, however this effect was reduced if MSCs were pre-treated with KGF siRNA (192). It also been shown that in murine models of direct lung injury from LPS or live *E. Coli*, administration of MSC-derived microvesicles (MVs) can attenuate lung injury and result in elevated levels of KGF in the broncho-alveolar lavage fluid(195, 196). MSC-MVs were found to contain KGF RNA, and pre-treatment of MSCs with KGF siRNA partially reduced the therapeutic effects of MSC-MVs(196). It has also been shown that type 2 alveolar epithelial cells take up MSC-MVs in a CD44 receptor dependant manner(195). However, there is no evidence in the literature that MSCs directly secrete KGF themselves. Based on the findings presented here and data from the literature, it was

postulated that stimulated MSCs secrete negligible levels of KGF protein, but do release MVs containing KGF RNA. When MSCs or MSC-CM are administered in murine or EVLP models of lung injury, type 2 alveolar epithelial cells take up these MSC-MVs containing KGF RNA, and subsequently secrete KGF protein. To confirm this, primary human type 2 alveolar epithelial cells will need to be cultured with CM from cytomix-stimulated MSCs, and after 24 hours KGF secretion will need to be quantified.

The MSC-THP1 macrophage co-culture experiments showed that prolonged MSC expansion did not have a significant effect on PGE₂ secretion in the presence of LPS. However, prolonged MSC expansion significantly decreased IL-10 secretion from LPS-stimulated MSC-THP1 macrophage co-cultures. A significant decrease was seen between P3 and P7, then again between P7 and P9. IL-10 is an anti-inflammatory cytokine secreted by M2 (pro-resolution) macrophages. Importantly, this indicates that the ability of MSCs to induce a pro-resolving phenotype in macrophages decreases with prolonged *in vitro* expansion. It has previously been shown that PGE₂ secretion by MSCs stimulates macrophages to release IL-10(187). However, IL-10 release appears to decrease in spite of PGE₂ levels remaining relatively constant. Therefore, there is either an additional pathway present by which MSCs stimulate macrophages to release IL-10, or these experiments are not sufficiently powered to detect a significant decrease in PGE₂ secretion with prolonged expansion. The effect of MSC-CM on macrophage efferocytosis had not previously been investigated in the literature. CM from untreated low-passage MSCs unexpectedly decreases alveolar macrophage (AM) efferocytosis. Interestingly, CM from cytomix-treated MSCs has no effect on AM efferocytosis at either P3 or P9.

The surface marker assessments and bi-lineage differentiation experiments prove that all MSCs maintain a stem cell phenotype following 10 passages and 32 days of *in vitro* culture. This was done to show that changes seen with prolonged MSC expansion were not due to differentiation of MSCs into another cell type.

There are several limitations to the work presented in this chapter. Firstly, by only investigating MSCs from 4 donors, it is inevitable that these experiments lacked sufficient power to detect a difference in secreted factors caused by prolonged expansion. A total of 5 donors would be required in order to detect a difference in VEGF secretion from cytomix-treated MSCs and in TGF- β 1 secretion from untreated MSCs caused by expansion (power 0.8, $p=0.05$). However, the Institute of Regenerative Medicine at Texas A&M, from where MSCs were purchased, had a limited number of donors and not all MSCs available were at the passage required (P2). Another limitation was the use of cell lines, such as A549 cells for wound healing experiments, and THP-1 cells to generate macrophages for co-culture experiments. Use of primary cells would have provided a closer model for human cellular interactions, as they behave in a more physiological manner than cell lines. However, if primary type 2 alveolar epithelial cells had been used for wound healing and primary alveolar macrophages for co-culture, there may have been significant variations in the response of primary cells from different donors. Use of cell lines removes this source of variability from the experiments. Also, due to infrequent supply of lung tissue resections, it could not be guaranteed that there would be primary alveolar macrophages available for co-culture when MSCs reached passage time points. With regards to future work, the relevant experiments would need to be undertaken with both cell lines and primary cells.

A further limitation of the study was using MSC-CM treatment of AMs and A549 cells, instead of transwell co-culture with MSCs (albeit for pragmatic reasons as described above). Transwell co-culture would have allowed dynamic communication between cell types by way of cytokine and MV release. MSCs can release microvesicles containing mitochondria, cytokines, mRNA and microRNA(195, 196, 215, 218). In a transwell co-culture model, the content of these MSC-MVs may have been dynamically altered due to the presence of the second cell type (AM or A549) within the environment. In contrast, MSC-CM contained MVs generated beforehand, without the presence of a second cell type within the environment, which may have altered the MV cargo composition.

Another limitation of this study is that it was not possible to assess the MSC secretome at passages 1-2. Previous studies investigating therapeutic use of MSC in patients with graft-versus-host disease have shown that efficacy decreases with passage: 1-year survival was 75% with passage 1-2 MSCs, decreasing to 21% with passage 3-4 MSCs(279). The same decrease in efficacy beyond 2 passages may therefore also be relevant when considering therapeutic use of MSCs in ARDS.

In summary, prolonged expansion of MSCs in the presence of inflammatory cytokines does not affect the ability of MSC-CM to promote epithelial wound healing. This is reflected by the fact that prolonged expansion of MSCs either increases (Ang-1) or has no effect on (VEGF, TGF- β 1) secretion of pro-repair mediators. However, prolonged MSC expansion does significantly decrease the ability of MSCs to induce IL-10 release by macrophages, following direct co-culture in the presence of LPS. This effect may occur independently of PGE₂ release by MSCs. These findings

indicate that some of the immuno-modulatory, anti-inflammatory effect of MSCs may decline with prolonged expansion.

**CHAPTER 5 : CREATION AND CHARACTERISATION
OF 11 β HYDROXYSTEROID DEHYDROGENASE
TYPE-1 TRANSGENIC MESENCHYMAL STEM CELLS**

5.1 INTRODUCTION

Numerous pre-clinical studies have shown that administration of mesenchymal stem cells (MSCs) can attenuate inflammation and promote repair in models of lung injury(280). MSCs have been shown to be efficacious in both small and large animal models of lung injury, and in both animal and human injured ex-vivo lung perfusion models(183, 222). Phase 1/2 clinical trials in patients with ARDS have shown that MSC administration is safe, feasible and well-tolerated, with no adverse effects(239-241). MSCs can be expanded *in vitro*, retain their efficacy following cryopreservation(205), and localise to the lungs following intravenous administration(187, 225). Allogenic MSCs can be administered without inducing an immune response(234). All these factors support the use of MSCs as a clinical therapy for ARDS, however further phase 2/3 trials are required to determine MSC efficacy in these patients. However, studies have shown that MSCs lose their intrinsic anti-inflammatory capabilities with repeated *in vitro* passage(238). Data presented in chapter 4 shows that prolonged *in vitro* expansion reduced the ability of MSCs to stimulate IL-10 secretion by macrophages. Therefore, the expansion required to generate sufficient MSCs for clinical therapy may also decrease their anti-inflammatory functions, thereby limiting their therapeutic potential.

Use of transgenic MSCs (tMSCs) in murine models of lung injury have shown that the synergistic anti-inflammatory action of combined cellular and gene therapy can be superior to that of cellular therapy alone. Transgenes delivered by tMSCs in lung injury models have included Interleukin-10(245) and Angiopoietin-1(242, 243). MSCs have also been considered as vectors for gene therapy in lung cancer. Professor Sam Janes' group at University College London (UCL) have created tMSCs

expressing TNF-related apoptosis-inducing ligand (TRAIL), using a lentiviral vector containing the Tet-on system(281, 282). The TRAIL transgene was under the control of a tetracycline promoter and only expressed in the presence of doxycycline. In this way, the Tet-on system allowed efficient control of TRAIL transgene expression. It was shown that TRAIL tMSCs can kill cancer cells in murine lung metastatic cancer and malignant mesothelioma models following intravenous administration(281, 283). Previous work has shown that ARDS patients have a relative alveolar cortisol deficiency due to reduced alveolar macrophage 11 β Hydroxysteroid Dehydrogenase Type-1 (HSD-1) reductase activity(91). *In vivo*, HSD-1 acts as a reductase converting the inactive glucocorticoid cortisone into its active counterpart cortisol, thereby locally amplifying glucocorticoid action within specific tissues(84). Previous studies have demonstrated that elevated HSD-1 reductase activity and cortisol activation promote anti-inflammatory macrophage polarisation, efferocytosis, and resolution of inflammation(175, 176, 284). Preliminary work undertaken prior to this PhD found that human MSCs have negligible mRNA expression of HSD-1. It was postulated that use of tMSCs expressing the HSD-1 transgene would restore normal alveolar HSD-1 reductase activity and correct alveolar cortisol levels in ARDS. The combination of increased alveolar cortisol and the innate immuno-modulatory abilities of the MSCs could act synergistically to enhance alveolar macrophage function and attenuate inflammation in ARDS.

Following creation of a HSD-1 expressing tMSC, it would be necessary to ensure that the cells retain an MSC phenotype. The International Society for Cellular Therapy (ISCT) has recommended a set of criteria to define MSCs(181), which an HSD-1 tMSC would have to be tested against: 1) MSCs must adhere to plastic under

standard culture conditions. 2) MSCs must express CD105, CD73 and CD90, and lack expression of surface markers including CD45, CD34, CD14, CD19 and HLA-DR. 3) MSCs must have the ability to differentiate into osteogenic, adipogenic and chondrogenic cell lines (mesenchymal lineages) under appropriate culture conditions in vitro.

In this chapter, the first aim was to create HSD-1 tMSCs using a lentiviral vector containing the Tet-on system. Doxycycline treatment of tMSCs would activate HSD-1 transgene expression, and tMSCs not treated with doxycycline would act as controls. The second aim was to determine whether the HSD-1 tMSCs maintained an MSC phenotype following transfection, and if functional HSD-1 was being expressed.

5.2 METHODS

5.2.1 Construction of recombinant HSD-1 lentiviral plasmid

The following work was done under the kind supervision of Dr Zhengquiang Yuan and Dr Krishna Kolluri at UCL. A lentiviral plasmid (pRRL-cPPT-hPGK-mcs-WPRE) into which the Tet-on system elements have been introduced previously(282), had the reporter gene MuSEAP excised and replaced with the IRES-eGFP sequence (Internal Ribosome Entry Site – Enhanced Green Fluorescent Protein from pENTR1A) by Professor Sam Janes' group at UCL(281). This modified lentiviral plasmid (a gift from Professor Sam Janes), was used as the backbone for the incorporation of human 11 β Hydroxysteroid Dehydrogenase Type-1 (HSD-1) DNA. Custom forward and reverse primers for amplifying HSD-1, including restriction sites for enzymes BstB1 and MluI, were purchased (Invitrogen, UK). Forward primer sequence was CGT ACG CGT GCC ACC ATG GCT TTT ATG AAA AAA TAT CTC CTC CC. Reverse primer sequence was GTC GTT CGA ACT ACT TGT TTA TGA ATC TGT CC. A schematic of the custom amplification primers for HSD-1 is shown in Figure 5.1.

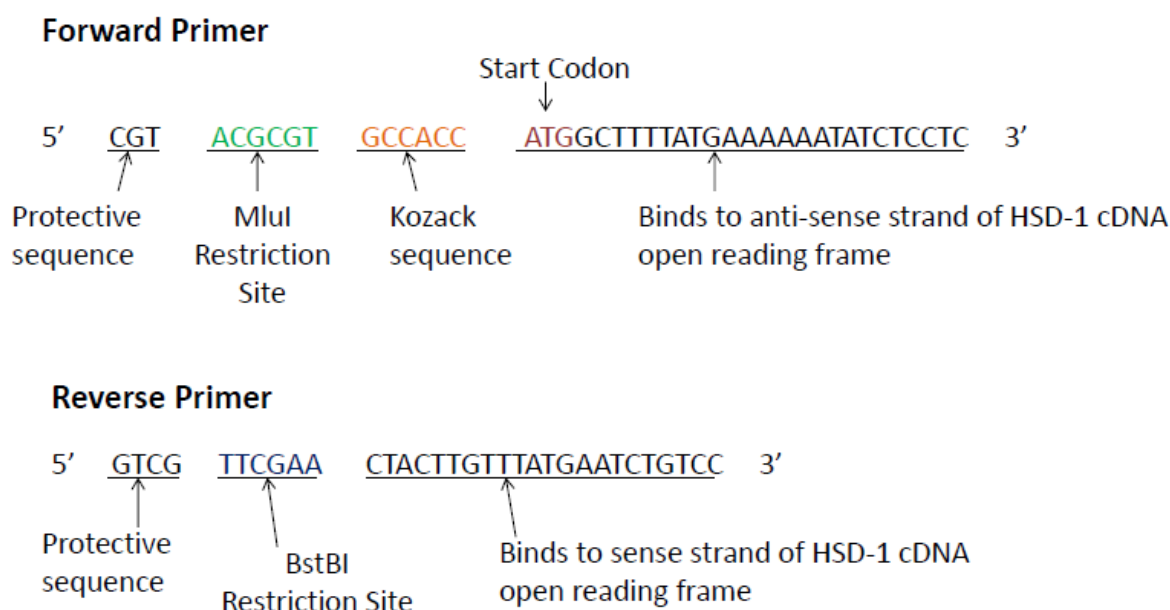


Figure 5.1: Schematic of custom primers for HSD-1 insert amplification

Polymerase chain reaction (PCR) was performed using these custom primers and Q5® High-Fidelity DNA Polymerase (New England Biolabs, USA) to amplify human HSD-1 cDNA (Origene, UK) and add restriction sites for MluI and BstB1. PCR was performed as per manufacturer's (New England Biolabs) protocol, using a gradient thermal cycler (PTC-225, MJ Research, USA). An initial denaturation step of 30 seconds at 98°C was followed by 25 cycles of: 10 seconds at 98°C, 30 seconds at 62°C (annealing step) and 30 seconds at 72°C (elongation step). This was followed by a final extension step of 72°C for 2 minutes. The HSD-1 PCR product was then purified using a Qiaquick PCR Purification Kit (Qiagen, UK) as per manufacturer's protocol. The purified PCR product was then run on an agarose gel electrophoresis to confirm amplification had occurred successfully.

To make agarose gel, 1g of agarose (Bioline) was dissolved in 100mls of Tris/Borate/Ethylenediaminetetraacetic acid (TBE, ThermoFisher) Buffer by heating. 3µl of gel red nucleic acid stain (Biotium) was added to the gel prior to cooling, and a comb used to create wells. The agarose gel was submerged in TBE buffer, and 4µl of 1 kilo base pair (kbp) DNA ladder or HSD-1 PCR product mixed 5:1 with DNA loading dye (Plasmid Factory, UK) was added to wells. A 166V current was passed across the agarose gel for 40 minutes to separate the DNA bands, before reading on a transilluminator (ChemiDoc MP, Bio-Rad, UK). The purified PCR product gave a band just under 900bp, which correlated with the HSD-1 open reading frame of 879bp, indicating that PCR amplification had been successful.

The purified HSD-1 PCR product and the lentiviral backbone plasmid were then both sequentially digested to create “sticky ends”. First digestion with MluI in 3:1 Buffer (New England Biolabs) was performed at 37°C for 12 hours. The digestion products were then purified using the QiaQuick PCR Purification Kit, before being digested with BstB1 in CutSmart Buffer (New England Biolabs) at 65°C for 2 hours. The digestion products were then purified again, and run on agarose gel as above to confirm the DNA was still intact at the correct size. Digestion of the lentiviral backbone plasmid created a break between the Tet-on promoter and the IRES-eGFP. The digested HSD-1 PCR product was then quantified using a spectrophotometer (Nanodrop, ThermoFisher Scientific) as per manufacturers protocol.

The digested HSD-1 PCR product (the “Insert”) was then ligated into the digested modified lentivirus plasmid (the “Vector”) next to the IRES-eGFP, using T4 DNA ligase and reaction buffer (New England Biolabs, USA) as per manufacturer’s

protocol. A 4:1 molar ratio of Insert to Vector was used, and the ligation mixture was incubated at 16°C for overnight. The resulting ligation reaction mixture contained the recombinant plasmid and was used directly for bacterial transformation. A summary of the molecular cloning experiments is shown in Figure 5.2.

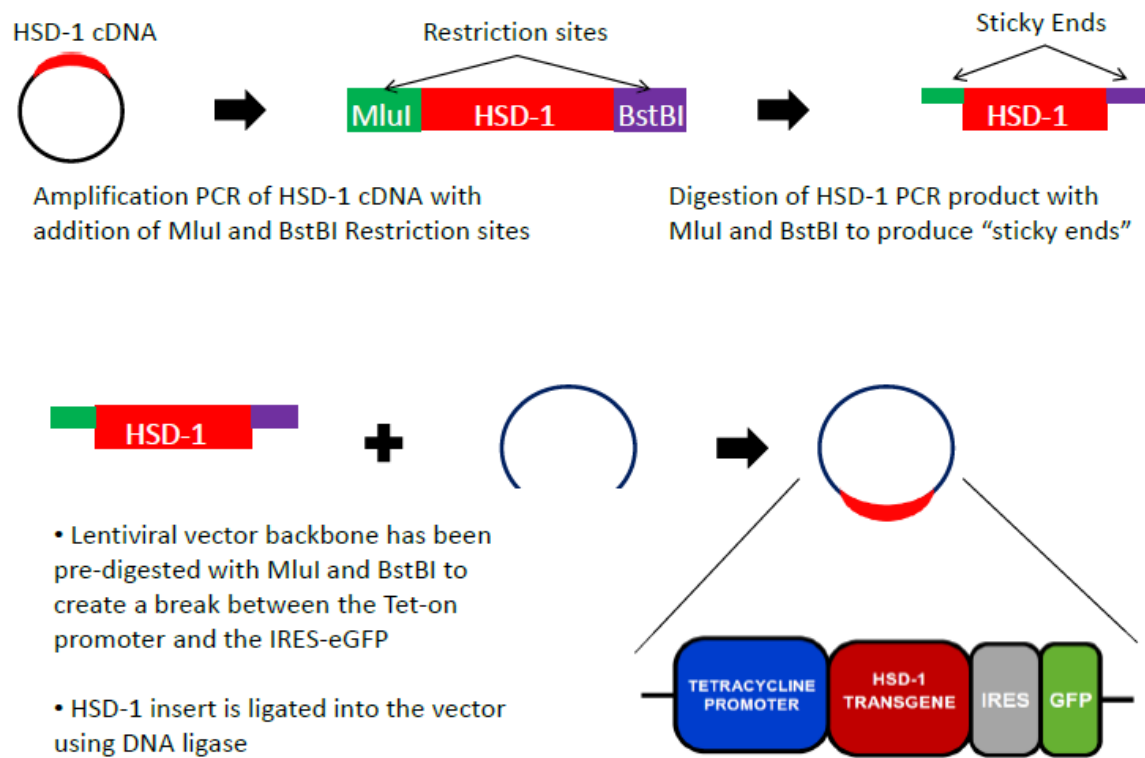


Figure 5.2: Summary of Molecular Cloning experiments

5.2.2 Bacterial Transformation and Plasmid Amplification

Heat-shock competent DH5 α *Escherichia coli* (*E. coli*, ThermoFisher Scientific, UK) were thawed on ice, and 50 μ l pipetted into microcentrifuge tubes. 5 μ l of ligation reaction mixture was added to 50 μ l of DH5 α *E. coli* before incubation on ice for 30 minutes. *E. coli* were then heat-shocked at 42°C for 45 seconds using a water bath. Tubes were then immediately placed on ice for 5 minutes, before 800 μ l Super Optimal medium (SOC medium, Thermofisher Scientific) was added and *E. coli* incubated at 37°C for 1 hour.

LB agar plates were prepared as described previously (section 3.9.7) but with the addition of 100 μ g/ml Ampicillin (Sigma-Aldrich, UK) prior to plating. 30 μ l or 100 μ l of heat-shocked *E. coli* were inoculated onto LB agar plates containing ampicillin, then incubated at 37°C for 24 hours. *E. coli* which had not been transformed were killed due to the ampicillin content of the LB agar plates. Only those *E. coli* which had been successfully been transformed by the recombinant plasmid could survive and form colonies on the plate, since the recombinant plasmid contained a β -lactamase enzyme which provides resistance against penicillins. One such plate is shown in Figure 5.3.

Lennox LB Broth (Sigma-Aldrich) was made by adding one tablet for every 48 mls water and autoclaving at 121°C for 20 minutes; while cooling, 100 μ g/ml ampicillin was added. Colonies of transformed *E. coli* were transferred from agar plates to 30mls ampicillin-supplemented LB broth in 50ml falcon tubes. Tubes were incubated on a 220rpm shaker at 37°C overnight. The next day, 1.5mls of transformed *E. coli* was taken from each tube, and plasmid DNA extracted using a QiaPrep Spin

Miniprep Kit (Qiagen) as per manufacturers protocol. Plasmid DNA concentrations were quantified and run on agarose gel as before, showing a band at ~11kbp (Vector 10kbp + Insert 879bp). To confirm whether extracted plasmid DNA contained the HSD-1 "Insert", and not only the "Vector", plasmids were digested with BstB1 and MluI, then amplification PCR was done using HSD-1 primers, as described above. The resulting mixture was run on agarose gel to confirm the plasmids contained the HSD-1 "Insert".

Next, the extracted plasmids underwent DNA sequence analysis (Source Bioscience, UK) which showed that no mutations had occurred in the HSD-1 Insert following bacterial transformation. Forward and reverse custom sequencing primers had been purchased from Invitrogen, UK: forward primer sequence was TTGCCCATGCTGAAGCAGA, reverse primer sequence was CACATGGTGAATATCATCATG.

The relevant transformed *E. coli* clones were then incubated in large volumes (3 x 150mls) of LB broth in Erlenmeyer flasks on a 150rpm shaker at 37°C overnight. Recombinant plasmid was then isolated using HiSpeed Plasmid Maxip kits (Qiagen, UK) as per manufacturers protocol, before being quantified and run on agarose gel.



Figure 5.3: Colonies of transformed DH5 α *E. coli* on an LB Agar Plate containing 100 μ g/ml Ampicillin

5.2.3 Production of Recombinant HSD-1 Lentivirus

The Human Embryonic Kidney 293T cell line (HEK 293T, Sigma-Aldrich) contains the Simian Vacuolating Virus 40 T antigen, which allows episomal replication of transfected plasmids and their release into the supernatant media. HEK 293T cells were cultured in T175 flasks (ThermoFisher Scientific) with Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) including 10% Fetal Bovine Serum (FBS), 100U/mL penicillin, 100ug/mL streptomycin and 2mM L-glutamine (all from Sigma-Aldrich) at 37°C and 5% CO₂ until reaching 80-90% confluency. Recombinant HSD-1 lentivirus was then produced by transfecting HEK 293T cells using a second generation packaging system. Culture medium was removed from flasks of HEK 293T cells, and replaced with 13mls of supplemented DMEM and 2mls of a 150nM

NaCl solution containing 80µl linear polyethylenimine (jetPEI transfection reagent, Polyplus transfection, USA), 20µg recombinant HSD-1 plasmid, 13µg packaging plasmid pCMV-dR8.74, and 7µg envelope plasmid pMD.G2. Both pCMV-dR8.74 and pMD.G2 were a kind gift from Professor Sam Janes, UCL. After 4 hours incubation at 37°C and 5%CO₂, the medium was removed and replaced with 15mls supplemented DMEM. At 24 hours post-transfection, media was changed again on all flasks, and 10µg/ml doxycycline added. At 48 hours post-transfection, flasks were viewed under fluorescence microscopy (Zeiss AxioVert A1, Zeiss, Germany) to detect presence of the Green Fluorescent Protein (GFP) transgene, which would indicate successful transfection (see Figure 5.4). Media containing recombinant lentivirus was then taken off, centrifuged at 300g for 10 minutes at 4°C (Eppendorf AG 5810R centrifuge, Germany) to pellet any cellular debris, and the supernatant media was filtered through a 0.45µm filter (Sartorius, Germany) before being stored at 4°C. A further 15mls of fresh supplemented DMEM was added to HEK 293T cells and the process repeated after a further 24hrs incubation. The combined filtered supernatant media was transferred to 38.5ml Thinwall Ultra-Clear™ ultracentrifuge tubes (Beckman, USA) and ultracentrifuged (SW28 rotor, Optima LE80K Ultracentrifuge, Beckman) at 52,000g and 4°C for 2 hours to concentrate the lentivirus. Supernatants were then discarded and the lentiviral pellet re-suspended in 100µl serum-free DMEM per ultracentrifuge tube, prior to storage at -80°C.

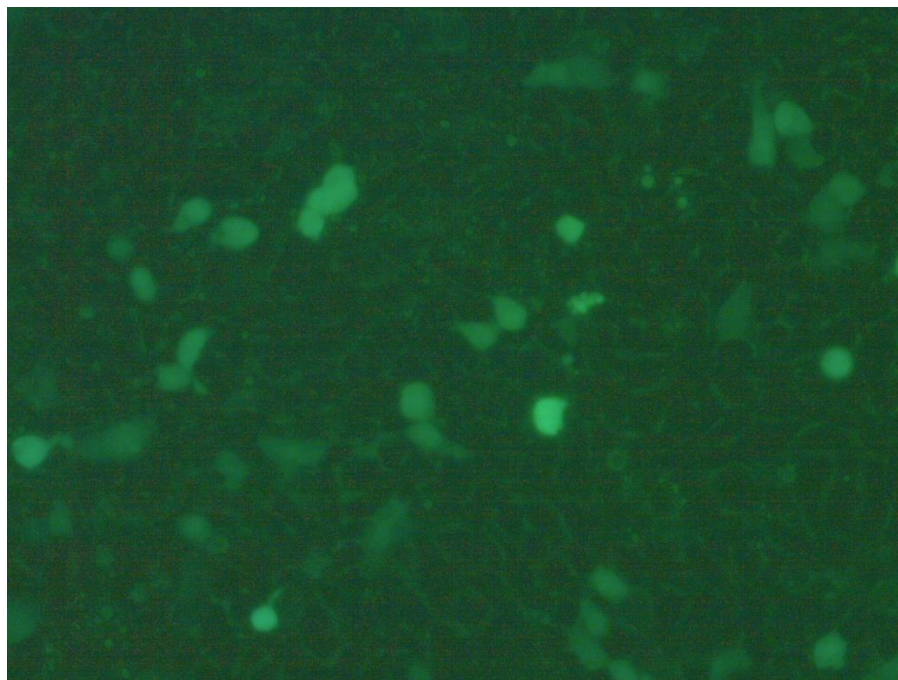


Figure 5.4: Transfected HEK 293T cells expressing GFP under fluorescence microscopy, 200x. Cells treated with 10µg/ml doxycycline for 2 days prior to image capture.

5.2.4. Determination of Viral Titre

HEK 293T cells were plated at 50,000 per well in a 12-well plate and incubated in supplemented DMEM at 37°C and 5%CO₂ overnight. All wells then received 8µg/ml hexamethadine bromide (Polybrene, Sigma-Aldrich, UK) transfection reagent. Recombinant HSD-1 Lentivirus was added to 6 wells by serial dilution: final volumes of lentivirus in each well were 4µl, 2µl, 1µl, 0.5µl, 0.25µl and 0.125µl. Two control wells received polybrene but no lentivirus. After 4 hour incubation as above, media was changed to supplemented DMEM. After a further 24 hours culture, media was changed to supplemented DMEM containing 10µg/ml doxycycline, which acted as the transcriptional activator for the HSD-1 and GFP transgenes. After a further 24hrs

culture, cells were washed in Dulbecco's Phosphate Buffered Saline (PBS, Sigma-Aldrich, UK), then incubated with 500µl of TrypLE express for 2 minutes at 37°C and 5%CO₂. 500µl of supplemented DMEM was then added to wells, and cell suspensions transferred to flow cytometry tubes before centrifuging at 400g for 5 minutes. Supernatants were discarded and cells re-suspended in 400µl PBS, before assessing for GFP expression using flow cytometry (LSR Fortessa X-20, BD Biosciences, USA). One of the control wells was also used to calculate cell number per well, as per section 3.3.5. After determining which volume of lentivirus resulted in approximately 20% of GFP positive cells, the following equation was then used to calculate viral titre:

$$\text{Viral Titre} = \frac{\text{Proportion of GFP positive cells} \times \text{Number of HEK 293T cells}}{\text{Volume of Virus in millilitres}}$$

5.2.5 Transfection of MSCs

Primary human MSCs were cultured in α-Minimal Essential Media (αMEM, ThermoFisher) containing 16% FBS as previously described in section 3.6.1. It was observed that MSCs from donor 8011 showed the most rapid expansion *in vitro*, therefore these MSCs were selected for transfection. Prior to and following transfection, these MSCs were cultured using tetracycline-free FBS (lot 42G9273K, ThermoFisher Scientific), to prevent uncontrolled expression of transgenes. Passage 2 (P2) MSCs from donor 8011 were plated at 100,000 per T75 flask and cultured overnight 37°C and 5%CO₂ to allow adherence. Media was removed from each flask

and replaced with 5mls α MEM containing 8 μ g/ml polybrene and 2.5 μ l lentivirus (200,000 viral transduction units). MSCs were transfected with a multiplicity of infection (MOI) of 2 virus particles for each cell. MSCs were then incubated for 4 hours before changing media to standard α MEM / FBS. After 24hrs incubation, media was changed again, and one flask also received 10 μ g/ml doxycycline. After a further 48 hours incubation, doxycycline treated cells were viewed under fluorescence microscopy to assess GFP expression. If transfection had been successful, Transgenic MSCs (tMSCs) would express both the HSD-1 and GFP transgenes following culture with a transcriptional activator such as doxycycline. Those tMSCs which had been exposed to doxycycline and expressed HSD-1 and GFP transgenes were referred to as transgene-activated tMSCs. Those tMSCs which had not been treated with doxycycline and did not express transgenes were referred to as transgene-inactive tMSCs.

5.2.6 Determination of Transfection Efficiency

Flow cytometry was used to determine the percentage of transgene-activated tMSCs expressing the HSD-1 transgene following intracellular staining. Transgene-active tMSCs and non-transfected MSCs were harvested and split into 3 FACS tubes each containing 1×10^5 cells. Two control tubes were used for each cell type: unlabelled and secondary antibody staining only. 2mls PBS were added to each tube, before centrifuging at 300g for 5 minutes. Supernatants were discarded and cells subsequently fixed in 4% paraformaldehyde in PBS at room temperature for 15 minutes. Cells were then washed in 2mls saponin wash buffer (PBS / 2% FBS / 0.1%

saponin), then made permeable by incubating in 1ml saponin buffer (PBS + 10% FBS + 0.1% saponin) for 1 hour. Cells were then centrifuged as above, before being re-suspended in 500µl saponin wash buffer and incubated for 2 hours at room temperature with primary rabbit anti-human HSD-1 monoclonal antibody (ab157223, Abcam, UK) at 1:100 dilution. Cells were washed as above before being re-suspended in 500µl saponin wash buffer and incubated with secondary donkey anti-rabbit IgG H&L antibody Alexa-fluor® 555 (ab150074, Abcam, UK) at 1:500 dilution for 1 hour at room temperature. Cells were then washed 3 times with saponin wash buffer. Cells were then re-suspended in 400µl PBS containing 2% bovine serum albumin (BSA, Sigma-Aldrich), before being run on flow cytometry (LSR Fortessa X-20, Becton-Dickinson). Alexa-fluor® 555 staining was detected on the phycoerythrin (PE) channel.

5.2.7 Immunofluorescence assessment of HSD-1 Transgene Expression

Immunofluorescence was used to assess expression of HSD-1 protein in transgene-activated tMSCs. 5×10^4 non-transfected MSCs and transgene-activated tMSCs were seeded in duplicate in chamber-well slides (Nunc, Lab-Tek, ThermoFisher Scientific). Cells were cultured for 24 hours in the presence of 10µg/ml doxycycline. Cells were then washed in PBS, and subsequently fixed in 4% paraformaldehyde in PBS at room temperature for 15 minutes. The supernatant was aspirated and cells were then washed in saponin wash buffer (PBS + 2% FBS + 0.1% saponin). Cells were then made permeable by incubating in permeablizing buffer (PBS + 10% FBS + 0.1%

saponin) for 1 hour. The supernatant was aspirated and cells were then incubated for 2 hours at room temperature with primary rabbit anti-human HSD-1 monoclonal antibody (ab157223, Abcam, UK) at 1:100 dilution in saponin wash buffer. Chamber slides were kept on a rocker during incubation, a final volume of 200µl was used per chamber. The supernatant was aspirated, and the cells washed 3 times with saponin wash buffer. Cells were then incubated with secondary donkey anti-rabbit IgG H&L antibody Alexa-fluor® 555 (ab150074, Abcam, UK) at 1:500 dilution in saponin wash buffer for 1 hour at room temperature. The supernatant was aspirated, and the cells washed 3 times with saponin wash buffer. Chamber walls were removed and mounting buffer containing 4',6-diamidino-2-phenylindole (DAPI Prolong Gold Antifade Reagent, ThermoFisher Scientific, UK) was added. Coverslips were placed on the slides and images taken with a fluorescent microscope using the AF555/Rhodamine and DAPI settings.

5.2.8 Western Blot assessment of HSD-1 Transgene Expression

Expression of HSD-1 protein in tMSCs was assessed by Western Blot. 1×10^6 non-transfected MSCs and tMSCs (previously cultured with 2µg/ml doxycycline) were harvested. Cells were washed in PBS, then lysed using 60µl RIPA buffer (Cell Signalling) and incubated on ice for 30mins. Cells were centrifuged at 1100rpm for 5 mins, and the supernatant was then aspirated. Protein concentrations of cell lysates were calculated using the bicinchoninic acid (BCA) assay as described in section 3.8.1.

To make Tris-buffered Saline (TBS), 48.4g Tris-base and 160g NaCl were dissolved in 1L ddH₂O. Then, 1ml Tween 20 (Sigma-Aldrich) was added to 1L TBS to make 0.1% TBS Tween (TBST). Cell lysates were prepared by vortexing 45µg protein with ddH₂O and 4x laemmli buffer (LB). The mixture was heated at 70°C for 10 minutes using a thermal cycler. Samples were vortexed again, then added to a Bolt 4-12% Bis-Tris-Plus 15-well gel (ThermoFisher) at 15µg or 22µg protein/well. A protein ladder was also added to the well furthest on the left. The gel was then run in a Bolt mini gel tank (ThermoFisher) at 165V for 35 minutes using MES SDS running buffer (2-(*N*-morpholino)ethanesulfonic acid, sodium dodecyl sulfate, ThermoFisher). The gel was then transferred onto a membrane using Novex iBlot transfer system (ThermoFisher). A 5% milk solution was made in TBS Tween (TBST). The membrane was blocked with 5% milk/TBST for 1 hour. Primary rabbit anti-HSD-1 monoclonal antibody (ab157223, Abcam, UK) was made up at a 1:10,000 dilution in 10mls of 5% milk/TBST. The membrane was incubated with primary antibody overnight using 50ml tubes at 4°C on a roller. The membrane was then washed 3 times in TBST; 10 minutes for each wash. The membrane was then incubated with Goat polyclonal anti-Rabbit HRP-conjugated antibody (P0448, Dako, USA) at 1:2000 dilution in 10mls of 5% milk/TBST for 1 hour at room temperature. Membrane was then washed 3 times in TBST; 10 minutes for each wash. The membrane was drained on tissue paper then placed on the detector tray. 1ml of Horseradish Peroxidase substrate (HRP substrate, Merck Millipore) was placed on membrane and incubated for 1 minute. The membrane was put in detector (Image Quant LAS 4000, GE, USA) and exposed between 10 seconds and 2 minutes. A multiformat image was created to see both protein ladder and bands

To assess total protein loading, we needed to strip and label the membrane for β -tubulin (component of microtubules, found in all Eukaryotic cells), to prove that all MSC lanes were properly loaded with an equal amount of protein. The membrane was washed in TBS, incubated with stripping buffer for 15 minutes, washed again in TBS, then blocked in 5% milk in TBST for 30 minutes at room temperature. The membrane was then incubated with anti- β -tubulin rabbit antibody (9F3, Cell Signalling, UK) at 1:1000 in 5% milk/TBST for 30 minutes at room temperature, before being washed 3 times in TBST. The membrane was then incubated with same secondary antibody as used previously (anti-Rabbit HRP-conjugated antibody at 1:2000 in 5% milk/TBST) for 1 hour at room temperature, before being washed 3 times in TBST. The membrane was dried, and 1ml of HRP substrate was added, before it was read on detector.

5.2.9 Assessment of transgenic mesenchymal stem cell phenotype

To determine whether tMSCs maintained a stem cell phenotype following transfection, adipogenic and osteogenic differentiation experiments were undertaken as previously describe in section 3.6.4. The tMSCs were also assessed for surface marker expression as previously described in section 3.6.5.

5.2.10 HSD-1 Functional Assays

To determine whether tMSCs were producing functional HSD-1 enzyme, HSD-1 functional assays were undertaken as previously described in section 3.5. In addition

to the previously described method, was that non-transfected MSCs were either cultured in standard media alone, or in the presence of 10µg/ml doxycycline, 50ng/ml tumour necrosis factor-α (TNF-α, Peprotech, UK) or 100ng/ml Lipopolysaccharide (LPS, Sigma-Aldrich) for 48 hours prior to HSD-1 functional assays being performed. To inhibit HSD-1 activity, cells were incubated with 10⁻⁷M glycyrrhetinic acid (Sigma-Aldrich, UK) for 24 hours prior to functional assays being performed.

5.2.11 Statistical Analysis

Data were analysed using Prism 8 software (GraphPad, USA). Normality of data was assessed using the D'Agostino & Pearson test. Differences between continuously distributed non-parametric data were assessed using Mann-Whitney tests. Differences between three or more non-parametric data sets were assessed using the Kruskal-Wallis one-way analysis of variance (ANOVA) and Dunn's multiple comparison tests. Two-tailed p-values of ≤0.05 were considered as significant. Results from parametric data are shown as mean and standard deviation. Results from non-parametric data are shown as median and interquartile range.

5.3 RESULTS

5.3.1 Creation of HSD-1 Recombinant Lentivirus

As described in section 4.2.1, the full sequence of human HSD-1 cDNA was successfully amplified with the addition of MluI and BstBI restriction sites through the use of custom primers and PCR. The size of the amplified HSD-1 PCR product was confirmed on agarose gel as shown in Figure 5.5A. The HSD-1 DNA 'Insert' was then digested and ligated into the lentiviral plasmid 'Vector' to generate a recombinant HSD-1 lentiviral plasmid.

Competent DH5 α *E. coli* underwent heat shock transformation with this recombinant HSD-1 lentiviral plasmid. *E. coli* were cultured on LB Agar plates containing ampicillin in order to select for colonies which had been successfully transformed. Only those *E. coli* which had been successfully transformed by the recombinant plasmid could survive and form colonies on the plate, since the recombinant plasmid contained a β -lactamase enzyme which provides resistance against penicillins. Transformed *E. coli* colonies were then transferred to LB broth containing ampicillin, and cultured to allow plasmid replication. To ensure that no mutations had occurred during the transformation process, plasmids from transformed *E. coli* were extracted and purified. Digested and intact plasmids were run on agarose gels to ensure that the recombinant plasmid still contained the HSD-1 insert, as shown in Figure 5.5B & 5.5C. Extracted recombinant plasmids from transformed *E. coli* were then sent for DNA sequencing, which following analysis showed no evidence of mutation in the HSD-1 insert. Transformed *E. coli* were then grown up by culturing in large volumes of LB Broth, before extracting the recombinant plasmid.

Recombinant HSD-1 lentivirus was then produced by transfecting HEK 293T cells with recombinant HSD-1 lentiviral plasmid and using a second generation packaging system. HEK 293T cells allow episomal replication of transfected plasmids and virus formation within their cytoplasm. The resulting recombinant lentiviruses are released into the supernatant media. Supernatant media from HEK 293T cells was then ultra-centrifuged to concentrate the lentivirus. Differing volumes of lentivirus were then used to transfect HEK 293T cells. Transfected cells were then cultured in the presence of doxycycline, which acts as a transcriptional activator for the HSD-1 and GFP transgenes. After 24 hours, GFP transgene expression was assessed using flow cytometry; the percentage of cells undergoing successful transfection (expressing GFP) was used to calculate lentiviral titre. Figure 5.6 shows that transfection of 75,000 HEK 293T cells using 0.25µl lentivirus results in 22% of cells expressing the GFP transgene. To calculate the number of lentiviral transduction units (TU) per millilitre, the following calculation was done: $(0.22 \times 75,000) / 0.25\mu\text{l} = 66,000 \text{ TU}/\mu\text{l}$. One lentiviral transduction unit is required to transfect one cell.

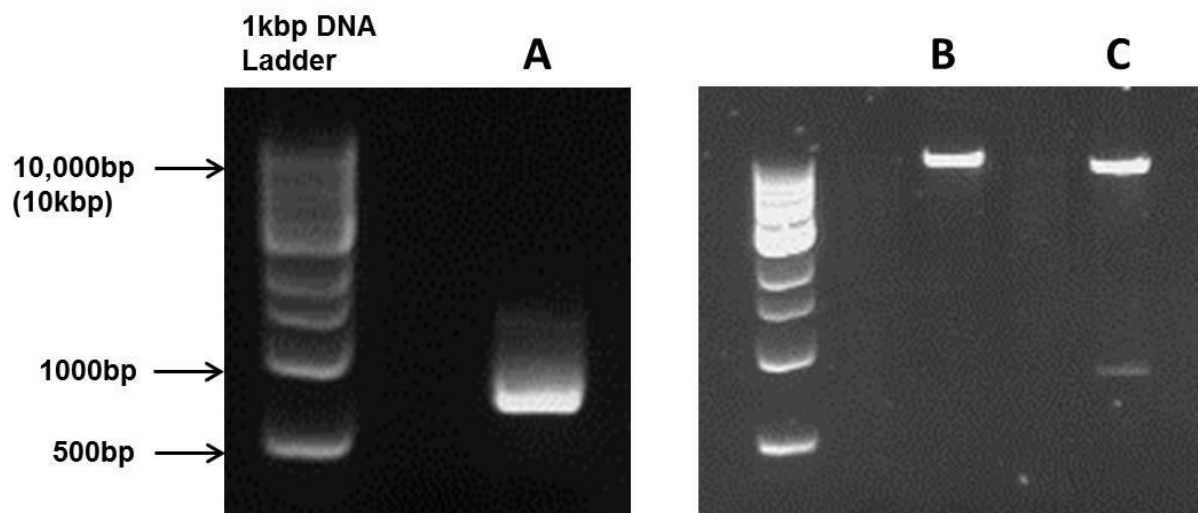


Figure 5.5: DNA electrophoresis using agarose gels.

A: Amplified and purified HSD-1 PCR product gives a band at 879bp. **B:** Recombinant HSD-1 lentiviral plasmid extracted from transformed *E. Coli* (combined Insert + Vector) give a band at ~11kbp. **C:** Recombinant plasmid from 'B' was digested with MluI and BstB1, then purified and run on agarose: shows 2 bands, the lentiviral Vector gives the brighter 10kbp band, and HSD-1 Insert gives the fainter 879bp band.

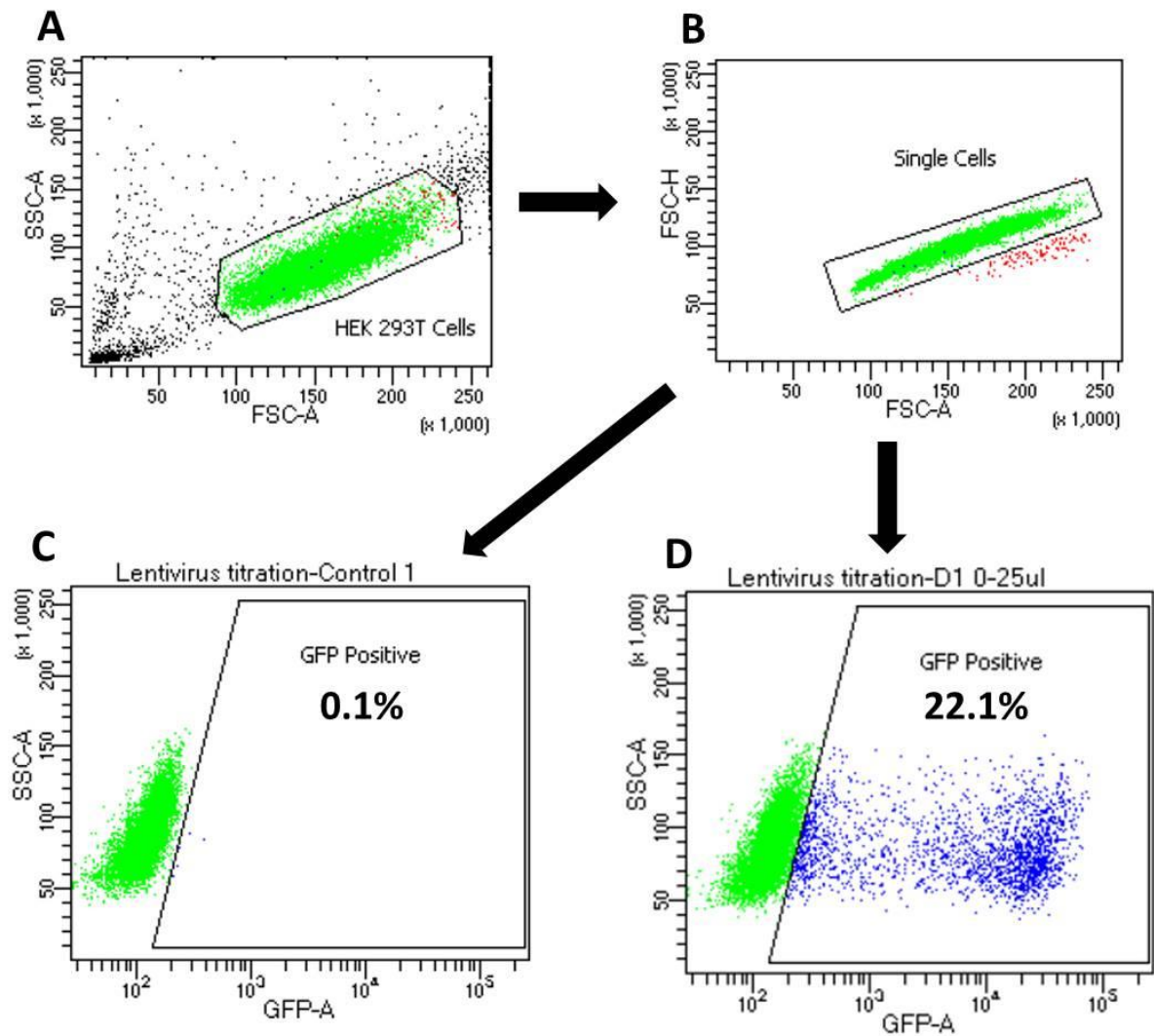


Figure 5.6: Flow cytometry gating strategy for lentiviral titration.

To determine the concentration of recombinant HSD-1 lentivirus, 7.5×10^5 HEK 293T cells were transfected with varying volumes of lentivirus in the presence of 10 μ g/ml doxycycline, which acts as a transcriptional activator for the HSD-1 and GFP transgenes. **A:** HEK 293T cells gated on a forward vs side scatter plot. **B:** Gating to exclude doublets. **C:** Control non-transfected cells used to position the GFP positive gate. **D:** Use of 0.25 μ l lentivirus resulted in successful transfection of 22% of cells, as indicated by GFP expression detected on the FITC channel.

5.3.2 Creation of HSD-1 Transgenic Mesenchymal Stem Cells

Human Mesenchymal Stem Cells (MSCs) from donor 8011 were chosen to be transfected since MSCs from this donor had shown the more rapid growth *in vitro*. To ensure maximum transfection efficiency, we used a multiplicity of infection (MOI) of 2. Therefore, to transfect 100,000 MSCs at a multiplicity of infection (MOI) of two, 3µl of lentivirus were used: $(100,000 / 66,000 \text{ TU}/\mu\text{l}) \times 2 = 3 \mu\text{l}$. Following transfection and culture with doxycycline, transgenic MSCs (tMSCs) were visualised under fluorescence microscopy to assess for GFP expression, as shown in Figure 5.7. Transgene-activated tMSCs were stained intracellularly for HSD-1 to assess for transfection efficiency, which was found to be 91.1% on flow cytometry (Figure 5.8).

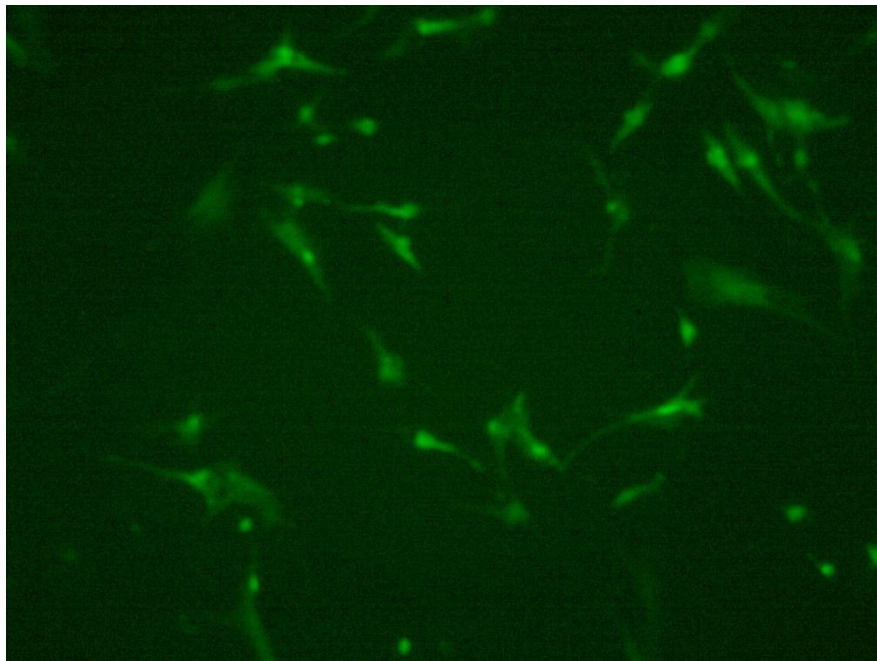


Figure 5.7: Transgene-activated tMSCs expressing GFP under fluorescence microscopy (200x)

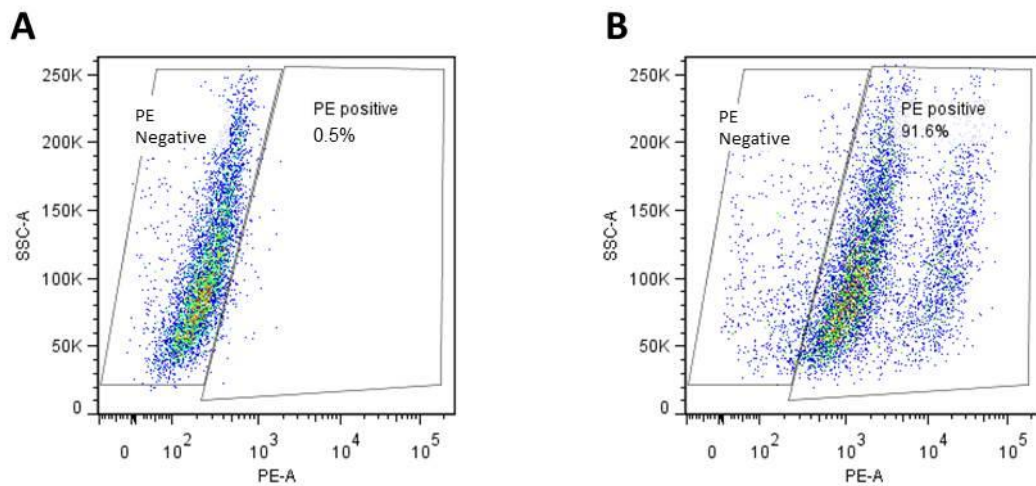


Figure 5.8: Representative flow cytometric assessment of tMSC transfection efficiency

Transgene-activated tMSCs were made permeable with saponin buffer prior to intracellular staining for HSD-1. **A:** tMSCs labelled with AlexaFluor 555-conjugated secondary antibody only. There is a 0.5% false positive value. **B:** tMSCs labelled with both primary (anti-HSD-1) and secondary antibodies. After the false positive value is subtracted ($91.6 - 0.5$), this shows 91.1% transfection efficiency of tMSCs.

5.3.3 Transgenic MSCs express Functional HSD-1

Following successful transfection, the next step was to confirm that transgene-activated tMSCs expressed the HSD-1 protein. Both immunofluorescence and western blot experiments (figures 5.9 and 5.10) found that HSD-1 protein was present in transgene-active tMSCs, and absent in non-transfected MSCs. Immunofluorescence also showed that HSD-1 protein localised to the cytoplasm of tMSCs. These results indicated that the transfection process had been successful and that tMSCs were able to produce the HSD-1 protein when cultured with doxycycline (the transcriptional activator). These experiments also confirmed that there was no expression of HSD-1 in non-transfected MSCs. which supports our group's previous finding that MSCs lack expression of HSD-1 mRNA.

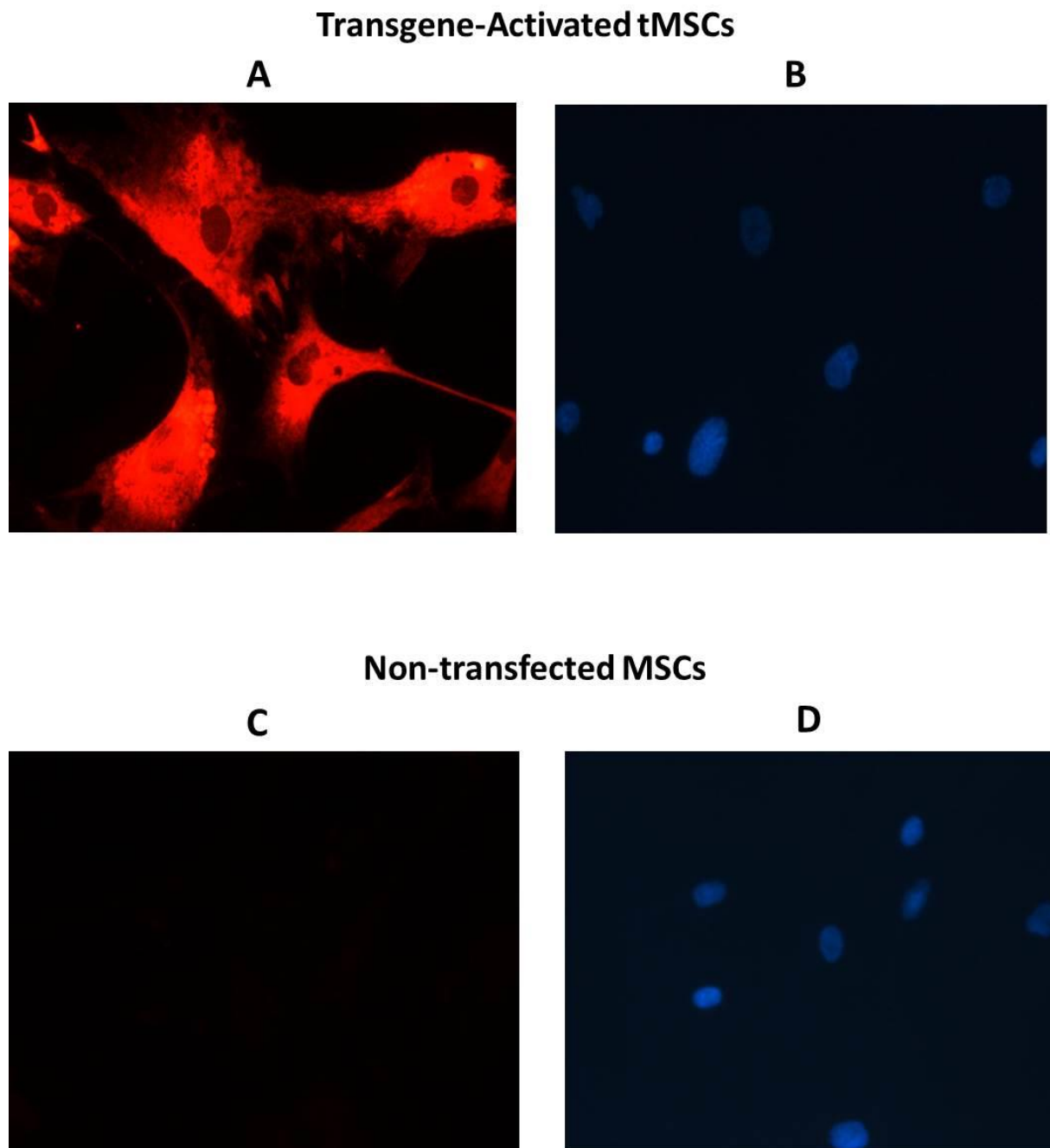


Figure 5.9: Assessment of HSD-1 expression in tMSCs by immunofluorescence
 Cells were made permeable with saponin buffer prior to intracellular staining for HSD-1. Cells were incubated with primary anti-HSD-1 antibody then secondary antibody conjugated to AlexaFluor 555. All images are at 400x magnification. **A:** Transgene-activated tMSCs show cytoplasmic expression of HSD-1 when viewed under the AF555 filter. **B:** DAPI nuclear staining of the same tMSCs shown when viewed under the DAPI filter. **C:** Non-transfected MSCs do not show any fluorescence under the AF555 filter indicating a lack of HSD-1 expression. **D:** DAPI nuclear staining of the same MSCs shown when viewed under the DAPI filter.

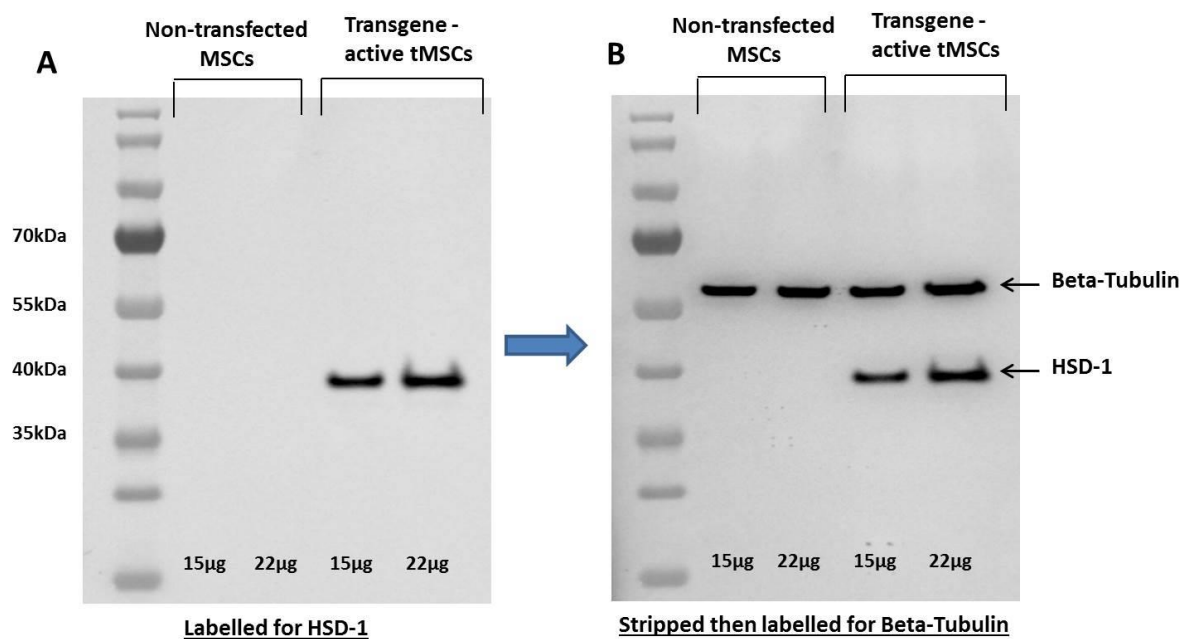


Figure 5.10: Western Blot showing expression of HSD-1 protein by transgene-activated tMSCs

Two different protein concentrations were used for each cell type: 15µg and 22µg. **A:** Protein from non-transfected MSCs and transgene-activated tMSCs run on Western Blot and labelled for HSD-1. The predicted band size of HSD-1 protein is 38kDa. Transgene-active tMSCs expressed HSD-1 protein whereas non-transfected MSCs did not. **B:** The same membrane was stripped (with partial success) and re-labelled for beta-tubulin. This shows that similar amounts of protein had been loaded for both cell types.

The next step was to determine if the HSD-1 protein expressed within tMSCs was functional. The tMSCs were initially treated with doxycycline for differing lengths of time before performing HSD-1 activity assays using thin layer chromatography. This experiment showed that duration of doxycycline treatment significantly affects functional HSD-1 reductase activity in tMSCs (figure 5.11A, Kruskal-Wallis 16.36, $p=0.001$). HSD-1 activity in tMSCs peaks after 48hrs exposure to doxycycline (median 8.23 pM/hour/100,000 cells). HSD-1 reductase activity was also seen in

doxycycline-naïve tMSCs (cultured in media containing tetracycline-free FBS); however this was significantly lower than reductase activity following doxycycline treatment for 48 hours (figure 5.11A, mean rank difference -11.5, $p=0.0025$) or 72 hours (figure 5.11A, mean rank difference -11.17, $p=0.0165$). On fluorescent microscopy and flow cytometry, these doxycycline-naïve tMSCs had undetectable expression of the GFP transgene (data not shown), which should be co-expressed with the HSD-1 transgene. After treating these doxycycline-naïve tMSCs with glycyrrhetic acid (GA) which inhibits HSD-1 activity, the reductase activity significantly diminished (figure 5.12, 2.92 vs 0.44 pM/hour/100,000 cells, $p=0.047$), indicating that the reductase activity observed was due to HSD-1 activity.

In further experiments, tMSCs were initially cultured with doxycycline for 48 hours, then doxycycline was removed from culture, and the duration for which tMSCs subsequently maintained functional HSD-1 activity was assessed. The tMSCs were found to maintain a constant level of functional HSD-1 reductase activity up to 72 hours after doxycycline removal (figure 5.11B, Kruskal-Wallis 3.64, $p=0.303$).

Non-transfected MSCs were either cultured alone, or with doxycycline, or with inflammatory mediators LPS and TNF α for 24 hours to mimic an inflammatory environment. None of these conditions resulted in HSD-1 activity being detected (figure 5.11C, Kruskal-Wallis 26.55, $p<0.0001$). Therefore, the presence of doxycycline or an inflammatory environment does not upregulate HSD-1 expression in non-transfected MSCs. Transgene-active tMSCs were found to exhibit 475 times greater HSD-1 reductase activity than an ARDS alveolar macrophages (figure 5.11C, 8.23 vs 0.0173 pM/hour/100,000 cells, $p=0.014$) and 41 times greater HSD-1 reductase activity than normal alveolar macrophages (8.23 vs 0.2 pM/hour/100,000

cells). Comparative alveolar macrophage HSD-1 activity data is taken from chapter 6 of this thesis. There was no detectable HSD-1 dehydrogenase activity observed in non-transfected MSCs; treatment with doxycycline, TNF α and LPS had no effect (data not shown).

The HSD-1 tMSCs showed significantly lower dehydrogenase activity compared to reductase activity, regardless of the duration of exposure to doxycycline (Figure 5.13). After 48hrs doxycycline treatment, dehydrogenase vs reductase activity was 3.38 vs 8.23 pM/hour/100,000 cells.

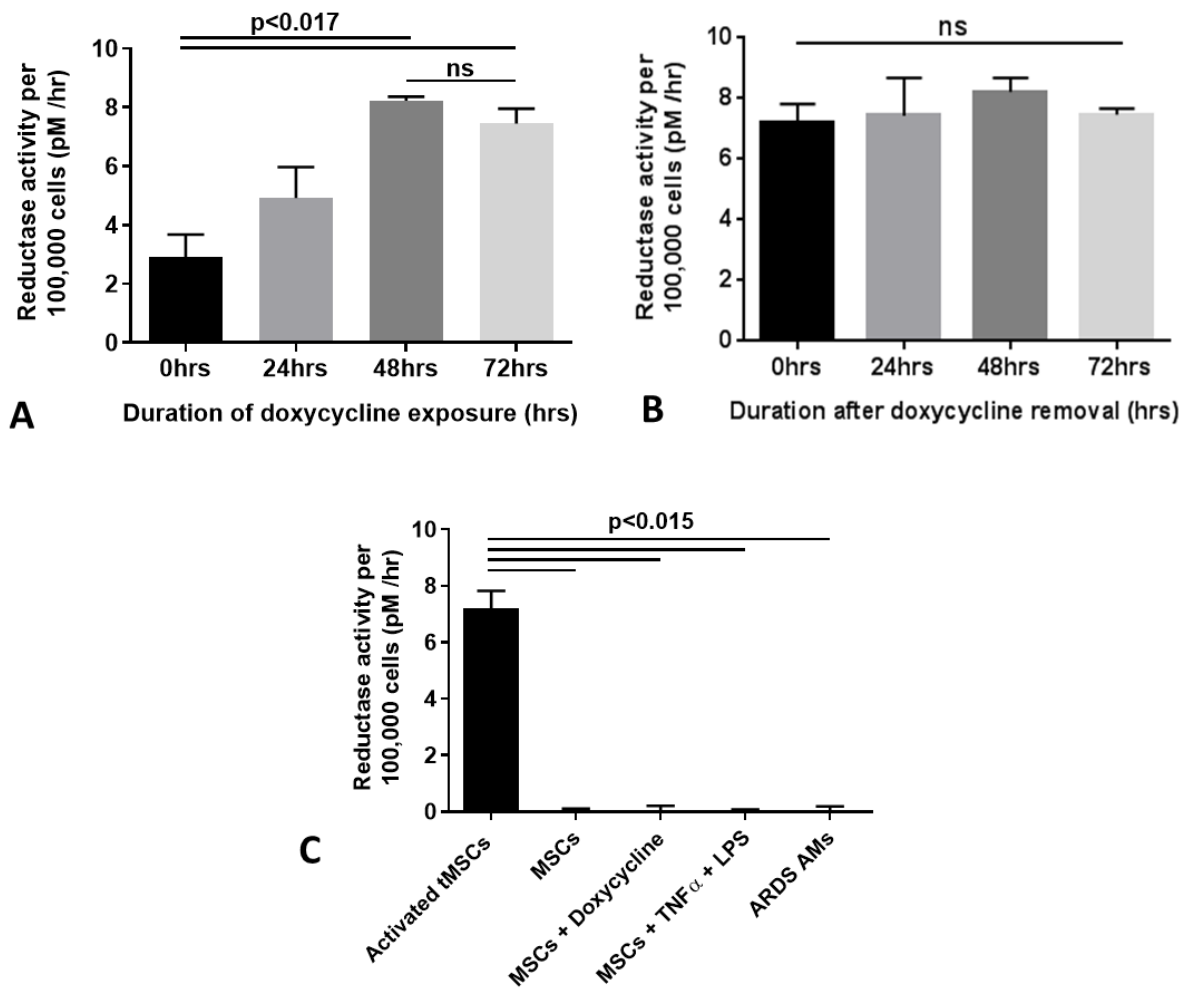


Figure 5.11: HSD-1 functional assays with transgenic MSCs

All data are shown as median and inter-quartile range, with $n \geq 4$ for all conditions. Statistical analysis by Kruskal-Wallis tests and Dunn's multiple comparison test. **A:** Reductase activity of HSD-1 tMSCs following differing durations of doxycycline exposure. Following exposure to doxycycline (which acts as a transcriptional activator), tMSCs express HSD-1 with functional reductase activity (Kruskal-Wallis $p=0.001$). HSD-1 reductase activity plateaus after 48 hours exposure to doxycycline. There is some baseline HSD-1 reductase activity seen in tMSCs without doxycycline exposure, however this is significantly lower than activity following 48 hours doxycycline treatment (mean rank difference -11.5, $p=0.0025$). **B:** This graph shows tMSCs which had previously been exposed to doxycycline for 48 hours, then cultured in doxycycline-free media prior to HSD-1 functional assay. It shows that tMSCs retain a constant level of functional HSD-1 reductase activity for 72 hours following doxycycline removal (Kruskal-Wallis $p=0.303$). **C:** This graph shows that HSD-1 reductase activity is negligible in non-transfected MSCs, and that exposure to doxycycline or an inflammatory environment (TNF α and LPS) have no effect on MSC HSD-1 activity (Kruskal-Wallis $p<0.0001$). The alveolar macrophage HSD-1 reductase data from ARDS patients (ARDS AMs) is taken from chapter 6 of this thesis.

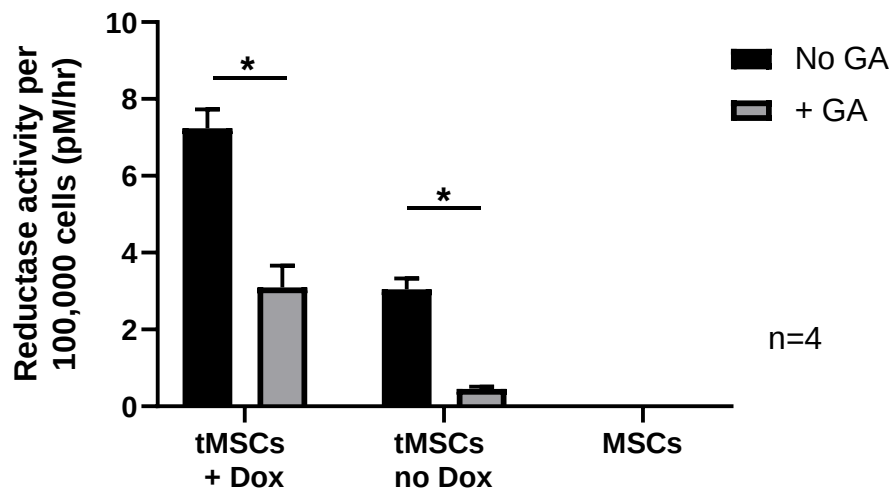


Figure 5.12: Effect of glycyrrhetinic acid on reductase activity of HSD-1 tMSCs
Incubation with 10^{-7} M glycyrrhetinic acid significantly reduces reductase activity of both doxycycline-treated and untreated tMSCs. GA = 24hr treatment of 10^{-7} M glycyrrhetinic acid. Dox = 72hr treatment of $10\mu\text{g/ml}$ doxycycline. Mann-Whitney U test, $n=4$ for all groups, $*p<0.05$

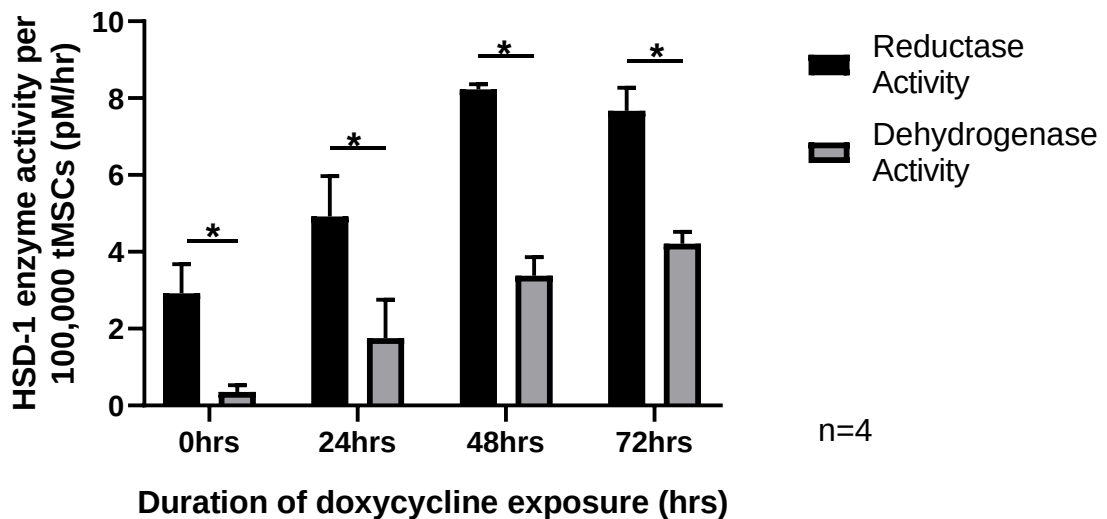


Figure 5.13: Reductase versus dehydrogenase activity of HSD-1 tMSCs
Assessment of HSD-1 reductase and dehydrogenase activity in tMSCs following differing durations of doxycycline exposure. At each time-point of doxycycline exposure, HSD-1 reductase activity is significantly higher than dehydrogenase activity in tMSCs. Mann-Whitney U test, $n=4$ for all groups, $*p<0.05$

5.3.4 HSD-1 Transgenic MSCs maintain an MSC phenotype

In order to show that HSD-1 tMSCs maintain a stem cell phenotype, differentiation capacity and surface marker expression was assessed as per International Society for Cellular Therapy (ISCT) criteria(181). Adipogenic and osteogenic differentiation experiments showed that tMSCs retain the capacity to differentiate down adipogenic and osteogenic lines, to the same degree as non-transfected MSCs (see figure 5.14). This indicates that tMSCs retain multipotency. Chondrogenic differentiation assays were also attempted, however due to technical difficulties associated with generating micromass cultures, these experiments were not completed. The tMSCs also adhere to plastic surfaces equally as well as non-transfected MSCs under standard culture conditions, which is another defining criteria of an MSC according to the ISCT.

Phenotypic assessment of HSD-1 tMSCs with flow cytometric analysis of surface markers was also performed (see figure 5.15). Flow cytometry revealed that tMSCs expressed positive markers CD90, CD105 and CD73. The tMSCs also lacked expression of negative markers CD14, CD19, CD34, CD45 and HLA-DR. Therefore, HSD-1 tMSCs retain the MSC surface marker configuration as per ISCT criteria. These results indicate that tMSCs retain an MSC phenotype and differentiation capabilities following transfection with recombinant HSD-1 lentivirus.

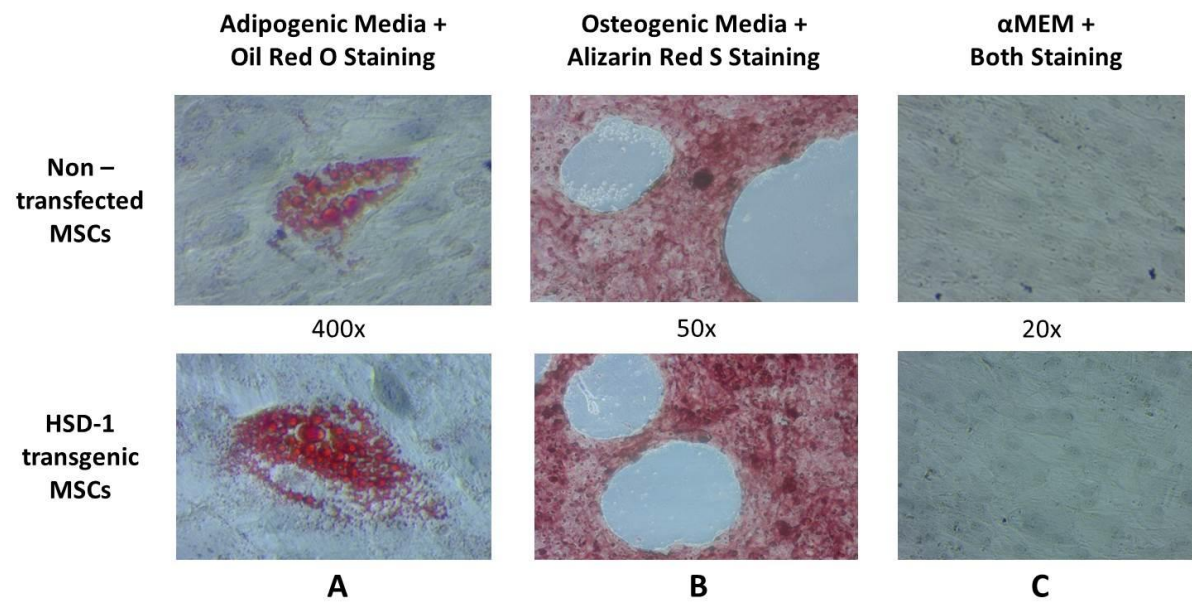


Figure 5.14: Adipogenic and Osteogenic differentiation of tMSCs

All cells shown are at passage 4. Non-transfected MSCs and tMSCs were successfully differentiated down adipogenic (**A**) and osteogenic (**B**) lineages. When cultured in α MEM including 16% FBS (control), both MSCs and tMSCs showed no signs of lineage differentiation on dual staining (**C**).

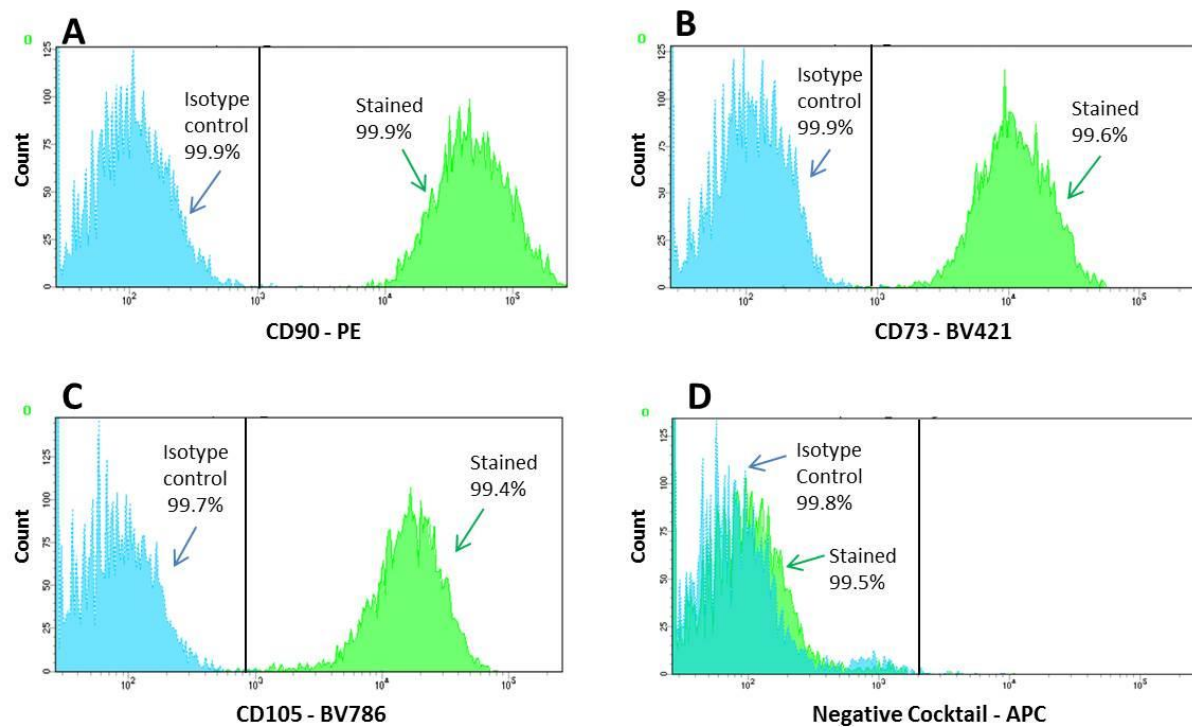


Figure 5.15: Transgenic MSC surface marker expression assessed by flow cytometry

A-C: Show overlaid fluorescence histograms of HSD-1 tMSCs labelled with antibodies against MSC positive markers (CD90, CD73 and CD105) or isotype controls. Distinct separation of histograms indicate that HSD-1 tMSCs do express surface markers CD90, CD73 and CD105. **D:** The negative staining cocktail contained APC-conjugated antibodies against CD14, CD19, CD34, CD45, and HLA-DR. When fluorescence histograms are overlaid, there is near-complete overlap, indicating that HSD-1 tMSCs lack expression of all these surface markers.

5.4 DISCUSSION

This chapter describes the successful creation of a recombinant lentivirus which contains the HSD-1 and GFP transgenes under the control of a tetracycline promoter. Human MSCs were subsequently transfected with this recombinant lentivirus to create HSD-1 tMSCs; a satisfactory transfection efficiency of 91% was achieved. Following exposure to doxycycline, the HSD-1 tMSCs were found to co-express HSD-1 and GFP protein. The HSD-1 protein produced by tMSCs was functional, predominantly as a reductase, converting inactive cortisone into active cortisol. tMSC HSD-1 reductase activity was found to peak and plateau after 48 hours culture with doxycycline. This identified the minimum time required for incubation with doxycycline before maximal HSD-1 reductase activity occurred, and guided the preparation of tMSCs for future experiments.

Use of doxycycline in co-culture experiments and models of lung injury would have a significant confounding factor due to its action as an antibiotic and matrix metalloproteinase (MMP) inhibitor(285). The anti-inflammatory effect of doxycycline is also mediated by downregulation of reactive oxygen species(286), nitric oxide(287) and inflammatory cytokines TNF α and IL-1(287, 288). Therefore, it was important to determine how long maximal HSD-1 activity would be maintained for after doxycycline was removed from tMSCs. It was found that after an initial 48 hours culture with doxycycline, if doxycycline is then removed from culture, the tMSCs maintain constant levels of HSD-1 reductase activity for at least a further 72 hours. Therefore, tMSCs can be pre-treated with doxycycline for 2 days to induce HSD-1 expression, then doxycycline can be removed from culture and HSD-1 activity will be

maintained for the duration of co-culture experiments or murine models of lung injury. This would avoid the model or co-culture system being exposed to doxycycline.

However, doxycycline also directly activates the intracellular PI3K pathway(289), which has a multitude of effects on cellular function including control of the cell cycle, proliferation, polarity, chemotaxis, and migration(290, 291). Doxycycline also inhibits cell migration by phosphorylation of focal adhesion kinase and MMP inhibition(292). Studies have also shown that doxycycline induces changes in metabolic gene expression and glucose metabolism within cells(293). A limitation of this study is that even though doxycycline was removed from tMSCs prior to their use, exposure to doxycycline may have triggered changes to intracellular signalling pathways resulting in differing proliferation, metabolism and function to doxycycline-naïve tMSCs.

The HSD-1 functional assays have confirmed that non-transfected MSCs do not express functional HSD-1. They have also shown that MSCs do not express functional HSD-1 in response to doxycycline, LPS or TNF- α treatment. This is important to clarify, as any functional HSD-1 activity seen in doxycycline-treated tMSCs is therefore due to expression of the HSD-1 transgene, and not due to upregulation of the HSD-1 gene from the native MSC DNA. The tMSCs will be used in co-culture experiments with ARDS alveolar macrophages, and administer in murine models of lung injury. These models will both contain high levels of pro-inflammatory mediators in the alveolar space, including LPS and TNF- α . Therefore, the HSD-1 activity seen in tMSCs exposed to an inflammatory environment is likely to be due to expression of the HSD-1 transgene, and not due to upregulation of the HSD-1 gene from the native MSC DNA.

The HSD-1 functional assays also showed some HSD-1 dehydrogenase activity in tMSCs, i.e. conversion of cortisol into cortisone. It is understood that *in vivo*, HSD-1 acts exclusively as a reductase due a 7-fold greater affinity to cortisone than cortisol(294). Dehydrogenase activity is thought to only occur *in vitro* when HSD-1 expressing cells are artificially exposed to cortisol only(294); which is the environment tMSCs were exposed to when dehydrogenase activity was assessed. The thin-layer chromatography assay used in this study to assess HSD-1 activity has limitations, the most significant being that cells are treated with either tritiated cortisone or cortisol, not a mixture of the two steroids as would be expected *in vivo*. Of the cells exposed to cortisol alone, the direction of action of the enzyme may be forced in the dehydrogenase direction, since cortisol is the only substrate available. Encouragingly, any tMSC dehydrogenase activity observed was significantly lower than reductase activity, following treatment with doxycycline for varying time periods.

When first using the HSD-1 functional assay, tMSCs were cultured with tritiated cortisone for over 18 hours (data not shown), and the detected reductase activity was surprisingly low. We concluded that this was because during a prolonged incubation, the tMSCs had converted all available cortisone (10^{-7}M) into cortisol, and then started acting as a dehydrogenase converting cortisol back into cortisone. The time course of the HSD-1 functional assay was then changed to 6 hours, to ensure that reductase activity could be measured without all the substrate (cortisone) being depleted.

Unexpectedly, HSD-1 reductase activity was observed in tMSCs which had been cultured in media containing tetracycline-free FBS, and never exposed to exogenous doxycycline (transgene-inactive tMSCs). On fluorescent microscopy and flow cytometry, these doxycycline-naïve tMSCs had undetectable expression of the GFP

transgene, which is co-expressed with the HSD-1 transgene. After treating these doxycycline-naïve tMSCs with glycyrrhetic acid (GA) which inhibits HSD-1 activity, the reductase activity significantly diminished, indicating that the observed reductase activity was due to HSD-1 activity. After discussion with Dr Kolluri at UCL, we postulated that there must be some minimal transgene expression in the absence of the transcriptional activator. In the case of the GFP transgene, this protein expression was so minimal as not to be detected on fluorescent microscopy and flow cytometry. However, it appeared that even this minimal expression of HSD-1 protein could result in detectable enzymatic activity; the degree of HSD-1 protein expression is not directly proportional to the functional activity observed. This effect was not seen with doxycycline-naïve TRAIL tMSCs created by the Janes group at UCL(294), because TRAIL is not an enzyme and therefore a minimal amount of protein expression would give an equally minimal effect. Ideally, to control for this effect a null-transfected tMSC would have been created using recombinant lentivirus containing the GFP transgene but not HSD-1. However, due to time constraints this option was unfortunately not possible. Instead, the decision was made to pre-treat “inactive” tMSCs with GA to minimise the underlying HSD-1 activity as much as possible prior to use in transwell co-culture and murine experiments.

It was shown that the tMSCs retain a stem cell phenotype and possess the associated multipotency; the transfection process had not caused differentiation of the MSCs. Therefore, by retaining an MSC phenotype, the retained intrinsic anti-inflammatory capabilities of the tMSCs could combine synergistically with elevated HSD-1 transgene expression to enhance alveolar macrophage (AM) function and reduce inflammation.

In summary, tMSCs which express functional HSD-1 enzyme have been successfully created. These tMSCs will be used in murine models of lung injury and *in vitro* transwell co-culture experiments with AMs from ARDS patients, to determine if this therapeutic strategy could attenuate inflammation in ARDS.

CHAPTER 6 : THE ALVEOLAR MACROPHAGE FUNCTION IN ARDS (AM-ARDS) CLINICAL STUDY

6.1 INTRODUCTION

Acute respiratory distress syndrome (ARDS) remains a major cause of mortality and morbidity in critically ill patients(3). Despite advances in supportive care and ventilation strategies, mortality remains at 26%, and there is no effective pharmacotherapy available(24). The main risk factor for ARDS is sepsis, which is thought to be the underlying aetiology in ~80% of cases(1, 9, 14, 17). We have begun to understand more about how ARDS develops: that it requires damage to the alveolar epithelium and endothelium(29), leading to reduced alveolar fluid clearance(30), increased permeability, exaggerated inflammation(32), and development of a neutrophilic alveolar oedema(31). However, the role of alveolar macrophages (AMs) in the pathogenesis of ARDS is not fully understood.

Administration of mesenchymal stem cells (MSCs) in pre-clinical models of ARDS have shown efficacy at reducing inflammation and aiding the restoration of epithelial / endothelial function(183). However, clinical trials of MSCs in ARDS patients have yet to show the same efficacy, although their administration was found to be safe(239-241). Data presented in chapter 4 shows that the prolonged expansion required to generate enough MSCs for a clinical dose, was associated with reduced immunomodulatory activity. Pre-clinical studies have also shown that use of a combined cellular and gene based therapy may be superior to cellular therapy alone(242-246).

Numerous clinical trials of intravenous glucocorticoids in ARDS have been undertaken, with varying results. A recently meta-analysis of these trials, concluded that low dose methylprednisolone started before day 14 of ARDS onset, and tapered off slowly after achieving unassisted breathing, could reduce mortality in ARDS

patients(295). However, concerns remain with regards to inducing immunosuppression with high dose systemic glucocorticoid therapy in patients whose underlying aetiology is sepsis.

As mentioned above, ARDS is associated with a massive neutrophil influx into the alveolar space. Murine models of ARDS have shown that following influx, large numbers of neutrophils must eventually undergo apoptosis and accumulate within the alveolar space(169). Therefore, it is likely that whilst the inflammatory alveolar environment of early ARDS may initially delay neutrophil apoptosis, these neutrophils will ultimately undergo apoptosis in the alveolar space and accumulate due to the massive influx observed in early disease. Efficient efferocytosis of these apoptotic neutrophils by AMs is critical for the resolution of inflammation in ARDS and promotion of tissue repair (121, 132, 169, 170). The “neutrophil hypothesis” of ARDS(171) suggests that apoptotic neutrophils accumulate in ARDS due to defective AM efferocytosis and/or excessive apoptosis which overwhelms AM efferocytosis capacity. These accumulated apoptotic neutrophils then undergo secondary necrosis, resulting in cell rupture and release of inflammatory cells contents (i.e. Damage Associated Molecular Patterns [DAMPs] including high mobility group box-1 [HMGB-1], heat shock proteins) into the surrounding tissues(117, 118). These mediators are postulated to prolong exaggerated inflammation, characteristic of ARDS.

There is a paucity of studies which have assessed the proportion of alveolar neutrophils which are apoptotic at the time of ARDS diagnosis, immediately post-BAL. Matute Bello et al(168) showed that *in vivo* alveolar neutrophil apoptosis rates were higher in ARDS patients at days 1 and 3 (1.5% and 3%, n=35) compared to at-

risk patients (0.5% and 1.6%, n=13), although absolute values were low and the difference was not significant. Lesur et al(166) showed that *in vivo* alveolar neutrophil apoptosis was reduced in ARDS patients (14.3%, n=9) compared to control critically ill patients (21.7%, n=10). These are the only two studies which assess *in vivo* alveolar neutrophil apoptosis rates at the time of BAL, however they give opposite results, and the absolute values are not comparable. Further studies are required to clarify the degree of alveolar neutrophil apoptosis in ARDS patients.

Thus far, no study has assessed AM efferocytosis in ARDS patients. However, studies have shown that ARDS patients have impaired MDM efferocytosis, and that treatment of healthy macrophages with ARDS patient broncho-alveolar lavage fluid (BALF) suppresses efferocytosis(91, 150). Elevated levels of HMGB-1 have been detected in ARDS patient BALF(91, 149, 150), and HMGB-1 is known to inhibit macrophage efferocytosis(154-156). These findings suggest there may be impaired AM efferocytosis in ARDS.

Work previously undertaken by Dr Christopher Bassford at the University of Birmingham showed that BALF cortisol: cortisone ratios are 52% lower compared to plasma ratios in ARDS patients(91). This would suggest ARDS patients have dysregulated alveolar steroid metabolism causing alveolar cortisol deficiency. HSD-1 is a reductase enzyme which converts cortisone into cortisol, thereby locally amplifying glucocorticoid action within specific tissues(83, 84). The main cell type in the adult lungs that express HSD-1 are AMs(85). Dr Bassford found that alveolar macrophages (AMs) from ARDS patients have impaired 11 β hydroxysteroid dehydrogenase type-1 (HSD-1) reductase activity early in the disease course(91). Cortisol is known to stimulate efferocytosis(147, 176), and macrophage HSD-1

deficiency is associated with impaired efferocytosis in a model of peritonitis(175). Therefore, ARDS AMs with impaired HSD-1 activity may also have impaired efferocytosis. Upregulation of HSD-1 in the alveolar space may restore AM efferocytosis, and therefore offer a potential therapeutic strategy for ARDS.

This chapter investigates the potential pathophysiological link between impaired alveolar macrophage HSD-1 activity, defective alveolar macrophage efferocytosis and resulting accumulation of apoptotic neutrophils in the alveolar space. This chapter also investigates whether a combined MSC and HSD-1 gene therapy could restore alveolar macrophage function and attenuate inflammation in ARDS. This strategy would aim to enhance local alveolar HSD-1 activity and thereby increase alveolar cortisol levels, which should restore normal alveolar macrophage efferocytosis and promote apoptotic neutrophil clearance, thereby attenuating inflammation in ARDS.

6.2 METHODS

For complete methods, see sections 3.1 - 3.5 and 3.8.

6.2.1 Ethical Approval for the AM-ARDS Clinical Study

National research ethical approval was sought for an observational clinical study entitled: “A research study to investigate whether transgenic mesenchymal stem cells expressing hydroxysteroid dehydrogenase type-1 can attenuate inflammation in Acute Respiratory Distress Syndrome” (AM-ARDS). A favourable ethical opinion was given following review by the Wales Research and Ethics Committee 1 (REC 16/WA/0169).

6.2.2 AM-ARDS Study Patient Recruitment

Patients were enrolled into the AM-ARDS study from the intensive care units (ICUs) of the Queen Elizabeth Hospital Birmingham and Birmingham Heartlands Hospital. Two groups of patients were recruited: patients with sepsis-related ARDS, and a control group of sepsis patients at risk of ARDS. In this study, patients would be unable to give informed consent due to alterations in conscious level caused by illness and therapeutic sedation. Therefore, their next of kin would be requested to give assent for the patient to be recruited into the study. If a potential participant had been identified and there was no next of kin available after 24 hours, then the Intensive Care consultant doctor responsible for the patient's clinical care was approached to act as a Nominated Consultee and give an opinion on whether the patient should be enrolled. Following recruitment, samples of blood, urine and broncho-alveolar lavage fluid (BALF) were taken from all patients. If patients

regained the capacity to comprehend the details of the study, retrospective consent was sought to include their data in the study.

6.2.3 AM-ARDS Study Inclusion and Exclusion Criteria

Inclusion Criteria for ARDS Patients

ARDS was diagnosed according to the Berlin criteria(11). Patients in which the suspected cause of ARDS was sepsis were selected, since this was the population that alveolar HSD-1 deficiency was previously identified in. The inclusion criteria for ARDS patients are shown below, all these criteria would need to be met for enrolment:

- Age greater than or equal to 18 years
- Admission to an Intensive Care Unit
- Patient undergoing mechanical ventilation
- Underlying aetiology of infection/sepsis
- Patient must fulfil the Berlin diagnostic criteria for ARDS:
 - Acute onset (within 1 week of clinical insult)
 - Bilateral infiltrates on chest radiograph
 - Respiratory failure not fully explained by heart failure or fluid overload
 - $\text{PaO}_2/\text{FiO}_2$ ratio <40 kPa with PEEP or CPAP ≥ 5 cm H₂O
- ARDS diagnosed within the last 48 hours

Inclusion Criteria for Critically Ill Patients At Risk of ARDS (Control Group)

A control group of patients was identified, who were undergoing mechanical ventilation on ICU, had sepsis, and were at risk of developing ARDS. The inclusion criteria for critically ill patients at-risk of ARDS patients are shown below, all these criteria would need to be met for enrolment:

- Age greater than or equal to 18 years
- Admission to an Intensive Care Unit
- Patient undergoing mechanical ventilation
- Underlying aetiology of infection/sepsis
- Patient NOT meeting all Berlin criteria for ARDS
- Fulfils the Systemic Inflammatory Response Syndrome (SIRS) criteria; must have had 2 or more of the following within the last 24hrs:
 - Temperature >38 or <36 °C
 - Heart rate >90 beats per minute
 - Respiratory rate >20 breaths per minute or $\text{PaCO}_2 <4.3$ kPa (prior to mechanical ventilation)
 - White cell count >12.0 or $<4.0 \times 10^4/\mu\text{l}$

Exclusion Criteria

Under certain circumstances, it was deemed inappropriate for a patient to be enrolled to the AM-ARDS study, even if they met the inclusion criteria. These exclusion criteria mainly related to patient welfare and assent. Patients who were clinically immunosuppressed, or on long term steroid therapy prior to ICU admission, were

also excluded on the basis that these would be confounding factors which would interfere with alveolar macrophage function and HSD-1 activity. Patients with significantly abnormal clotting function were excluded since bronchoscopy could not be safely performed. The vast majority of patients eligible for recruitment to both groups received intravenous hydrocortisone to treat for septic shock on admission to ICU. Excluding patients who received acute corticosteroid treatment would have made recruitment to this study impracticable. Therefore, a pragmatic approach was taken in which patients who had received acute corticosteroid treatment for less than 24 hours at the time of screening would be considered eligible for recruitment. The full exclusion criteria are shown below, meeting any of these criteria would exclude a patient from being enrolled:

- Imminent treatment withdrawal
- Patient on long term steroid therapy prior to current acute illness
- Abnormal clotting function (INR > 3.0 or platelets < 20)
- Clinically relevant immunosuppression for any reason
- Personal consultee, when available, does not provide assent
- Designated consultee, if used, does not provide assent

6.2.4 Clinical Sample Collection and Severity Scoring

Blood, urine and BALF were collected as described in section 3.2. At least 5 mls of BALF was sent to the hospital microbiology department for analysis. The remainder of the sample was placed on ice and transported to the laboratory for processing. Bronchoscopy and collection of BALF was performed within 24 hours of enrolment to the study (day 1). Physiological data including the Sepsis-related Organ Failure

Assessment (SOFA) Score and Acute Physiology and Chronic Health Evaluation II (APACHE II) scores were recorded for each patient as described in section 3.1.4.

6.2.5 Clinical Sample Processing

Blood and urine samples were processed as described in sections 3.3.1 and 3.3.2. BALF was filtered through sterile surgical gauze to remove mucus, into a sterile 50ml centrifuge tube (Greiner Bio-One, Austria). Cell count, viability check and differential cell count were performed as described in sections 3.3.5 and 3.3.6. A sample of filtered BALF cells was used to assess apoptosis on flow cytometry as described in section 3.3.7. The filtered BALF was centrifuged at 560g for 10 minutes at 4°C (Eppendorf AG 5810R, Germany). Acellular BALF supernatant was aspirated and stored in 1ml aliquots at -80°C for future analysis. The cell pellet was re-suspended in 10 mls of RPMI 1640 media (Sigma-Aldrich) containing 10% Foetal Bovine Serum (ThermoFisher). Alveolar macrophages were isolated from this cell suspension using a Lymphoprep™ (StemCell Technologies, Canada) density gradient, then plated according to assay (as described in section 3.3.8). A cell count, viability check and differential count were repeated (see sections 3.3.5 and 3.3.6). AMs were cultured for 24 hours at 37°C with 5% CO₂ to allow adherence to the bottom of the wells. After 24 hours incubation, conditioned media was removed and the AMs were used in functional assays. The conditioned media was centrifuged at 1000g for 5 minutes to pellet cellular debris, before the supernatant was aliquoted and stored at -80°C.

6.2.6 Alveolar Macrophage Functional Assays

Alveolar macrophages (AMs) were used in efferocytosis, phagocytosis, and HSD-1 functional assays (for full details see sections 3.4.1, 3.4.2, and 3.5). Functional assays were started 24hrs after adding AMs to culture plates.

6.2.7 Transwell co-culture of HSD-1 transgenic mesenchymal stem cells with alveolar macrophages

AMs from ARDS patients were co-cultured with HSD-1 transgenic mesenchymal stem cells (tMSCs) at passage 4 prior to efferocytosis assays. The creation of tMSCs is described in chapter 5. Following isolation, AMs were cultured at 2.5×10^5 per well in 24-well plates for 24 hours. Conditioned media was then collected, wells were washed with PBS and media changed to 600 μ l serum-free RPMI 1640. Exogenous cortisone (Sigma-Aldrich) was added to give a final concentration of 10^{-7} M in all wells; this is the physiological circulating concentration of cortisone in humans(296). Transwell® permeable inserts containing a 0.4 μ m polycarbonate microporous membrane (Costar, Corning, USA) were added to each well. Then, 100 μ l of serum-free RPMI containing either 6.25×10^4 transgene-activated tMSCs, transgene-inactive tMSCs or no cells were added to the upper compartment. The ratio of AMs to tMSCs was 4:1. This ratio was chosen to replicate the predicted ratio of MSCs to AMs in the lungs of ARDS patients undergoing clinical trials of MSC therapy(239, 241, 297). Plates were then incubated for 6 hours at 37°C and 5% CO₂. The basis of the 6hr co-culture duration was twofold: Firstly, HSD-1 functional assays on HSD-1 tMSCs performed in chapter 5 revealed that 6 hours allowed 100,000 tMSCs to convert ~50% of the available cortisone into cortisol. As detailed in chapter 5, due to the

artificial nature of this *in vitro* experiment a prolonged culture of more than 8 hours would lead to the risk of the HSD-1 enzyme in tMSCs acting as a dehydrogenase (a function not seen *in vivo*), converting the newly generate cortisol back into cortisone. Secondly, previous murine studies investigating the pharmacodynamics of MSCs following intravenous injection have shown that MSCs do not remain in the lungs for longer than a few hours: Rocheforte et al(298) found that MSC numbers in the lung halved between 1 and 3 hours post-infusion, whereas Eggenhofer et al(236), found an almost 10-fold decrease between 1 and 24 hours post-infusion. Therefore, to recapitulate the duration that tMSCs would be present in the lungs if used as an intravenous clinical therapy, a co-culture duration of several hours was used. The transwell inserts to which tMSCs had adhered to were then removed, and the efferocytosis assay was performed as described in section 3.4.1.

6.2.8 Biochemical Assays on Acellular Samples

Paired BALF and plasma samples were assessed for protein concentration using a bicinchoninic acid assay (section 3.8.1). The protein permeability index was then calculated as the ratio of BALF protein to plasma protein. Luminex[®] performance assays were used to calculate cytokine concentrations in BALF, plasma and AM conditioned media (section 3.8.4). The following cytokines were measured in plasma: Interleukin (IL)-6, IL-8, IL-10, IL-1 receptor antagonist (IL-1ra) and tumour necrosis factor (TNF)- α . These cytokines were also measured in BALF and AM conditioned media, with the addition of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein (MCP)-1. Concentrations of cortisol and cortisone were measured in BALF and serum using mass spectrometry (section 3.8.5).

6.2.9 Statistical Analysis

Dr Christopher Bassford at the University of Birmingham(91) previously showed that ARDS BALF treatment suppressed AM efferocytosis by 20%. Therefore, for the AM-ARDS study to detect a 10% difference in AM efferocytosis between sepsis patients with and without ARDS, with a power of 80% ($p = 0.05$, standard deviation 2.4%), at least 11 patients would be required in each group. Additional patients were recruited to allow for cases in which bronchoscopy was abandoned due to safety concerns, and difficulties with isolating AMs from BALF. An assessor who had been blinded to patient identity assessed neutrophil apoptosis by morphological criteria on BALF cytopins; this was performed in addition to my own blinded assessment.

Data were analysed using Prism 8 software (GraphPad, USA). Normality of data was assessed using the D'Agostino & Pearson test. Differences between continuously distributed data was assessed using unpaired t-tests for parametric data, or Mann-Whitney tests for non-parametric data. Differences between non-parametric paired data were assessed using the Wilcoxon matched-pairs signed rank test. Two-tailed p-values of ≤ 0.05 were considered as significant. Results from parametric data are shown as mean and standard deviation. Results from non-parametric data are shown as median and interquartile range. Linear associations were assessed using Spearman's correlation coefficient. Bonferroni's correction was used to correct significance for multiple correlations.

6.3 RESULTS

6.3.1 Patient recruitment and characteristics

Between December 2016 and February 2019, a total of 38 critically ill sepsis patients were recruited to the AM-ARDS study. Of these, 21 patients had ARDS and 17 patients did not have ARDS. A CONSORT diagram for the AM-ARDS study is shown in figure 6.1. Out of the 38 patients recruited, day 1 BALF was successfully collected from 31 patients. Of these, BALF neutrophil apoptosis was assessed in 21 patients – this laboratory assay was not part of the original study protocol and was added to the protocol after patient recruitment had already begun. Of those patients who had BALF collected, AM yield was sufficient to perform efferocytosis in 22 patients. The average AM yield was 1 million cells per patient. Therefore, it was often difficult to perform both efferocytosis and HSD-1 functional assays for every patient. The efferocytosis assay was given priority, and all AMs isolated were used in these assays. If there were AMs remaining after allocation for efferocytosis assay, then HSD-1 functional assays would be performed. HSD-1 functional assays were performed on AMs from 6 sepsis patients with ARDS, and 6 sepsis patients without ARDS. Ethical approval had been given for a second bronchoscopy and BALF collection at day 4-7, however in practice no patients underwent a second bronchoscopy in this study. This was decided with the senior Intensive Care doctors at both sites: there was thought to be no clinical benefit for these patients once a BALF microbiology sample had already been obtained during day 1 sampling and a potential unnecessary risk to repeat the bronchoscopy. Since only 1 BALF sample was collected per patient, all data henceforth pertaining to alveolar macrophage

function, BALF neutrophil apoptosis and BALF cytokine / protein / steroid concentrations refers to samples taken on day 1 of recruitment.

Reasons for not collecting a blood or urine sample at day 4 - 7 include the patient having died prior to this time point, or the patient having become anuric. Baseline demographic and physiological characteristics for the recruited patients are summarised in table 6.1. There was no significant difference in age, gender, smoking status, ethnicity, oxygenation, mode of lung injury, hospital length of stay (LoS) or inpatient mortality between sepsis patients with and without ARDS ($p > 0.05$ for all). However, SOFA and APACHE II scores were higher in sepsis patients with ARDS, and these differences approached significance ($p = 0.053$ and $p = 0.091$ respectively). As expected, sepsis patients who develop ARDS have more severe organ impairment and worse prognosis than sepsis patients without ARDS. The Murray Lung Injury Score was significantly higher in sepsis patients with ARDS ($p = 0.0085$) than those without ARDS, reflecting the reduced lung compliance and increased pulmonary infiltrates observed in ARDS patients. Sepsis patients who developed ARDS also had significantly reduced ventilator-free days to day 28 ($p = 0.0038$) compared to those without ARDS, indicating that ARDS development was associated with an increased duration of invasive mechanical ventilation. This finding was also supported by increased ICU LoS in sepsis patients who developed ($p = 0.0026$). The percentage of BALF polymorphonuclear cells was higher in sepsis patients with ARDS, reflecting the neutrophil influx into the alveolar compartment normally observed in ARDS patients, but did not reach significance ($p = 0.075$).

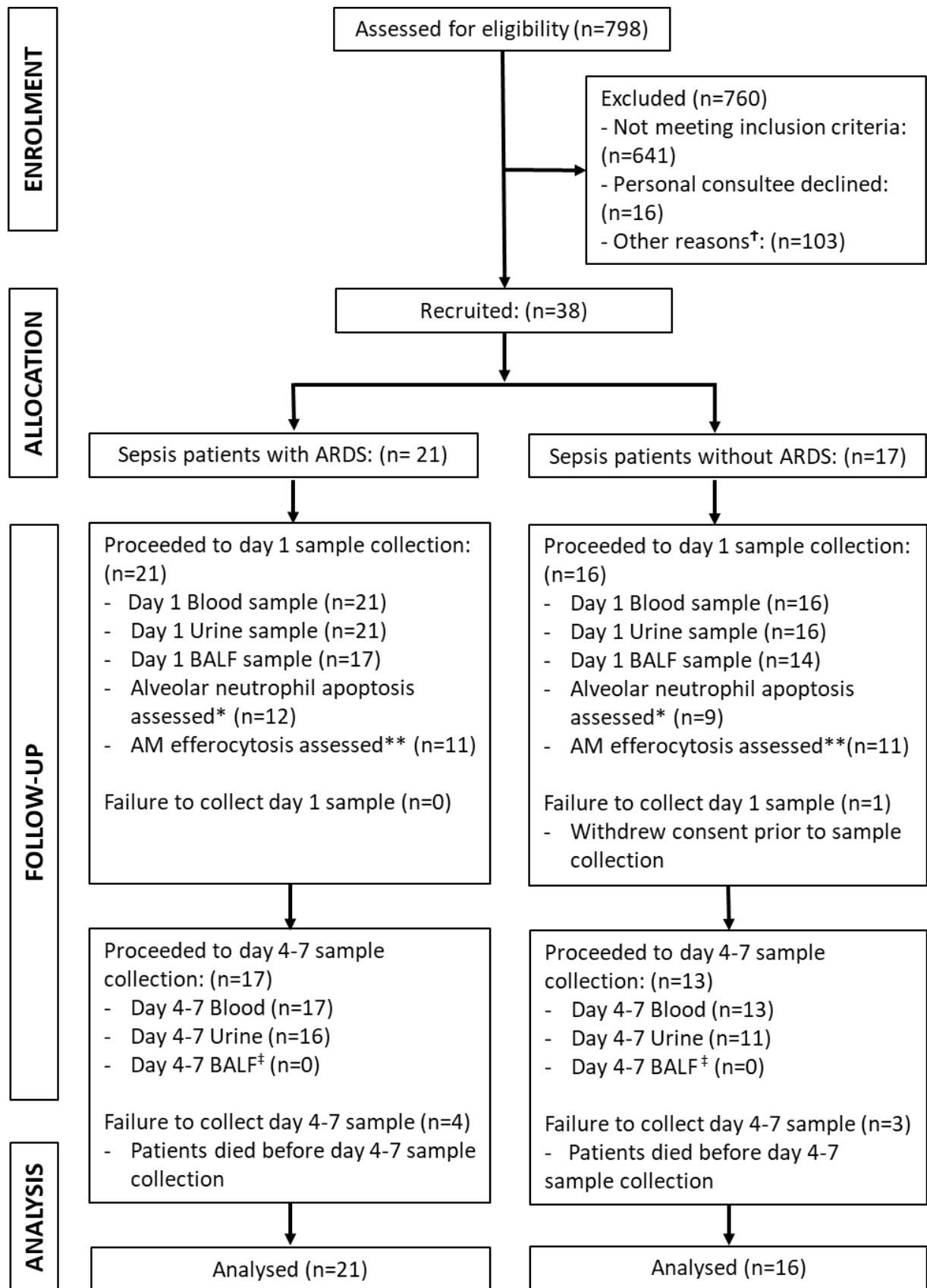


Figure 6.1: CONSORT diagram for AM-ARDS study.

BALF = Broncho-alveolar lavage fluid. AM = alveolar macrophage. †The main 'other reason' for excluding patients was if the ICU consultant responsible for their clinical care did not approve a bronchoscopy. *Neutrophil apoptosis was not initially assessed and was only added to the study protocol after recruitment had already begun. **The average AM yield was 1 million per patient. Therefore, it was often difficult to perform both efferocytosis and HSD-1 assays. The efferocytosis assay was given priority; if there were AMs remaining after allocation for efferocytosis assay, then HSD-1 functional assays would be performed. HSD-1 functional assays were performed on AMs from 6 sepsis patients with ARDS, and 6 sepsis patients without ARDS. If AM yield was insufficient to perform efferocytosis or HSD-1 assays, conditioned media was still collected. ‡A second BALF sample was not obtained from any of the patients, following discussion with the ICU consultant in charge of the patients' clinical care. There was thought to be no clinical benefit and potential unnecessary risk to repeat bronchoscopy in these patients once a BALF microbiology sample had already been obtained during day 1 sampling.

	Sepsis patients with ARDS (n=21)	Sepsis patients without ARDS (n=17)
Age in years: mean (SD)	59.2 (13.9)	55.1 (16.3)
Male Gender: n (%)	15 (71%)	11 (65%)
Smoking status: n (%)		
- Current	7 (33%)	5 (29%)
- Ex-smoker	9 (43%)	6 (35%)
- Never smoker	2 (10%)	1 (6%)
- Unknown	3 (14%)	5 (29%)
Ethnicity:		
Caucasian - n (%)	21 (100%)	17 (100%)
Day 1 P/F ratio: mean (SD)	21.8 (4.9)	24.1 (6.8)
Day 1 SOFA score: mean (SD)	12.5 (3.8)	10.3 (2.7)
Day 1 APACHE II score mean (SD)	18.6 (5.5)	15.2 (5.8)
Day 1 Lung Injury Score mean (SD)	2.57 (0.50)	2.13 (0.46)
Ventilator Free Days to day 28: mean (SD)	6.9 (9.2)	15.9 (8.3)
ICU LoS in days: mean (SD)	24.0 (13.2)	12.8 (7.1)
Hospital LoS in days: mean (SD)	39.0 (31.7)	30.4 (22.0)
Source of infection:		
Pneumonia: n (%)	19 (90)	12 (71)
Inpatient Mortality: n (%)	7 (33.3)	3 (17.6)
BALF leukocyte count x10⁶: median (IQR)	15.8 (7.4 – 31.3)	6.4 (3.8 – 27.0)
PMNs in BALF: % mean (SD)	69.9 (21.5)	49.0 (30.6)

Table 6.1: Patient baseline demographic and physiological characteristics

P/F ratio = ratio of arterial oxygen partial pressure to fractional inspired oxygen. SD = standard deviation. SOFA = Sepsis-related Organ Failure Assessment. APACHE II = Acute Physiology and Chronic Health Evaluation II. IQR = Interquartile range. LoS = Length of stay. ICU = Intensive care unit.

6.3.2 Alveolar macrophage functional assays

The alveolar macrophage (AM) efferocytosis index is significantly impaired in sepsis patients who develop ARDS compared to sepsis patients without ARDS (figure 6.2, medians 6.1% vs 22.7%, $p=0.001$).

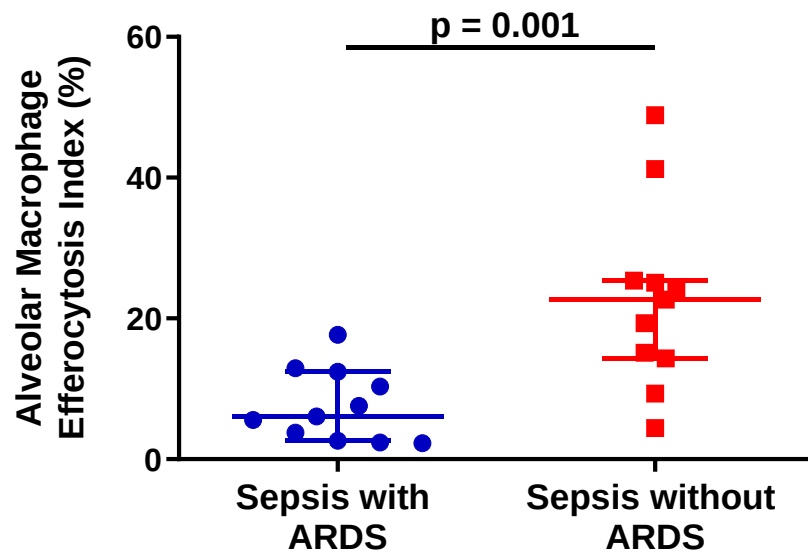


Figure 6.2: Alveolar macrophage efferocytosis index in sepsis patients with or without ARDS

Error bars shown as median and interquartile range. Statistical analysis by Mann Whitney Test, $n=11$ in both groups. Alveolar macrophages from sepsis patients with ARDS have significantly reduced efferocytosis index compared to sepsis patients without ARDS (medians 6.1 vs 22.7, $p=0.001$).

There was no significant correlation observed between AM efferocytosis and age, P/F ratio, SOFA score, APACHE II score and smoking status across both patient groups ($p>0.05$ for all, data not shown). After combining mortality data from sepsis patients with and without ARDS, it was found that patients who survived hospital admission had a significantly higher AM efferocytosis index compared to patients who died during their admission (figure 6.3A, medians 15.1% vs 5.6%, $p = 0.019$),

and at 90 days (figure 6.3B, medians 15.1 vs 5.6%, $p=0.021$). For all sepsis patients, AM efferocytosis index correlated directly with ventilator-free days to day 28 (figure 6.3C, Spearman $r = 0.443$, $p=0.039$).

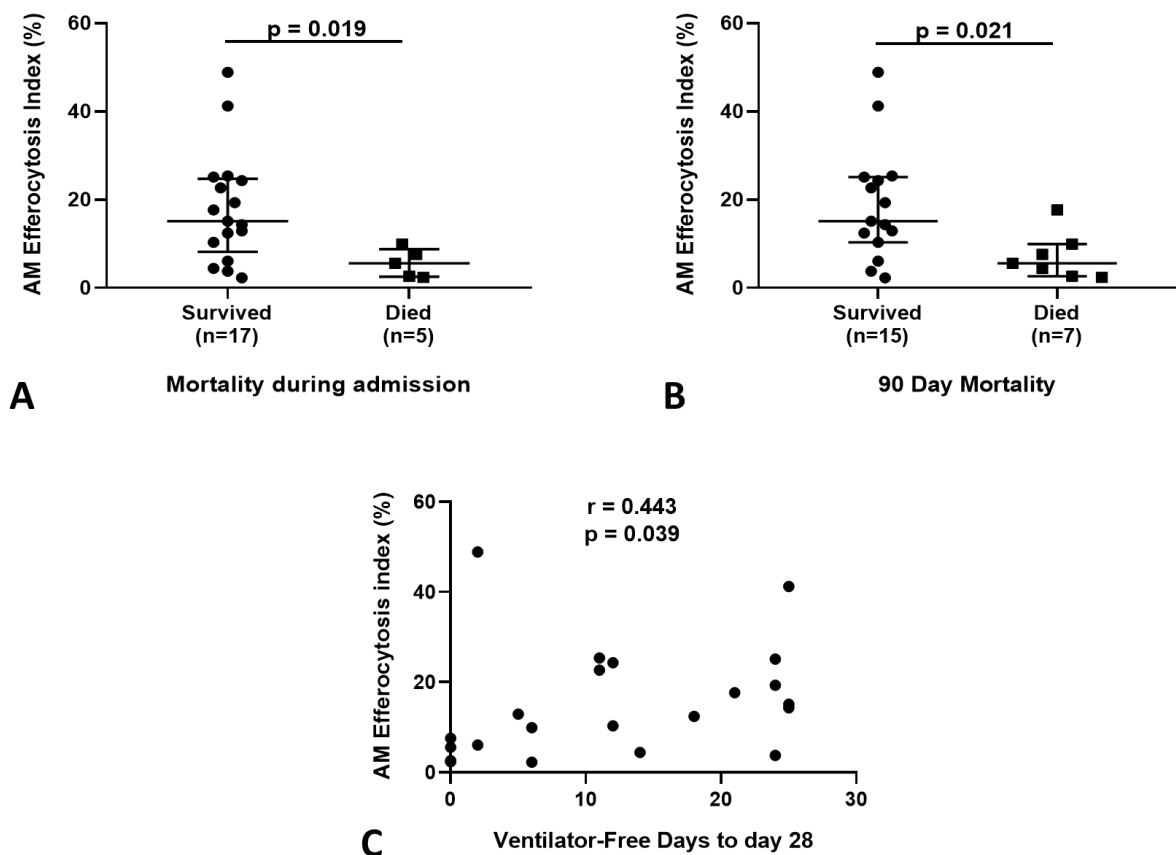


Figure 6.3: Alveolar macrophage efferocytosis index vs mortality and ventilator-free days in sepsis patients with and without ARDS.

AM = Alveolar macrophage. For sepsis patients with and without ARDS, AM efferocytosis index was higher in those who survived admission (**A**: medians 15.1 vs 5.6%, $p=0.019$) and those who survived for at least 90 days (**B**: medians 15.1 vs 5.6%, $p=0.021$). For **A** & **B**, Error bars shown as median and interquartile range, statistical analysis by Mann Whitney test. **C**: In sepsis patients with and without ARDS, AM efferocytosis index correlates directly with ventilator-free days to day 28 (Spearman $r = 0.443$, $p=0.039$).

In the AM-ARDS study, there was no significant difference in AM phagocytic index between sepsis patients with and without ARDS (figure 6.4). This was observed with phagocytosis of both *E. coli* (medians 707.3 vs 580.2, $p = 0.776$), and *S. Aureus* (576.0 vs 410.5, $p = 0.776$) pHrodo® bio-particles.

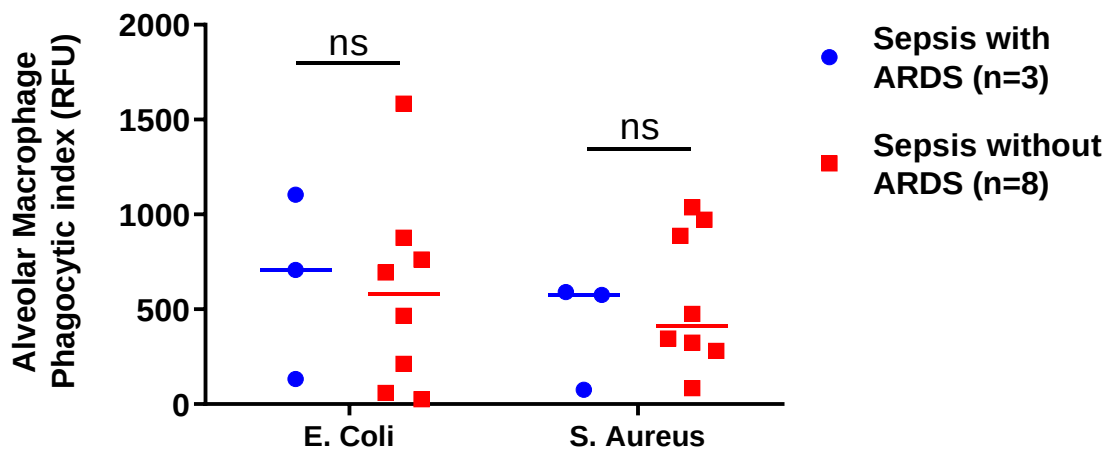


Figure 6.4: Alveolar macrophage phagocytosis in sepsis patients with or without ARDS.

RFU = Relative fluorescence units. ns = non-significant. Error bars shown as medians, statistical analysis by Mann Whitney Test. No significant difference between alveolar macrophage phagocytic index between sepsis patients with and without ARDS. This was observed with both *E. coli* (medians 707.3 vs 580.2, $p = 0.776$), and *S. Aureus* (576.0 vs 410.5, $p = 0.776$) pHrodo® bio-particles.

AMs from sepsis patients with ARDS have significantly reduced HSD-1 reductase activity compared to sepsis patients without ARDS (figure 6.5A, medians 0.031 vs 1.41 pM/hr, $p=0.028$). After combining data from both patient groups, a significant positive correlation was observed between AM efferocytosis index and AM HSD-1 reductase activity (figure 6.5B, $r = 0.803$, $p = 0.012$).

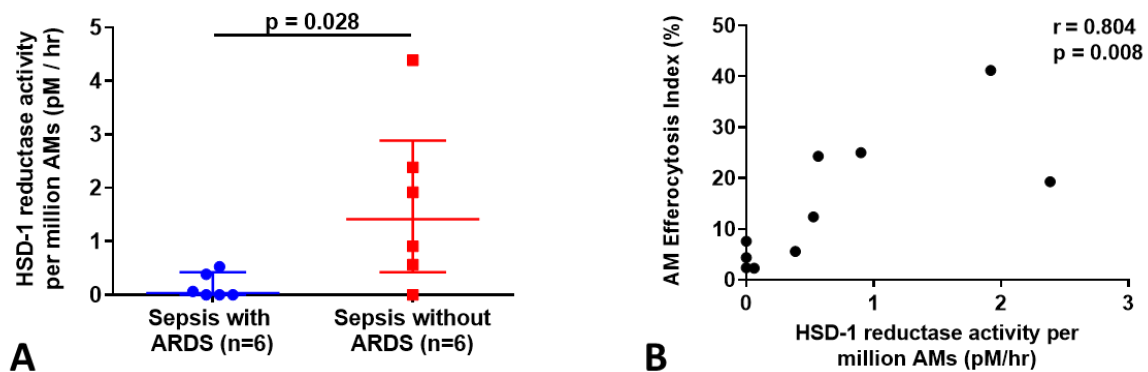


Figure 6.5: Alveolar macrophage HSD-1 reductase activity in sepsis patients with or without ARDS.

AM = alveolar macrophage. HSD-1 = 11 β hydroxysteroid dehydrogenase type-1. **A:** AM HSD-1 reductase activity in sepsis patients with and without ARDS. AMs from sepsis patients with ARDS have significantly reduced HSD-1 reductase activity compared to sepsis patients without ARDS (medians 0.031 vs 1.41 pM/hr, $p=0.028$). Error bars shown as median and interquartile range. Statistical analysis by Mann Whitney Test. **B:** AM efferocytosis index vs AM HSD-1 reductase activity in sepsis patients with and without ARDS. Statistical analysis by Spearman's correlation coefficient. Positive correlation observed between AM efferocytosis index and AM HSD-1 reductase activity ($n = 10$, $r = 0.804$, $p = 0.008$).

As described above, due to the limited number of AMs isolated from BALF, it was not possible to perform HSD-1 functional assays in every patient. Therefore, in order to assess the relationship between AM HSD-1 reductase activity and mortality, the data from the AM-ARDS study was combined with data previously collected by Dr Christopher Bassford at the University of Birmingham as part of the "Resolution of Inflammation Study"(91). The patient inclusion / exclusion criteria and HSD-1 functional assay protocol in both studies were identical. Patient characteristics are compared between both studies in table 6.2. For sepsis patients with ARDS, those recruited to the Bassford study had significantly higher APACHE-II scores in ICU admission, and higher inpatient mortality (table 6.2A). For sepsis patients without

ARDS, those recruited to the AM-ARDS study had worse lung injury score on ICU admission, however those recruited to the Bassford study had a worse APACHE-II score on ICU admission (table 6.2B). Higher APACHE-II scores on admission for patients from both groups recruited to the Bassford study indicate that these patients had more severe critical illness and a worse prognosis on ICU admission. This is reflected in the increased inpatient mortality for sepsis patients with ARDS recruited to the Bassford study. For sepsis patients without ARDS, the difference in lung injury scores indicates that more patients had pneumonia than other causes of sepsis in the AM-ARDS study.

A

Sepsis patients with ARDS		
	AM-ARDS (n=21)	Bassford (n=20)
Age in years (mean and SD)	59.2 (13.9)	64.5 (18.2)
Gender:		
Male - n (%)	15 (71%)	12 (60%)
Day 1 Lung Injury Score (mean and SD)	2.57 (0.50)	2.72 (0.46)
Day 1 SOFA score (mean and SD)	12.5 (3.8)	11.1 (2.1)
Day 1 APACHE II score (mean and SD)	18.6 (5.5)	24.7 (5.4)
Source of infection:		
Pneumonia - n (%)	19 (90%)	13 (65%)
Inpatient Mortality – n (%)	7 (33.3%)	14 (70%)

B

Sepsis patients without ARDS		
	AM-ARDS: (n=17)	Bassford: (n=5)
Age in years (mean and SD)	55.1 (16.3)	71 (13.3)
Gender:		
Male - n (%)	11 (65%)	3 (60%)
Day 1 Lung Injury Score (mean and SD)	2.13 (0.46)	1.13 (0.77)
Day 1 SOFA score (mean and SD)	10.3 (2.7)	10.6 (2.6)
Day 1 APACHE II score (mean and SD)	15.2 (5.8)	24.8 (5.0)
Source of infection:		
Pneumonia - n (%)	12 (71%)	3 (60%)
Inpatient Mortality – n (%)	3 (17.6%)	2 (40%)

Table 6.2: Comparison of patient demographics between the AM-ARDS study and Dr Bassford's study.

A: Comparison of sepsis patients with ARDS. **B:** Comparison of sepsis patients without ARDS. SD = standard deviation. SOFA = Sepsis-related Organ Failure Assessment. APACHE II = Acute Physiology and Chronic Health Evaluation II.

The AM-ARDS study contributed 12 patients and Dr Bassford's study contributed 24 patients, giving a total of 36 patients in this dataset (figure 6.6). Dr Bassford's study had previously identified a significantly reduced AM HSD-1 reductase activity in sepsis patients who developed ARDS (compared to those without ARDS), but had not shown a significant relationship between AM HSD-1 activity and mortality. The combined study dataset supported findings from the AM-ARDS study: that AMs from sepsis patients with ARDS have significantly reduced HSD-1 reductase activity compared to sepsis patients without ARDS (figure 6.6A, medians 0.079 vs 0.91 pM / hr, $p=0.0002$). Analysing data from both patient groups across both studies, it was found that all sepsis patients (with and without ARDS) who survived hospital admission had AMs with significantly higher HSD-1 reductase activity than those who died (figure 6.6B, medians 0.546 vs 0.0585 pM / hr, $p=0.0084$). However, when analysing data from patients with sepsis-related ARDS only across both studies, there was no significant difference in AM HSD-1 activity in patients who survived hospital admission compared to those who died (figure 6.6C, medians 0.203 vs 0.052 pM / hr, $p=0.061$).

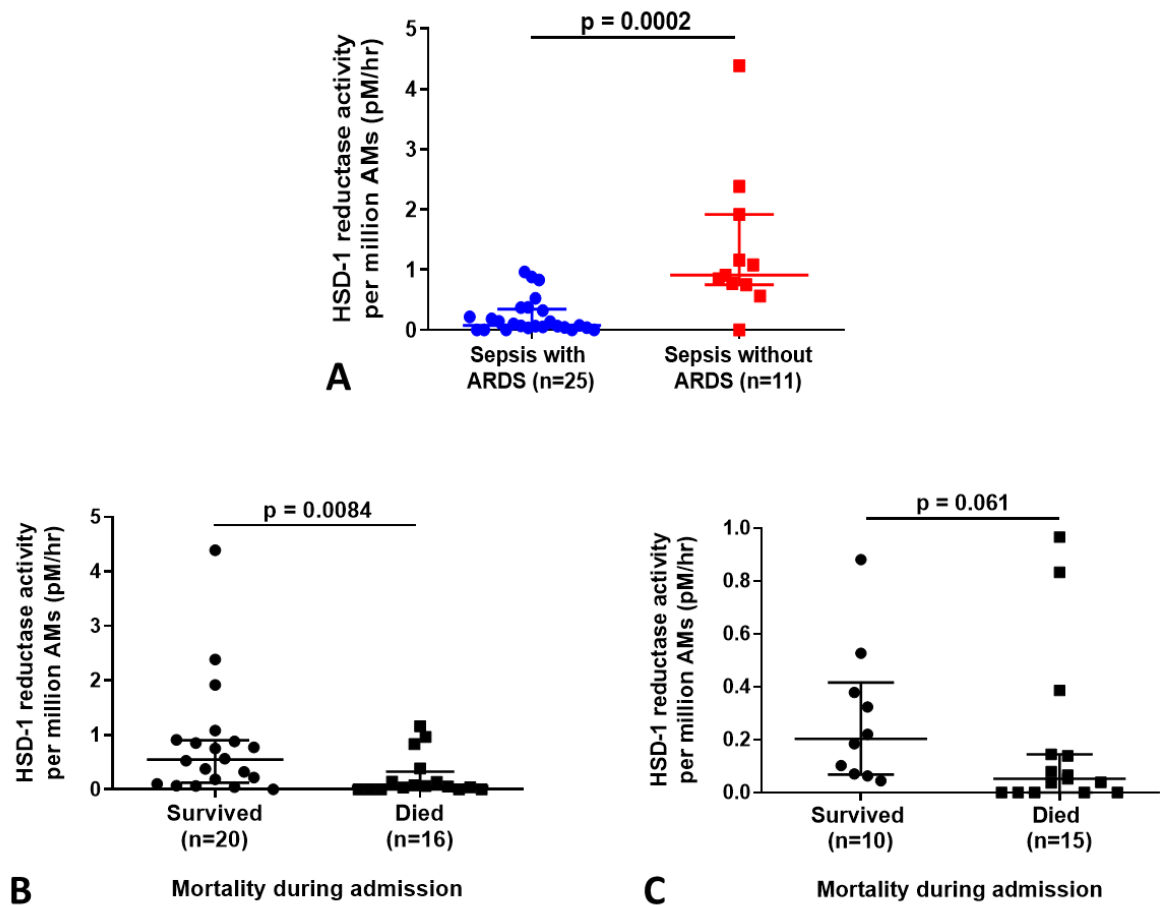


Figure 6.6: Combined alveolar macrophage HSD-1 reductase activity and mortality data from AM-ARDS and the Resolution of Inflammation in ARDS studies.

All HSD-1 activity data in this figure is combined from the AM-ARDS study and the “Resolution of Inflammation in ARDS” study previously undertaken by Dr Christopher Bassford(91). The AM-ARDS study contributed 12 patients and Dr Bassford’s study contributed 24 patients, giving a total of 36 patients in this dataset. Error bars shown as median and interquartile range. Statistical analysis by Mann Whitney Test. HSD-1 = 11 β hydroxysteroid dehydrogenase type-1. AM = alveolar macrophage. **A:** AM HSD-1 reductase activity in sepsis patients with and without ARDS. AMs from sepsis patients with ARDS have significantly reduced HSD-1 reductase activity compared to sepsis patients without ARDS (medians 0.079 vs 0.91 pM / hr, $p=0.0002$). **B:** AM HSD-1 reductase activity vs inpatient mortality in sepsis patients with and without ARDS. Sepsis patients with and without ARDS who survived admission had AMs with significantly higher HSD-1 reductase activity than those who died (medians 0.546 vs 0.0585 pM / hr, $p=0.0084$). **C:** AM HSD-1 reductase activity vs inpatient mortality in sepsis patients with ARDS only. Sepsis patients with ARDS who survived admission did not have significantly different AM HSD-1 reductase activity than those who died (medians 0.203 vs 0.052 pM / hr, $p=0.061$).

6.3.3 Alveolar Neutrophil Apoptosis

BALF neutrophil apoptosis was significantly greater in sepsis patients who developed ARDS compared to sepsis patients without ARDS (figure 6.7A, means 41.3% vs 14.1%, $p=0.0001$). After combining data from both patient groups, a significant negative correlation was observed between AM efferocytosis index and BALF neutrophil apoptosis (figure 6.7B, $r = -0.525$, $p = 0.05$). Analysing data from all patients, there was no significant difference in BALF neutrophil apoptosis between patients who survived hospital admission compared to those who died (means 27.9 vs 35.3%, $p=0.522$).

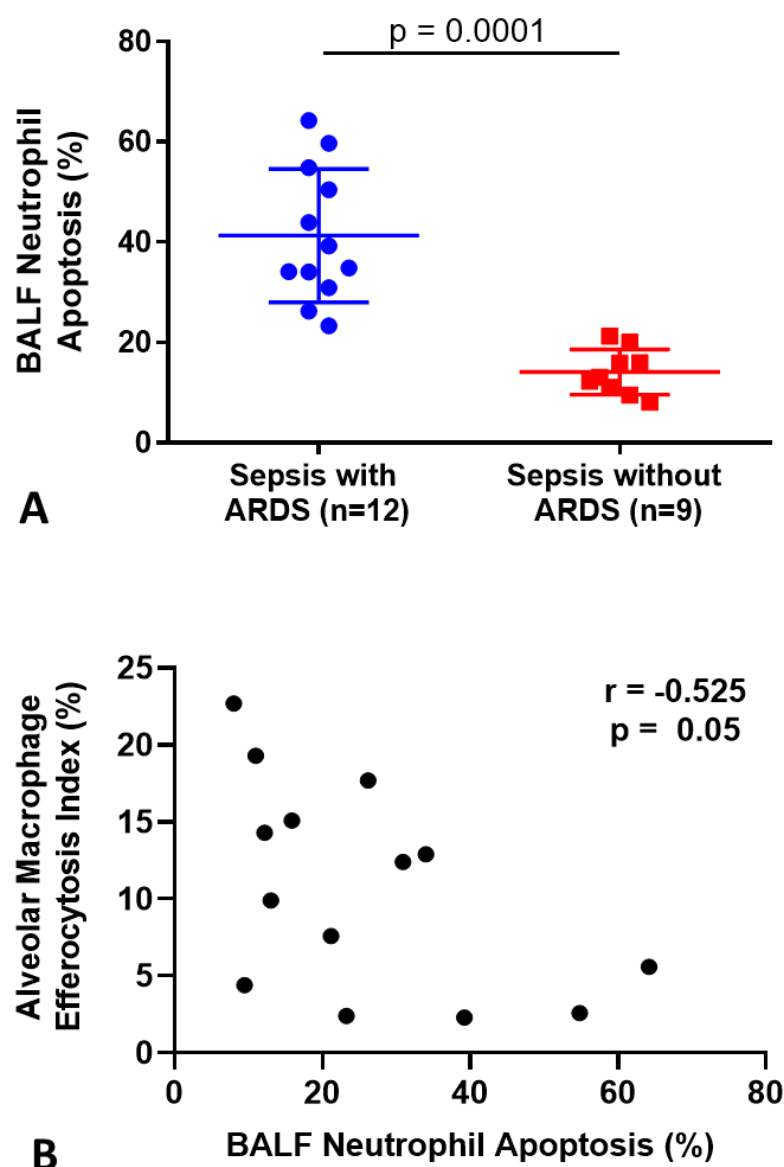


Figure 6.7: BALF neutrophil apoptosis in sepsis patients with and without ARDS

BALF = broncho-alveolar lavage fluid. Neutrophil apoptosis assessed within 1 hour of BALF collection. Some patients did not have neutrophil apoptosis measured since this was only added to the study protocol after recruitment had already begun. **A:** Percentage of BALF neutrophils which are apoptotic, in sepsis patients with and without ARDS. Error bars shown as mean and standard deviation. Statistical analysis by Welch's t-test. Sepsis patients with ARDS have significantly greater percentage of apoptotic neutrophils in BALF compared to sepsis patients without ARDS (medians 41.3 vs 14.1%, $p=0.0001$). **B:** Alveolar macrophage efferocytosis index versus BALF neutrophil apoptosis in sepsis patients with and without ARDS. Statistical analysis by Spearman's correlation coefficient. Negative correlation observed between alveolar macrophage efferocytosis and BALF neutrophil apoptosis ($n = 14$, $r = -0.525$, $p=0.05$).

6.3.4 Biochemical Assays

Sepsis patients with ARDS had significantly higher concentrations of IL-8 (medians 4163 vs 248 pg/ml, $p=0.019$) and MCP-1 (medians 1048 vs 253 pg/ml, $p=0.012$) in their BALF compared to sepsis patients without ARDS (table 6.3). However, there was no significant difference in BALF concentrations of IL-6, IL-1ra, IL-1 β , TNF- α , IL-10 and VEGF between both patient groups (table 6.2, $p>0.05$ for all).

	Sepsis patients with ARDS (n=17)	Sepsis patients without ARDS (n=14)	Mann Whitney Test
IL-6 (pg/ml)	74.6 (25.9 – 724.4)	84.0 (9.2 – 115.4)	$p = 0.401$
IL-8 (pg/ml)	4163 (652 – 18432)	248 (109 – 2842)	$p = 0.019$
IL-1ra (pg/ml)	4023 (1067– 10980)	834 (289 – 7329)	$p = 0.110$
IL-1β (pg/ml)	46.7 (7.8 – 471.5)	9.4 (7.7 – 82.4)	$p = 0.331$
TNFα (pg/ml)	6.7 (0 - 22.6)	0 (0 – 8.9)	$p = 0.153$
IL-10 (pg/ml)	5.6 (5.6 – 9.0)	5.6 (5.6 – 5.6)	$p = 0.404$
VEGF (pg/ml)	208.2 (84.7 – 441.0)	109.4 (16.6 – 354.5)	$p = 0.173$
MCP-1 (pg/ml)	1048 (499 – 3175)	253 (101 – 1359)	$p = 0.012$

Table 6.3: BALF cytokines from sepsis patients with and without ARDS on day 1 of recruitment

All data presented as median and interquartile range. IL = interleukin. TNF α = tumour necrosis factor α . VEGF = vascular endothelial growth factor. MCP-1 = monocyte chemoattractant protein-1.

Analysis of data across both patient groups (figure 6.8) showed significant negative correlation between AM efferocytosis index and BALF concentrations of IL-8 ($r = -0.707$, $p=0.0003$) and IL-1ra ($r = -0.601$, $p=0.004$) following Bonferroni's correction. Following Bonferroni's correction, there was no significant correlation between AM efferocytosis index and BALF concentrations of IL-6, IL-1 β , TNF- α , IL-10, VEGF and MCP-1 (data not shown, $p>0.00625$ for all). Analysis of data across both patient groups showed no significant correlation between BALF neutrophil apoptosis and BALF concentrations of IL-8, IL-1ra, IL-6, IL-1 β , TNF- α , IL-10, and MCP-1 (data not shown, $p>0.0625$ for all) following Bonferroni's correction. Analysis of data across both patient groups showed no correlation between AM HSD-1 reductase activity and BALF cytokine concentrations.

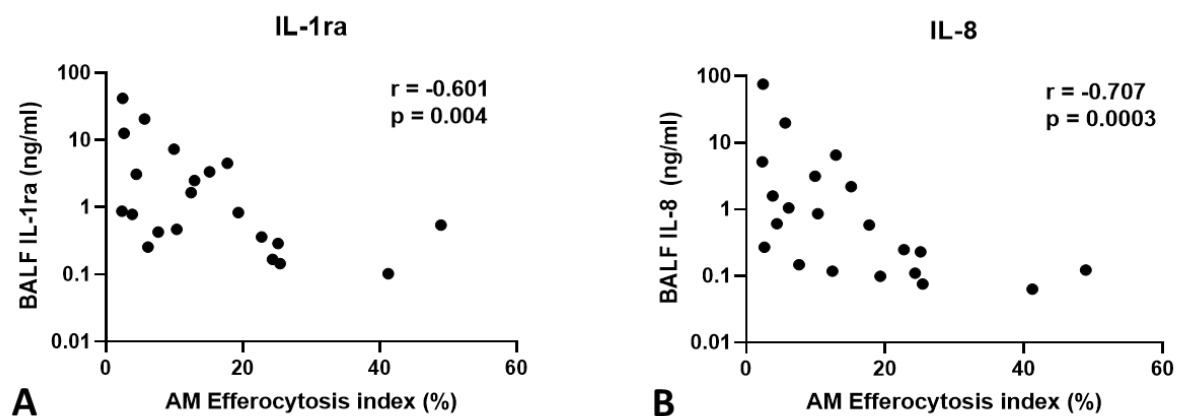


Figure 6.8: Correlations between alveolar macrophage efferocytosis and BALF cytokines in sepsis patients with and without ARDS

AM = alveolar macrophage. BALF = broncho-alveolar lavage fluid. IL-1ra = Interleukin 1 receptor antagonist. Log scales used for both graphs, $n=21$ for all plots. Spearman's correlation coefficient with Bonferroni's correction used for all statistical analysis. Bonferroni corrected significance $p<0.00625$. **A-B:** There is significant negative correlation between alveolar macrophage efferocytosis index and BALF concentrations of IL-1ra ($r = -0.601$, $p=0.004$) and IL-8 ($r = -0.707$, $p=0.0003$) in sepsis patients with and without ARDS.

Day 1 protein permeability index was not significantly higher in sepsis patients with ARDS compared to those without ARDS (figure 6.9, medians 1.12 vs 1.11, $p=0.23$).

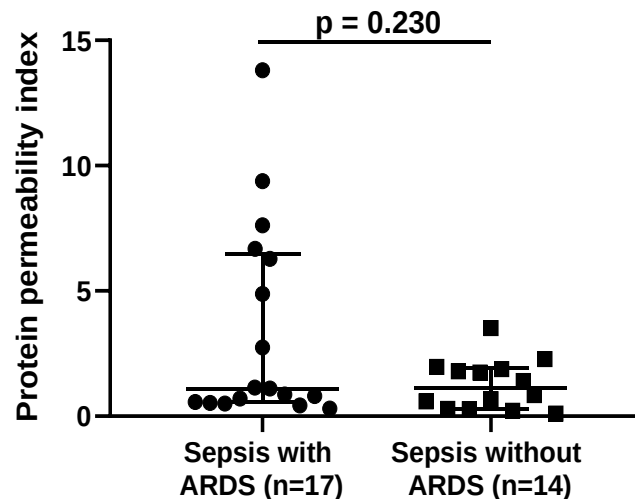


Figure 6.9: Day 1 protein permeability index in sepsis patients with and without ARDS

Error bars shown as median and interquartile range. Statistical analysis by Mann-Whitney test. There is no significant difference in PPI between sepsis patients with or without ARDS (1.12 vs 1.11, $p=0.230$).

Cytokines measured in AM conditioned media were IL-6, IL-8, IL-1ra, IL-1 β , TNF- α , IL-10 and MCP-1. All cytokines were released in greater quantities by AMs from sepsis patients without ARDS, compared to those with ARDS, however, this only reached statistical significance for IL-8 and IL-1ra ($p<0.03$ for both, table 6.4). There was no significant correlation between any AM release of cytokines and AM efferocytosis (data not shown, $p>0.05$ for all). There was no significant correlation between any of the cytokines measured in AM conditioned media and AM HSD-1 reductase activity, or BALF neutrophil apoptosis.

	Sepsis patients with ARDS (n=16)	Sepsis patients without ARDS (n=12)	Mann Whitney Test
IL-6 (pg/ml)	18.5 (7.7 – 51.1)	43.1 (9.2 – 91.2)	p = 0.415
IL-8 (pg/ml)	6084 (1877 – 10111)	30644 (5830 – 48038)	p = 0.029
IL-1ra (pg/ml)	3025 (545 – 5277)	6541 (2451 – 32408)	p = 0.025
IL-1β (pg/ml)	12.4 (7.7 – 22.7)	16.6 (9.8 – 59.7)	p = 0.339
TNFα (pg/ml)	18.4 (7.5 – 43.4)	31.7 (8.6 – 105.6)	p = 0.285
IL-10 (pg/ml)	0 (0 – 19.1)	10.1 (0 – 32.8)	p = 0.075
MCP-1 (pg/ml)	669 (47 – 3688)	3328 (619 – 6197)	p = 0.136

Table 6.4: Cytokine release by alveolar macrophages from sepsis patients with and without ARDS

TNFα = tumour necrosis factor α. MCP-1 = monocyte chemoattractant protein-1. IL = interleukin. Concentration of alveolar macrophages in conditioned media was 500,000 per ml. Conditioned media was collected after the initial 24hrs of *in vitro* culture. All data presented as median and interquartile range. Mann Whitney test used for all statistical analyses.

Cytokines measured in plasma were IL-6, IL-8, IL-1ra, IL-1β, and TNF-α. There was no significant difference in plasma cytokine concentrations between sepsis patients with or without ARDS, at day 1 or day 4-7 (table 6.5, p>0.05 for all). No detectable IL-1β was found in any of the plasma samples tested. There was no significant correlation between any cytokines measured in day 1 plasma and AM efferocytosis (data not shown, p>0.05 for all).

		Sepsis patients with ARDS (n=17)	Sepsis patients without ARDS (n=13)	Mann Whitney Test
IL-6 (pg/ml)	Day 1	124.1 52.5 – 360.7	115.8 61.3 – 215.9	p = 0.735
	Day 4 - 7	34.6 3.3 – 65.5	68.3 2.9 – 133.3	p = 0.552
IL-8 (pg/ml)	Day 1	68.6 31.4 – 227.4	38.5 31.5 – 70.1	p = 0.104
	Day 4 - 7	40.0 25.4 – 89.0	32.8 4.1 – 50.8	p = 0.111
IL-1ra (pg/ml)	Day 1	3592 2227 - 6495	6587 3352 – 11045	p = 0.086
	Day 4 - 7	4431 1998 – 9243	3841 2294 – 7009	p = 0.779
TNFα (pg/ml)	Day 1	12.9 2.8 – 24.1	10.7 3.3 – 29.7	p = 0.923
	Day 4 - 7	4.2 0.4 – 12.5	2.9 0.5 – 18.1	p = 0.877

Table 6.5: Plasma cytokines from sepsis patients with and without ARDS on day 1 and day 4 - 7 of recruitment

IL = interleukin. TNF α = tumour necrosis factor α . All data presented as median and interquartile range. Mann Whitney test used for all statistical analyses. No detectable IL-1 β was found in any plasma sample.

6.3.5 Glucocorticoid concentrations and ratios in BALF and Serum

There were no significant differences in day 1 BALF cortisol concentration, cortisone concentration or cortisol: cortisone ratio between sepsis patients with or without ARDS (figure 6.10A-C, $p>0.05$ for all). Alveolar epithelial lining fluid (ELF) concentrations of cortisone and cortisol were estimated (as described in section 3.8.6), however no significant differences were observed between sepsis patients with or without ARDS ($p>0.05$ for all). There were no significant differences in day 1 serum cortisol concentration, cortisone concentration or cortisol: cortisone ratio between sepsis patients with or without ARDS (figure 6.10D-F, $p>0.05$ for all). There were no significant differences in day 4-7 serum cortisol concentration, cortisone concentration or cortisol: cortisone ratio between sepsis patients with or without ARDS (figure 6.10G-I, $p>0.05$ for all).

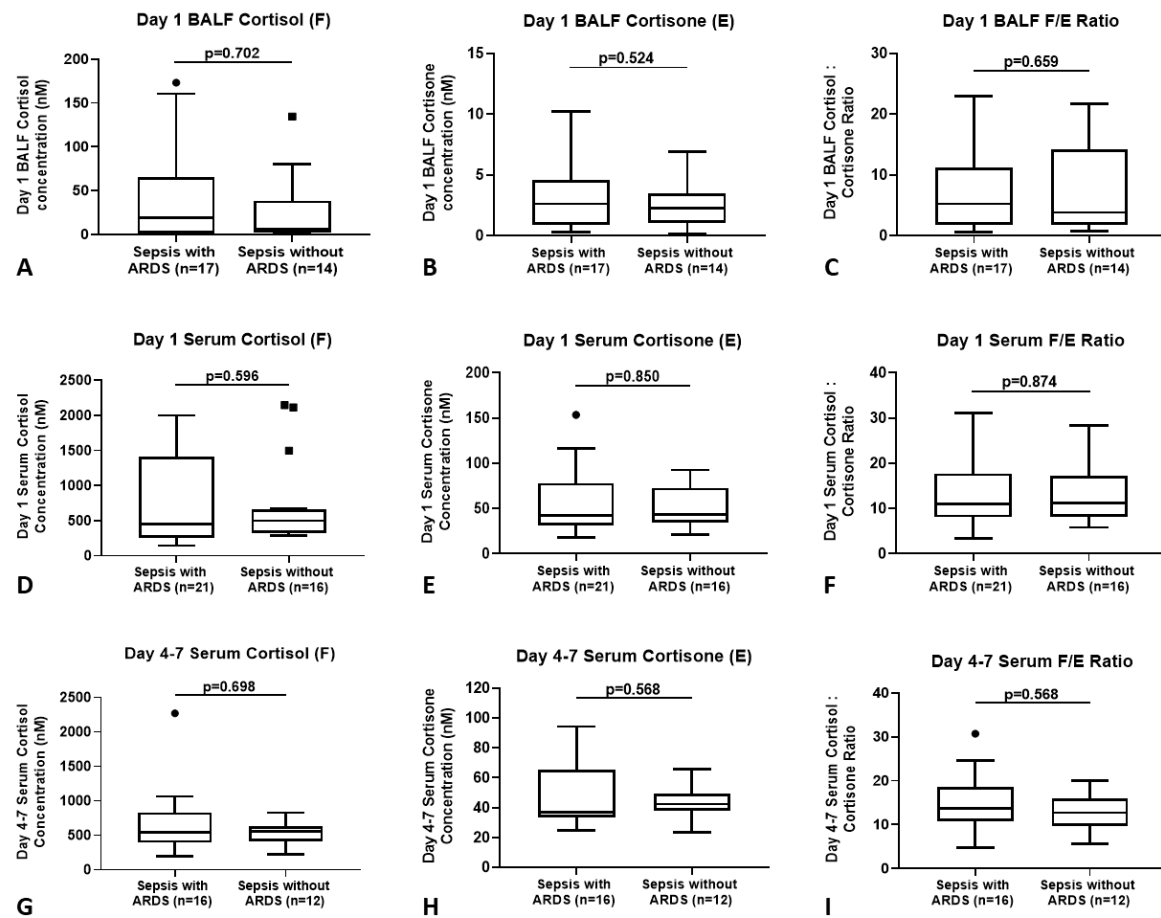


Figure 6.10: BALF and Serum glucocorticoid concentrations in sepsis patients with and without ARDS

Statistical analysis by Mann-Whitney tests, ns = non-significant. **A-C:** No significant differences in day 1 BALF cortisol concentration, cortisone concentration or cortisol: cortisone ratio between sepsis patients with or without ARDS ($p > 0.05$ for all). **D-F:** No significant differences in day 1 serum cortisol concentration, cortisone concentration or cortisol: cortisone ratio between sepsis patients with or without ARDS ($p > 0.05$ for all). **G-I:** No significant differences in day 4-7 serum cortisol concentration, cortisone concentration or cortisol: cortisone ratio between sepsis patients with or without ARDS ($p > 0.05$ for all).

There was no significant change in serum cortisol: cortisone ratio between day 1 and day 4-7 in sepsis patients with ARDS (figure 6.11A, n=16, median of differences = 2.95, p=0.207). There was no significant change in serum cortisol: cortisone ratio between day 1 and day 4-7 in sepsis patients without ARDS (figure 6.11B, n=12, median of differences = 3.15, p=0.423).

In sepsis patients with ARDS, day 1 cortisol: cortisone ratios are significantly higher in serum compared to BALF (figure 6.12A, median of differences = 4.4, p<0.0001. In sepsis patients without ARDS, day 1 cortisol: cortisone ratios are significantly higher in serum compared to BALF (figure 6.12B, median of differences = 6.15, p=0.0017).

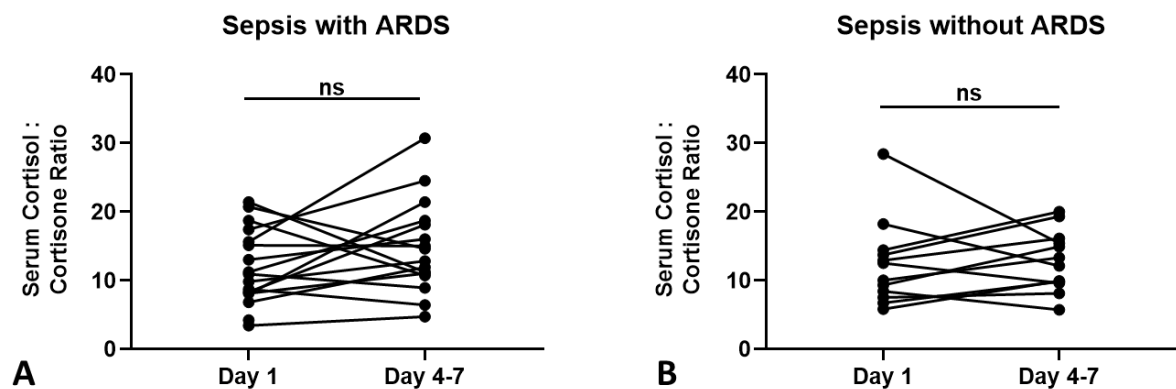


Figure 6.11: Differences in serum cortisol: cortisone ratio between day 1 and day 4-7 in both patient groups.

Statistical analysis by Wilcoxon test, ns = not significant. **A:** No significant change in serum cortisol: cortisone ratio between day 1 and day 4-7 in sepsis patients with ARDS (n=16, median of differences = 2.95, p=0.207). **B:** No significant change in serum cortisol: cortisone ratio between day 1 and day 4-7 in sepsis patients without ARDS (n=12, median of differences = 3.15, p=0.423).

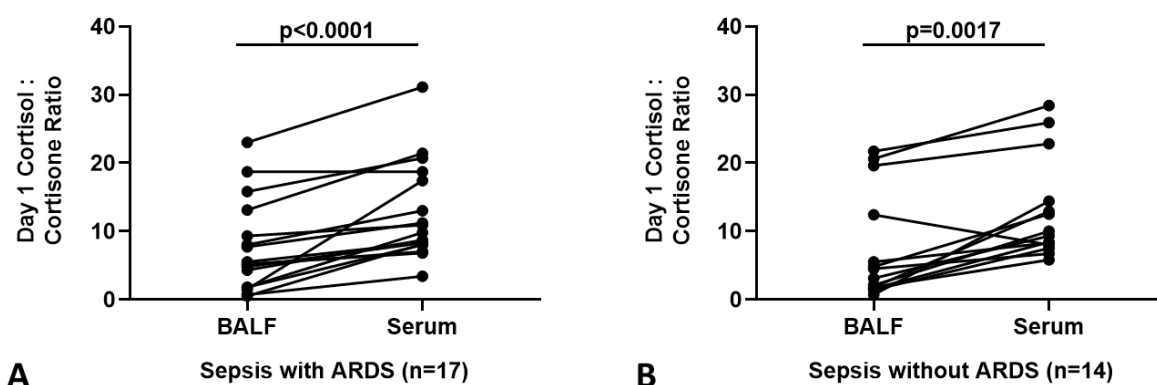


Figure 6.12: Day 1 Cortisol: Cortisone Ratios in BALF and Serum for both patient groups.

Only paired BALF and Serum data shown. Statistical analysis by Wilcoxon tests. **A:** In sepsis patients with ARDS, day 1 cortisol: cortisone ratios are significantly higher in serum compared to BALF (median of differences = 4.4, $p < 0.0001$). **B:** In sepsis patients without ARDS, day 1 cortisol: cortisone ratios are significantly higher in serum compared to BALF (median of differences = 6.15, $p = 0.0017$).

Across both patient groups, AM efferocytosis index had no significant correlation with day 1 BALF cortisone concentration, day 1 BALF cortisol concentration, and day 1 BALF cortisol: cortisone ratio, following Bonferroni's correction (data not shown, $p > 0.0167$ for all). Day 1 ELF cortisol and cortisone concentrations correlated directly with APACHE-II severity score in sepsis patients with ARDS (figure 6.13A-B) following Bonferroni's correction.

Across both patient groups, APACHE-II score had no significant correlation with day 1 serum cortisone concentration, day 1 serum cortisol concentration, and day 1 serum cortisol: cortisone ratio, following Bonferroni's correction (data not shown, $p > 0.0167$ for all).

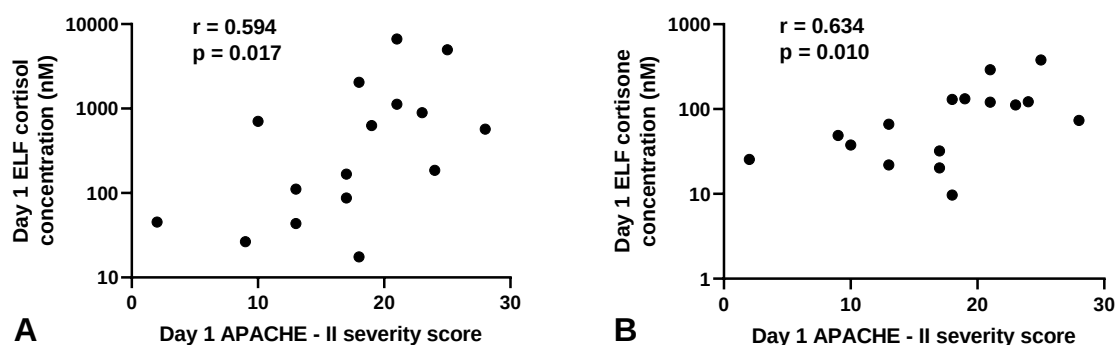


Figure 6.13: Correlations between day 1 ELF glucocorticoid concentrations and day 1 APACHE-II severity score in sepsis patients with ARDS

ELF = Alveolar epithelial lining fluid. Log scales used for ELF glucocorticoids. Statistical analysis by Spearman's correlation coefficient and Bonferroni's correction. Bonferroni corrected significance $p < 0.025$. **A:** Day 1 ELF cortisol concentrations showed significant correlation with day 1 APACHE-II severity score ($n=16$, $r = 0.594$, $p = 0.017$). **B:** Day 1 ELF cortisone concentrations showed significant correlation with day 1 APACHE-II severity score ($n=16$, $r = 0.634$, $p = 0.010$).

In order to determine if a correlation could be observed between steroid ratios and mortality in ARDS patients, ARDS patient data from the AM-ARDS study was combined with data previously collected by Dr Christopher Bassford at the University of Birmingham as part of the "Resolution of Inflammation Study"(91). The patient inclusion / exclusion criteria and HSD-1 functional assay protocol in both studies were identical. A comparison of patient demographics between the AM-ARDS and Bassford studies is shown in table 6.2. The AM-ARDS study contributed 17 day 1 BALF samples, 21 day 1 serum samples and 16 day 4-7 serum samples. Dr Bassford's study contributed 20 day 1 BALF samples, 20 day 1 plasma samples and 30 day 4 plasma samples (figure 6.16). Validation studies previously undertaken in the Institute for Metabolism and Systems Research at the University of Birmingham found that cortisol: cortisone ratios were identical between human serum and plasma

samples when measured by tandem mass spectrometry. Therefore, we were able to combine cortisol: cortisone ratio data from both studies.

There was no significant difference in day 1 BALF cortisol: cortisone ratio and day 1 circulating cortisol: cortisone ratio in ARDS patients who survived compared to those who died within 30 days (figure 6.14A-B, $p>0.05$ for both). The change in circulating cortisol: cortisone ratio between day 1 and day 4-7 was not significantly different between ARDS patients who survived compared to those who died within 30 days (figure 6.14C, difference between medians 1.05, $p=0.292$). There is no significant correlation between day 1 BALF cortisol: cortisone ratio and SOFA or APACHE-II severity scores in ARDS patients (figure 6.14D-E, $n=36$, $p>0.05$ for both). There is no significant correlation between day 1 BALF cortisol: cortisone ratio and AM HSD-1 reductase activity (figure 6.14F, $n=28$, $r = -0.189$, $p=0.335$). There is no significant correlation between day 1 circulating cortisol: cortisone ratio and SOFA severity scores in ARDS patients (figure 6.14G, $n=40$, $r=0.227$, $p=0.159$). There is no correlation between day 1 circulating cortisol: cortisone ratio and APACHE-II severity scores in ARDS patients (figure 6.14H, $n=40$, $r=0.340$, $p=0.0341$) following Bonferroni's correction (significant $p<0.025$).

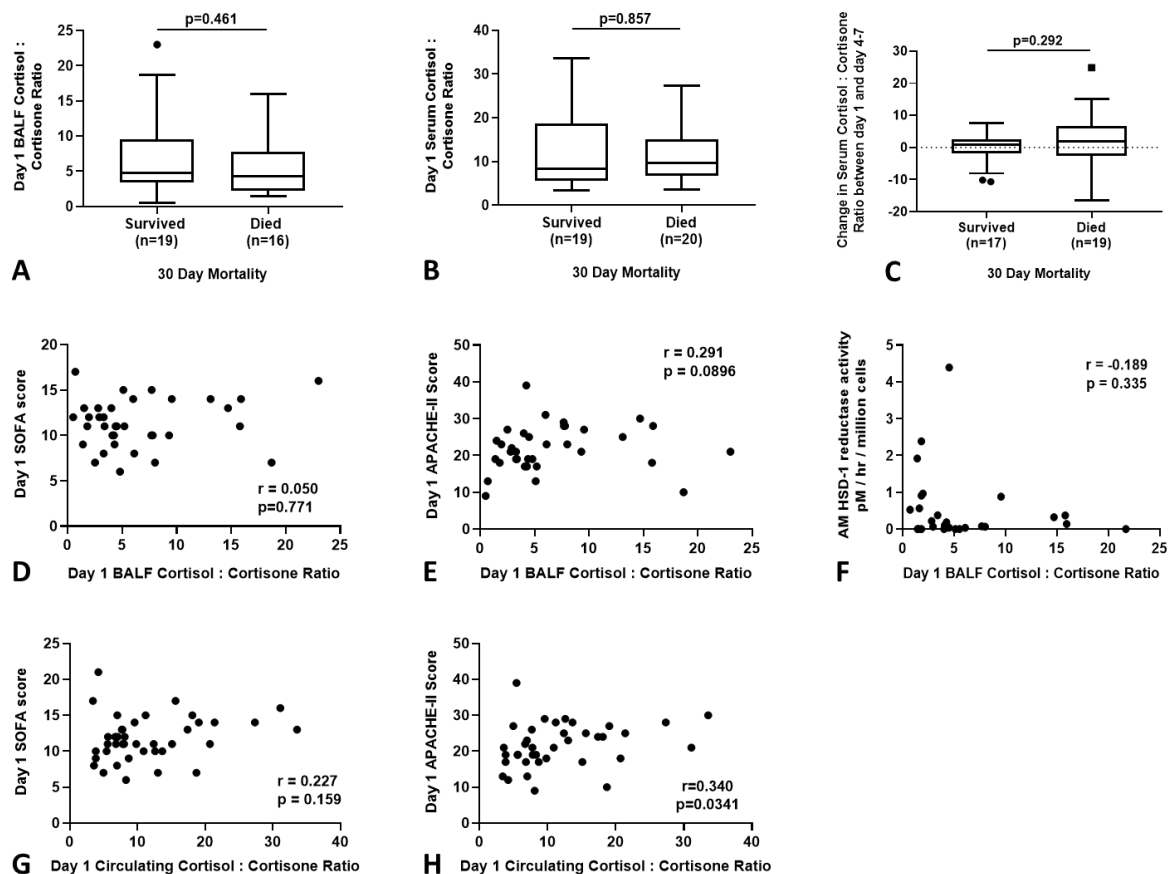


Figure 6.14: Combined cortisol: cortisone ratio data from ARDS patients recruited to the AM-ARDS and Resolution of Inflammation in ARDS studies

All data from sepsis patients with ARDS enrolled across both studies. Statistical analysis for **A-C** by Mann Whitney test; **D-H** by Spearman's Correlation Coefficient. **A-B**: There was no significant difference in day 1 BALF cortisol: cortisone ratio and day 1 circulating cortisol: cortisone ratio in ARDS patients who survived compared to those who died within 30 days ($p > 0.05$ for both). **C**: The change in circulating cortisol: cortisone ratio between day 1 and day 4-7 was not significantly different between ARDS patients who survived compared to those who died within 30 days (difference between medians 1.05, $p = 0.292$). **D-E**: There is no significant correlation between day 1 BALF cortisol: cortisone ratio and SOFA or APACHE-II severity scores in ARDS patients ($n = 36$, $p > 0.05$ for both). **F**: There is no significant correlation between day 1 BALF cortisol: cortisone ratio and AM HSD-1 reductase activity ($n = 28$, $r = -0.189$, $p = 0.335$). **G**: There is no significant correlation between day 1 circulating cortisol: cortisone ratio and SOFA severity scores in ARDS patients ($n = 40$, $r = 0.227$, $p = 0.159$). **H**: There is no correlation between day 1 circulating cortisol: cortisone ratio and APACHE-II severity scores in ARDS patients ($n = 40$, $r = 0.340$, $p = 0.0341$) following Bonferroni's correction.

6.3.6 Effect of HSD-1 tMSC Co-culture on ARDS Alveolar Macrophage Efferocytosis

AMs from sepsis patients with ARDS were co-cultured with transgene-inactive or transgene-active HSD-1 tMSCs prior to assessment of efferocytosis index (figure 6.15). There was no significant difference in the AM efferocytosis index following transwell co-culture with either transgene-inactive tMSCs (median of differences 0.385, $p=0.156$) or transgene-active tMSCs (median of differences 0.081, $p=0.219$). There was no significant difference between the ability of inactive vs active tMSCs to promote AM efferocytosis (median of differences 0.055, $p=0.99$).

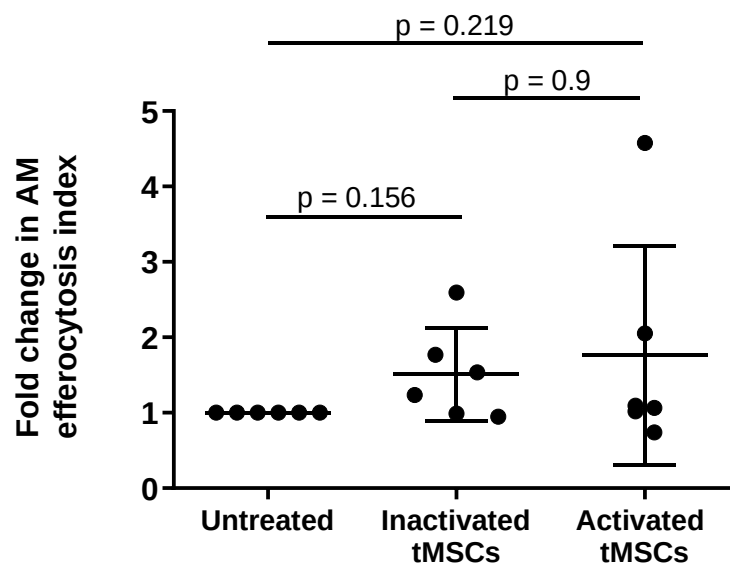


Figure 6.15: Fold change in the efferocytosis index of alveolar macrophages from ARDS patients following co-culture with HSD-1 transgenic MSCs

Statistical analysis by Wilcoxon matched-pairs signed rank test, $n=6$. tMSC = 11β hydroxysteroid dehydrogenase type 1 transgenic mesenchymal stem cell. There was no significant change in the alveolar macrophage efferocytosis index following transwell co-culture with either transgene-inactive tMSCs (median of differences 0.385, $p=0.156$) or transgene-active tMSCs (median of differences 0.081, $p=0.219$). There was no significant difference between the ability of inactive vs active tMSCs to promote alveolar macrophage efferocytosis (median of differences 0.055, $p=0.99$).

6.4 DISCUSSION

The main findings in this chapter are that development of sepsis-related ARDS is associated with decreased AM HSD-1 reductase activity, decreased AM efferocytosis, and increased alveolar neutrophil apoptosis. In all sepsis patients, AM efferocytosis directly correlates with AM HSD-1 reductase activity, and inversely correlates with BALF neutrophil apoptosis. Decreased AM efferocytosis is associated with increased alveolar inflammatory cytokines, duration of invasive ventilation and mortality. However, transwell co-culture of transgene-inactive or transgene-active HSD-1 tMSCs had no significant effect on the efferocytosis index of AMs from patients with sepsis-related ARDS. These findings support the first part of the hypothesis: that AMs in early ARDS have a dysfunctional phenotype with impaired HSD-1 reductase activity and impaired efferocytosis, resulting in persistent inflammation from secondary neutrophil necrosis, which influences clinical outcome (e.g. duration of invasive ventilation and mortality). The results indicate that AM dysfunction may contribute to ARDS pathogenesis. Although limited data was collected, it appears that AM bacterial phagocytosis may remain preserved in ARDS.

The AM-ARDS study is the first to show that alveolar macrophage efferocytosis is impaired in patients with sepsis-related ARDS, compared to matched ventilated patients with sepsis. Gregoire et al(150) recently showed that monocyte-derived macrophages from ARDS patients had impaired efferocytosis, which supports these findings. However, the AM-ARDS study provides greater insight into the pathology of ARDS: Firstly, because the identification of a defect in alveolar macrophage function is more relevant to the pathogenesis of ARDS than assessment of peripheral monocyte function, since the disease process originates in the alveolar space.

Secondly, the control group used in the study by Gregoire et al(150) included patients healthy enough to undergo outpatient bronchoscopy, as opposed to the critically ill sepsis patients requiring mechanical ventilation in the AM-ARDS study. Therefore, the AM-ARDS study was able to determine that the decrease in AM efferocytosis associated with the development of sepsis-related ARDS, occurs independently of sepsis, critical illness and mechanical ventilation.

The AM-ARDS study is also the first to show that apoptotic neutrophils accumulate in the alveolar space of patients with sepsis-related ARDS, compared to control ventilated patients with sepsis. The accumulation of apoptotic alveolar neutrophils in early ARDS could be due to increased apoptosis, and / or decreased clearance. In contrast, previous studies have shown that ARDS BALF treatment of neutrophils from healthy volunteers causes delayed apoptosis following *ex-vivo* culture for 18-24 hours (150, 166, 168, 172). Juss et al (172) have also shown that alveolar and circulating neutrophils from ARDS patients show delayed apoptosis following *ex-vivo* culture for 20 hours, compared to circulating neutrophils from healthy volunteers. However, these results cannot be directly compared with those from the AM-ARDS study, in which alveolar neutrophils were not cultured *ex vivo* prior to assessment of apoptosis. Instead, the AM-ARDS study investigated the proportion *in vivo* apoptotic alveolar neutrophils immediately after BALF collection. Taken together, these data suggest that alveolar neutrophil apoptosis is initially delayed due to the pro-inflammatory contents of ARDS BALF, however once apoptosis occurs the neutrophils are not cleared efficiently due to impaired AM efferocytosis.

Only two studies have previously investigated the proportion of *in vivo* apoptotic alveolar neutrophils in ARDS patients immediately after BALF sample collection. Firstly, Matute Bello et al(168) showed that *in vivo* alveolar neutrophil apoptosis was higher in ARDS patients at days 1 and 3 post-diagnosis (1.5% and 3%, n=35) compared to at-risk patients (0.5% and 1.6%, n=13), although absolute values were low and the difference was not statistically significant. The trend observed at day 1 is in keeping with results from the AM-ARDS study. However, this study used cyto-spin morphology as the only method of determining apoptosis in most patient samples, whereas subsequent studies have used two methods. Secondly, Lesur et al(166) showed that *in vivo* alveolar neutrophil apoptosis was reduced in ARDS patients (mean 14.3%, n=9) compared to control critically ill patients (mean 21.7%, n=10), however this difference was not statistically significant. These two previous studies give contradictory results, the absolute values are not comparable, and the differences were not statistically significant. Therefore, only limited comparisons can be drawn with results from the AM-ARDS study.

Measurement of glucocorticoid concentrations found that sepsis patients with or without ARDS have significantly reduced cortisol: cortisone ratios in their BALF compared to serum at day 1. This indicates that early in the disease course for all critically ill sepsis patients, there is reduced alveolar HSD-1 reductase activity compared to systemic HSD-1 reductase activity. Previous studies have shown that increased severity of critical illness is associated with increased cortisol release(299, 300). The direct correlation observed between ELF glucocorticoid concentrations and APACHE-II severity score in sepsis patients with ARDS supports these previous findings.

The inability of HSD-1 tMSCs to significantly upregulate AM efferocytosis following co-culture may be due to the limitations of this experiment. Glucocorticoids increase macrophage efferocytosis(68, 176) partly by upregulating expression of the efferocytosis receptor Mer(177, 178) and downregulating expression of the inhibitory receptor Signal Regulatory Protein- α (SIRP α)(147) on the surface of macrophages. Since the duration of co-culture was only 6 hours (for reasons detailed in section 6.2.7) and efferocytosis was assessed immediately after, this may not have allowed enough time for the elevated cortisol concentrations to influence macrophage surface receptor expression. However, despite limitations, the failure of HSD-1 tMSC therapy to upregulate AM efferocytosis indicates there are likely to be both HSD-1 dependant(175) and independent mechanisms of impaired AM efferocytosis in ARDS. Other potential mechanisms of impaired efferocytosis in ARDS include: 1) Elevated concentrations of inflammatory cytokines (e.g. TNF- α)(160, 301) and Damage Associated Molecular Patterns (e.g. high mobility group box-1 [HMGB-1])(154-156) within the alveolar space, which have previously been shown to inhibit macrophage efferocytosis. The AM-ARDS study did show a negative correlation between alveolar inflammatory cytokine concentrations and AM efferocytosis. 2) Cleavage of macrophage efferocytic receptors such as Mer by elevated levels of metalloproteinase in the alveolar space(302). 3) Phenotypic shift away from a pro-resolution “M2” macrophage towards a pro-inflammatory “M1” macrophage(101, 110).

Multiple studies have shown evidence that at ARDS initiation there is a massive infiltration of pro-inflammatory “M1” monocytes into the alveolar space. Rosseau et al(110) identified a massive influx of circulating monocytes into the alveolar space of

patients with early ARDS. A significant direct correlation was found between BALF MCP-1 concentrations, alveolar monocyte influx, severity of respiratory failure and mortality(110). The infiltrating monocytes were found to have increased expression of M1 markers (CD14, CD11b, 72E10) compared to resident mature AMs in ARDS patients(110). Mould et al(100) found that the transcriptional and metabolic profiles of resident and recruited alveolar macrophages differed in a murine model of lung injury. Soon after initiation of lung injury, the majority of inflammatory mediators (IL-6, IL-1 β and KC [murine homologue of IL-8]) were released by recruited (monocyte-derived) AMs, however the resident AMs took on an anti-inflammatory phenotype and produced minimal levels of inflammatory mediators(100). The AM-ARDS study did not differentiate between resident and recruited AMs; instead global AM efferocytosis capacity was measured. Therefore, the decreased efferocytosis in ARDS may be due to the massive influx of recruited monocytes / immature AMs polarised to a pro-inflammatory M1 phenotype, which is associated with a reduced efferocytosis capacity(101).

It has been shown that monocytes have negligible expression of HSD-1 and that expression of this enzyme is only induced upon differentiation to macrophages(86). Also, M1 macrophages have reduced HSD-1 reductase activity compared to M2 macrophages(86). Therefore, the monocyte influx into the alveolar space(303) would also explain the reduced AM HSD-1 reductase activity detected in ARDS patients.

As expected, inflammatory cytokine concentrations (e.g. IL-8) are higher in the BAL of sepsis patients with ARDS compared to those without ARDS (although not reaching statistical significance). Inflammatory cytokine release by AMs isolated from sepsis patients with ARDS was impaired compared to AMs from patients without

ARDS; the opposite trend to that observed in BALF. A similar dichotomy has previously been observed in sepsis patients, in which impaired inflammatory cytokine release by monocytes contrasts with elevated plasma cytokine concentrations (304). The findings indicate that AMs in patients with sepsis-related ARDS may have undergone endotoxin tolerance. The phenomenon of endotoxin tolerance is well documented in sepsis; repeated endotoxin (lipopolysaccharide) exposure impairs the response of macrophages and monocytes to further endotoxin, thereby reducing inflammatory cytokine release (305). AMs from COPD patients show a similar defective immune responsiveness with reduction in cytokine release(306). In patients with sepsis-related ARDS, global AM dysfunction (characterised by impaired HSD-1 reductase activity, efferocytosis and cytokine release) may be caused by continuous exposure to high concentrations of endotoxin and inflammatory cytokines within the alveolar space. The development of AM endotoxin tolerance may therefore partly determine which sepsis patients develop ARDS.

Future studies are required to assess the ARDS AM phenotype and correlate with function to help determine if the hypothesised pro-inflammatory “M1” phenotypic shift is responsible for AM dysfunction in ARDS. Recent advances in immunometabolism have discovered that macrophage phenotypic polarisation is associated with metabolic changes, e.g. switch to a pro-inflammatory M1 phenotype is associated with decreased mitochondrial respiration and increased glycolysis(307). Altered AM metabolism is associated with development of a pro-fibrotic phenotype in murine models of lung fibrosis(308). AMs from COPD patients have dysregulated mitochondrial metabolism, leading to a defective phenotype with decreased intracellular bacterial killing(309). AM metabolism can be influenced by multiple

factors within the alveolar environment, including cytokines (310, 311). Therefore, altered AM metabolism may result in the dysfunctional phenotype observed in ARDS patients, with impaired efferocytosis and cytokine release. Assessment of ARDS AM metabolic function along with phenotype and function would allow further elucidation of the role of AMs in ARDS pathogenesis, and potentially reveal alternative methods of restoring AM function.

Strategies to upregulate of AM efferocytosis would reduce secondary necrosis of alveolar neutrophils, thereby attenuating inflammation in ARDS. Despite the failure of HSD-1 tMSCs to upregulate AM efferocytosis, there are other strategies which may have the potential to achieve this: 1) Blockade of factors in BALF which are known to inhibit efferocytosis (e.g. HMGB-1 and TNF α). Anti-HMGB1 antibodies have previously been used to restore monocyte-derived macrophage efferocytosis following ARDS BALF treatment *in vitro*(150). 2) Blockade of intracellular pathways which inhibit efferocytosis. Rho Associated Protein Kinase (ROCK)-inhibitors have previously been shown to increase efferocytosis in monocyte-derived and alveolar macrophages from patients with chronic obstructive pulmonary disease(312). 3) Use of existing medications which have been shown to have a pro-efferocytic effect: anti-oxidants (e.g. N-acetylcysteine)(313), macrolide antibiotics(314, 315), and statins(163).

The AM-ARDS study had several major limitations which meant that all the experiments necessary to investigate the role of AMs in ARDS pathogenesis could not be performed. ARDS patient recruitment was challenging because prior steroid use, immunosuppression or immunodeficiency were all exclusion factors, however many patients diagnosed with ARDS had a transplant background. Even if meeting

the inclusion criteria, not all patients could safely receive a bronchoscopy, mainly due to their ventilation status. For these reasons, patient recruitment was slow and total numbers low. Following bronchoscopy on ARDS patients, the BALF was often highly neutrophilic, making it difficult to isolate AMs. Even if AMs were isolated, the average number isolated was 1.2 million, meaning that often only one type of functional assay could be performed per patient. In these situations, the efferocytosis assay was given priority. It has been shown that treatment of monocyte-derived macrophages with pooled ARDS BALF impairs efferocytosis(150). Owing to difficulties associated with isolating AMs from ARDS patients, an *in vitro* model of ARDS was set up using AMs from lung resection tissue and pooled ARDS BAL to recapitulate the dysfunctional AM phenotype seen *in vivo*. These experiments are detailed in chapter 7. There were also limitations associated with using data from Dr Bassford's study; patients recruited to his study had more severe disease on ICU admission, and increased mortality rates. The lower mortality rate in the AM-ARDS cohort may be related to improvements in ventilation strategies and outcomes over the last decade. Also, circulating glucocorticoid concentrations in his patients were assessed in serum, whereas this was assessed in plasma for the AM-ARDS cohort. This meant that absolute concentrations of circulating glucocorticoids could not be directly compared between studies due to inter-assay variability.

In summary, the development of sepsis-related ARDS is associated with decreased AM HSD-1 reductase activity and efferocytosis, which likely contribute to the increased alveolar neutrophil apoptosis, alveolar inflammation, hypoxia and mortality observed. However, transwell co-culture of HSD-1 tMSCs had no significant effect on *ex vivo* ARDS AM efferocytosis, indicating the presence of HSD-1 independent

mechanisms of impaired efferocytosis in ARDS. The ability of tMSCs to enhance AM function and attenuate inflammation in both *in vitro* and *in vivo* models of ARDS will be investigated in chapters 7 and 8.

CHAPTER 7 : ALVEOLAR MACROPHAGE FUNCTION IN AN *IN VITRO* MODEL OF ARDS

7.1 INTRODUCTION

Data presented in chapter 6 shows that the onset of ARDS in patients with sepsis is associated with significantly impaired alveolar macrophage (AM) efferocytosis. However, the clinical study had several major limitations which meant that all the experiments necessary to investigate the role of AMs in ARDS pathogenesis could not be performed. As described in section 6.4, ARDS patient recruitment was challenging because exclusion factors included prior steroid use, immunosuppression and immunodeficiency, thereby limiting the number of eligible patients. Even if meeting the inclusion criteria, not all patients could safely receive a bronchoscopy. The broncho-alveolar lavage fluid (BALF) was often highly neutrophilic, making it difficult to isolate AMs. Even if AMs were isolated, the average number isolated was 1.2 million, making it difficult to perform more than one type of functional assay.

Owing to these difficulties with isolating AMs from ARDS patients, an *in vitro* model of ARDS was set up using AMs from lung resection tissue. A previous study has shown that treatment of monocyte-derived macrophages with pooled ARDS BALF impairs macrophage efferocytosis(150), and that this is partially mediated by elevated high mobility group box-1 (HMGB-1) levels within ARDS BALF. It was therefore postulated that treatment of lung resection tissue-derived AMs with pooled ARDS BALF would similarly reduce efferocytosis. Since ARDS BALF contains high concentrations of pro-inflammatory cytokines and HMGB-1, it was hypothesised this could drive AMs away from a pro-resolving M2 phenotype and towards a pro-inflammatory M1 phenotype, which is associated with reduced efferocytosis capacity and reduced HSD-1 reductase activity(86, 175). It was also hypothesised that ARDS BALF

treatment would decrease expression of Mer (a pro-efferocytic receptor) and increase expression of signal-inhibitory protein- α (SIRP α , an anti-efferocytic receptor). The aim of this chapter was to investigate the changes in AM function, phenotype and 11 β hydroxysteroid dehydrogenase type 1 (HSD-1) reductase activity induced by ARDS BALF treatment. It has previously been shown that cytokine treatment of monocyte-derived macrophages can induce phenotypic polarisation (101): Treatment with interferon- γ (IFN γ) and lipopolysaccharide (LPS) can induce an M1 pro-inflammatory phenotype. Treatment with interleukin (IL)-4 and IL-13 can induce an M2 anti-inflammatory phenotype(316). However, few studies have investigated phenotypic polarisation in AMs(93). An additional aim was to determine the ability of ARDS BALF components (e.g. HMGB-1, IFN γ , LPS) and anti-inflammatory cytokines to influence AM efferocytosis, phenotype, and HSD-1 reductase activity.

A further aim was to determine whether HSD-1 transgenic mesenchymal stem cell (HSD-1 tMSC) transwell co-culture with ARDS BALF treated AMs could rescue impaired function and altered phenotype. However, transwell co-culture of HSD-1 tMSCs with ARDS patient AMs was found to be unsuccessful at enhancing efferocytosis (see section 6.3). Therefore, an investigation was undertaken into whether intracellular pathway inhibitors would be more successful at restoring efferocytosis in the *in vitro* model of ARDS. Following activation of SIRP α by surfactant protein A or D, intracellular signalling results in activation of RhoA, Rho-associated protein kinase (ROCK) and Phosphatase and tensin homolog (PTEN), which form a complex and inhibit phosphatidylinositol 3'-OH kinase (PI3K) signalling. This has the downstream effect of downregulating Ras-related C3 botulinum toxin substrate 1 (Rac1) guanosine triphosphatase (GTPase) expression, which mediates

the cytoskeletal rearrangement required for efferocytosis. Therefore, it was hypothesised that inhibition of ROCK or PTEN would promote expression of Rac1, thereby upregulating efferocytosis.

7.2 METHODS

Full methods can be found in sections 3.2 - 3.5 and 3.7.

7.2.1 Isolation of Alveolar Macrophages from Lung Tissue Resections

National research ethical approval was given for a study on human lung tissue samples, as described in section 3.1.1. The Lung Resection study recruited adult patients scheduled to have surgery to remove lung tissue as part of their clinical treatment at Birmingham Heartlands Hospital. The aim was to collect human lung tissue samples surplus to histopathological requirements following planned thoracic surgery. Baseline demographics including smoking history were collected. Lung resection samples were collected as described in section 3.2.4.

Following arrival in the laboratory, lung resection samples were immediately washed through with 500 – 2000 mLs of sterile 0.9% saline (Baxter, UK) using a 14 gauge needle (Vasofix®, Braun, Germany). The washed through lavage fluid was collected and placed into sterile 50ml centrifuge tubes, before being centrifuged in centrifuge (Eppendorf AG 5810R, Germany) at 4°C and 560g for 10 minutes. The supernatants were discarded and the cell pellets pooled and re-suspended in 10mls of RPMI 1640 media containing 10% FBS. The cell suspension was then overlaid onto 10 mls of Lymphoprep™ (StemCell Technologies, Canada) in a 50 ml centrifuge tube. The tube was centrifuged at 4°C and 800g for 30 minutes with minimal acceleration and no brake. The interphase layer containing mononuclear cells was aspirated using a 3 ml Pasteur pipette (Alphalabs, UK) into a sterile 50 ml centrifuge tube containing PBS

supplemented with 10% FBS. Cells were then centrifuged at 300g for 10 minutes at 4°C to enable removal of platelets and wash off any remaining Lymphoprep. Alveolar macrophages (AMs) were re-suspended in RPMI 1640 media supplemented with 10% FBS, 100U/mL penicillin, 100ug/mL streptomycin and 2mM L-glutamine (Sigma-Aldrich). A cell count, viability check and cytospin differential count were done (as per sections 3.3.5 and 3.3.6). AMs were diluted in supplemented RPMI 1640 media and plated according to assay (specified in section 3.3.8), prior to culture in an incubator at 37°C with 5% CO₂ in preparation for functional studies. AMs adhered to the flat bottom wells of the culture plates. After 24 hours incubation, media was removed from the cultured AMs and replaced with supplemented RPMI 1640 media.

7.2.2 Use of alveolar macrophages in an *in vitro* model of ARDS

AMs from the lung resections of never or long-term (>5 years) ex-smoker patients without COPD were plated at concentrations specified in section 3.3.8. BALF taken from 6 ARDS patients (sections 3.2.3 and 3.3.3) was pooled and mixed in a 1:1 ratio with RPMI-1640 including 10% FBS. To elicit functional changes associated with ARDS, the AMs were treated with this 50% ARDS BALF mixture. As a vehicle control treatment, AMs were also treated with a 1:1 mixture of 0.9% Saline (Baxter, UK) and RPMI-1640 including 10% FBS. Other treatments given in conjunction with 50% ARDS BALF or saline included 200nM Y-27632 dihydrochloride (Rho-associated protein kinase inhibitor, Apexbio, USA), 2μM SF1670 (Phosphatase and tensin homolog inhibitor, Selleckchem, USA), dimethyl sulfoxide (DMSO, vehicle control for Y-27632 and SF1670, Sigma-Aldrich) at a 1:50,000 dilution, and 500ng/ml recombinant human high mobility group box-1 (HMGB-1, Abcam, UK). ROCK and

PTEN inhibitor treatment doses determined by dose response on untreated AM efferocytosis (see figure 7.1). HMGB-1 dose was determined by concentrations previously detected in ARDS BALF(91, 150). Other treatments not combined with 50% ARDS BALF or saline included 50ng/ml IFN γ (Peprotech, UK), 1 μ g/ml LPS (Sigma-Aldrich), 40ng/ml IL-4 (Peprotech, UK), and 40ng/ml IL-13, Peprotech, UK). Cytokine concentrations were based on published methods(317). The 1 μ g/ml dose of LPS was based on the lowest dose required to elicit tumour necrosis factor- α (TNF α) production (see figure 7.2). Efferocytosis, phagocytosis, apoptosis, viability, HSD-1 functional assays and RNA extraction for gene expression (sections 3.3.7, 3.4.1, 3.4.2, 3.5 and 3.7) were performed 24 hours after treatment with 50% ARDS BALF. Phenotyping (section 3.4.4) was performed 48 hours after treatment.

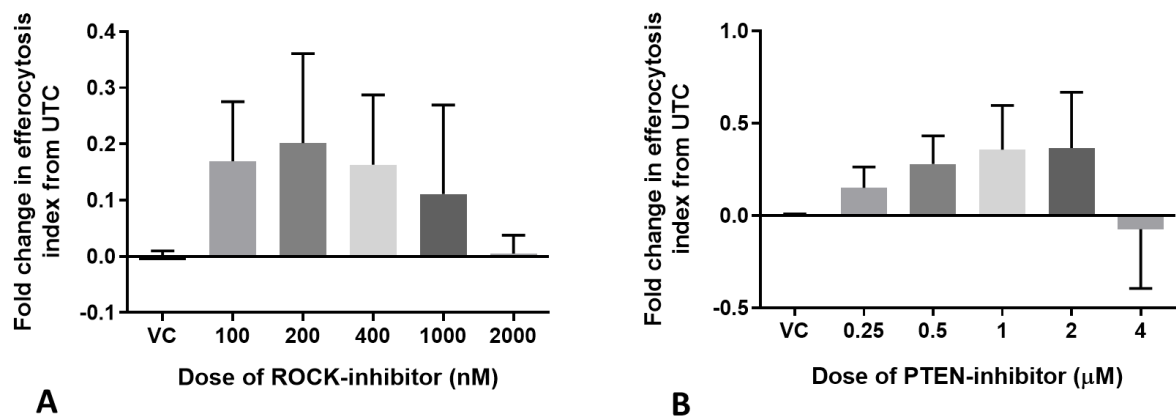


Figure 7.1: Dose response of ROCK-inhibitor and PTEN-inhibitor on the efferocytosis index of uninjured alveolar macrophages

Data shown as mean and standard deviation, n=4 all groups. UTC = Untreated control. VC = Vehicle Control (Dimethyl Sulfoxide [DMSO] at 1:50,000 dilution). ROCK-inhibitor = 200nM Y-27632 dihydrochloride; Rho-associated protein kinase inhibitor. PTEN-inhibitor = 2 μ M SF1670; Phosphatase and tensin homolog inhibitor. **A:** The dose of ROCK-inhibitor which elicited the greatest fold change in the efferocytosis index of uninjured alveolar macrophages was 200mM. According to the supplier (Apexbio, USA), the IC₅₀ (50% of the maximal inhibitory concentration) of ROCK-inhibitor Y-27632 is 140nM, approximately in keeping with my findings. **B:** The dose of PTEN-inhibitor which elicited the greatest fold change in the efferocytosis

index of uninjured alveolar macrophages was 2 μ M, equal to IC₅₀ (as per the supplier Selleckchem, USA). My findings appear to contradict the IC₅₀ value, which should be 1 μ M based on my data.

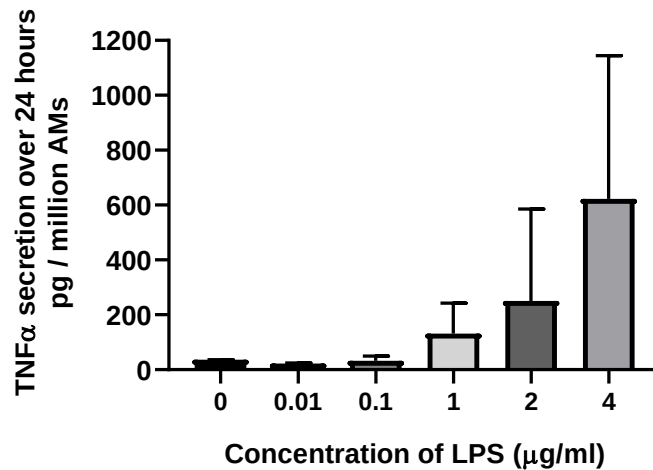


Figure 7.2: Dose response of LPS on alveolar macrophage secretion of TNF α
 UTC = Untreated control. n=3. LPS only stimulated AM secretion of TNF α at doses $\geq$ 1 μ g/ml.

7.2.3 Transwell co-culture of HSD-1 transgenic mesenchymal stem cells with alveolar macrophages

To assess the impact of HSD-1 transgenic mesenchymal stem cells (tMSCs) on restoring AM function after treatment with 50% ARDS BALF, a 6 hour Transwell co-culture of passage 4 tMSCs with AMs was performed between removal of ARDS BALF and efferocytosis or phenotyping assays. The creation of tMSCs is described in chapter 4. AMs were cultured at 2.5×10^5 per well in RPMI 1640 containing 10% FBS in 24-well plates at 37°C and 5% CO₂. In the morning of the efferocytosis assay,

wells were washed with PBS and media changed to 600µl serum-free RPMI 1640. Exogenous cortisone (Sigma-Aldrich) was added to give a final concentration of 10^{-7} M in all wells. Then, Transwell® permeable inserts containing a 0.4µm polycarbonate microporous membrane (Costar, Corning, USA) were added to each well. Then, 100µl of serum-free RPMI containing either 6.25×10^4 MSCs, transgene-activated tMSCs, transgene-inactive tMSCs or no cells was added to the upper compartment. The ratio of AMs to tMSCs was 4:1. Plates were then incubated for 6 hours at 37°C and 5% CO₂. The transwell inserts to which tMSCs had adhered to were removed, and the efferocytosis or phenotyping assay was performed as described in sections 3.4.1 and 3.4.4.

7.2.4 Statistical Analysis

Data were analysed using Prism 8 software (GraphPad, USA). Normality of data was assessed using the D'Agostino & Pearson test. Differences between continuously distributed data was assessed using unpaired t-tests for parametric data, or Mann-Whitney tests for non-parametric data. Two-tailed p-values of ≤ 0.05 were considered as significant. Differences between three or more paired parametric data sets were assessed using the repeated measures Analysis of Variance (ANOVA) followed by Tukey's or Dunnett's multiple comparison tests. Differences between three or more paired parametric data sets were assessed using Friedman ANOVA followed by Dunn's multiple comparison tests. Results from parametric data are shown as mean and standard deviation. Results from non-parametric data are shown as median and interquartile range.

7.3 RESULTS

7.3.1 Impact of ARDS BALF and cytokine treatments on alveolar macrophage function

The efferocytosis index of untreated AMs isolated from the lung tissue of never-smokers or ex-smokers has considerable variability between individuals (figure 7.3, n=11, mean 32.2%, range 18.1 – 50.7%). AM treatment with 50% ARDS BALF significantly reduced efferocytosis compared to treatment with 50% saline vehicle control (figure 7.3, mean of differences 12.97%, n=11, p=0.0006). AM treatment with 50% saline (vehicle control) had no effect on efferocytosis compared to untreated control (figure 7.3, mean of differences 7.57%, n=11, p=0.053).

Treatment of AMs with 50% saline vehicle control or 50% ARDS BALF had no significant effect on viability (figure 7.4A, n=5, repeated measures ANOVA p=0.573). Treatment of AMs with 50% saline vehicle control or 50% ARDS BALF had no significant effect on apoptosis (figure 7.4B, n=5, repeated measures ANOVA p=0.146).

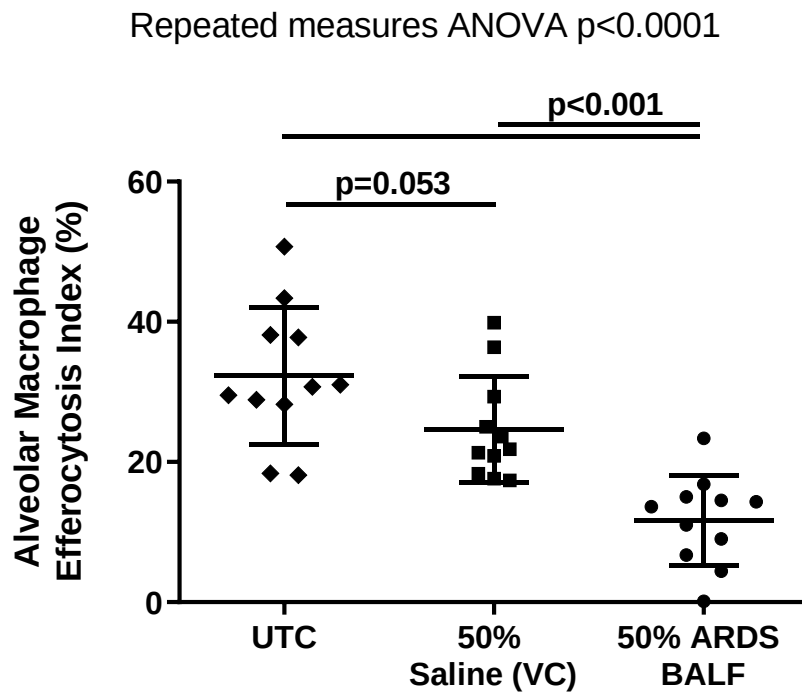


Figure 7.3: Effect of 50% pooled ARDS BALF treatment on alveolar macrophage efferocytosis

UTC = Untreated Control (cultured in RPMI + 10% FBS). VC = Vehicle control (50% saline). Data shown as mean and standard deviation, $n=11$ for all groups. Statistical analysis by repeated measures ANOVA and Tukey's multiple comparisons test. Repeated measures ANOVA $p < 0.0001$. 50% ARDS BALF treatment significantly reduced AM efferocytosis compared to VC (mean of differences 12.97%, $p=0.0006$) and UTC (mean of differences 20.55%, $p=0.0009$). Treatment of AMs with VC had no effect on efferocytosis compared to UTC (mean of differences 7.57%, $p=0.053$).

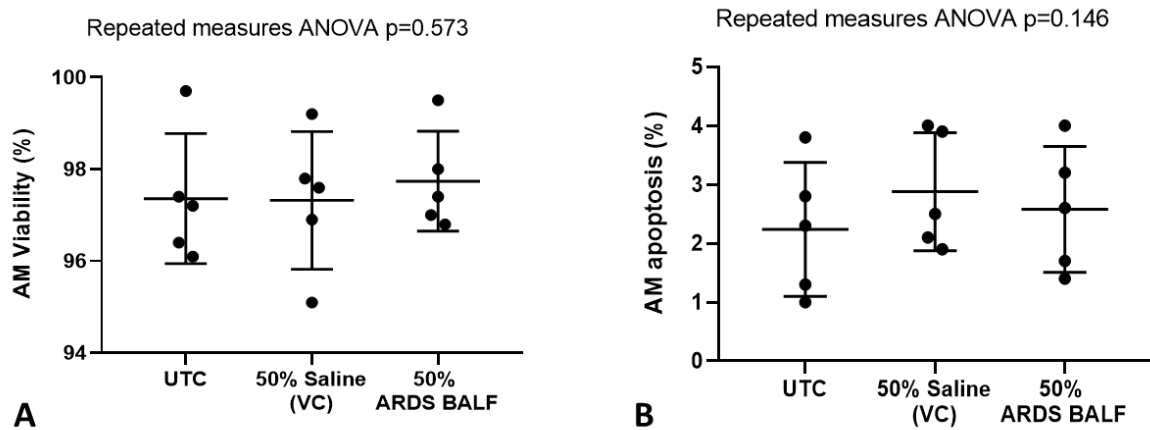


Figure 7.4: Effect of ARDS BALF treatment on alveolar macrophage viability and apoptosis.

UTC = Untreated control (RPMI + 10% FBS). AM = Alveolar macrophage. VC = Vehicle control (50% Saline). Data shown as mean and standard deviation, n=5 for each group. **A:** VC treatment and 50% ARDS BALF treatment have no significant effect on AM viability (repeated measures ANOVA p=0.573). **B:** VC treatment and 50% ARDS BALF treatment have no significant effect on AM apoptosis (repeated measures ANOVA p=0.146).

AM treatment with HMGB-1 at concentrations previously observed in ARDS BALF(91) significantly decreased efferocytosis compared to treatment with vehicle control (figure 7.5A, mean of differences 9.41%, n=7, p=0.0025). AM treatment with LPS + IFN γ significantly decreased efferocytosis compared to treatment with vehicle control (figure 7.5B, mean of differences 16.47%, n=7, p=0.031). AM treatment with IL-4 + IL-13 has no significant effect on efferocytosis compared to treatment with vehicle control (figure 7.5C, mean of differences 1.83%, n=7, p=0.34)

AM treatment with 50% ARDS BALF significantly reduced Rac1 mRNA expression, compared to treatment with 50% saline vehicle control (figure 7.6, median of differences 0.475, n=8, p=0.0156).

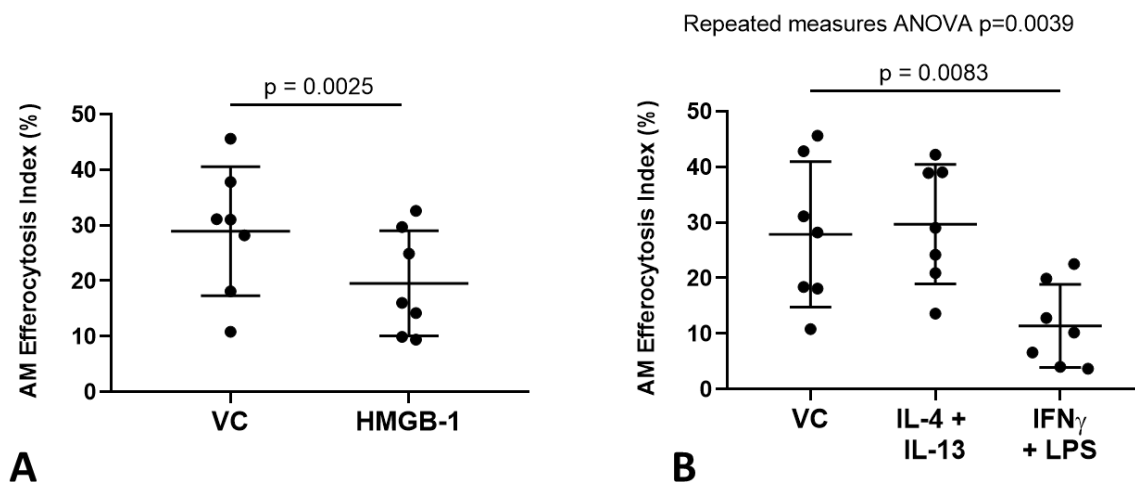


Figure 7.5: Effect of pro- and anti-inflammatory mediators on alveolar macrophage efferocytosis

VC = Vehicle Control (distilled water added at 1:500 to RPMI + 10% FBS). IL-4 + IL-13 = interleukins 4 & 13 used at 40ng/ml each. IFN γ = 50ng/ml interferon γ . LPS = 1 μ g/ml lipopolysaccharide. HMGB-1 = 500ng/ml recombinant human high motility group box-1. VC values are non-identical in graphs **A** and **B**. Data shown as mean and standard deviation, n=7 for all groups. **A:** HMGB-1 treatment significantly reduced AM efferocytosis compared to VC (paired t-test mean of differences 9.41%, p=0.0025). **B:** Cytokine treatment significantly affected AM efferocytosis (repeated measures ANOVA p=0.0089). IFN γ and LPS treatment significantly reduced AM efferocytosis compared to VC (Dunnett's multiple comparisons test mean difference 16.47%, p=0.0083). IL-4 and IL-13 had no significant effect on AM efferocytosis compared to VC (Dunnett's multiple comparisons test mean difference -1.83%, p=0.897).

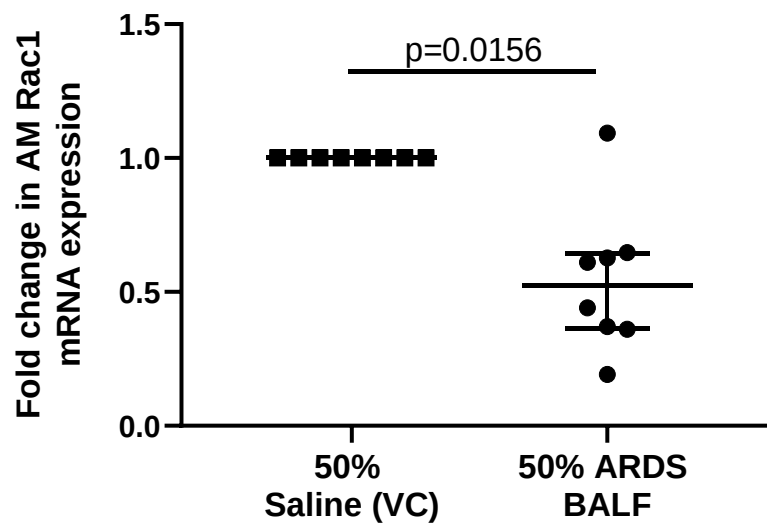


Figure 7.6: Effect of ARDS BALF treatment on Rac1 gene transcription in alveolar macrophages

Data shown as fold change in AM Rac1 mRNA expression from 50% saline treatment. Statistical analysis by Wilcoxon matched-pairs signed rank test, n=8. VC = vehicle control (50% saline). Treatment with 50% ARDS BALF significantly reduced Rac1 mRNA expression in AMs, compared to VC treatment (median of differences 0.475, p=0.0156).

AM treatment with 50% ARDS BALF significantly increased phagocytosis of *S. Aureus* pHrodo® bioparticles compared to treatment with 50% saline vehicle control (figure 7.7A, median of fold change differences = 0.324, n=6, p=0.031). AM treatment with 50% ARDS BALF increased phagocytosis of *E. coli* pHrodo® bioparticles compared to treatment with 50% saline vehicle control, however this did not quite reach statistical significance (figure 7.7B, median of fold change differences = 0.589, n=6, p=0.063).

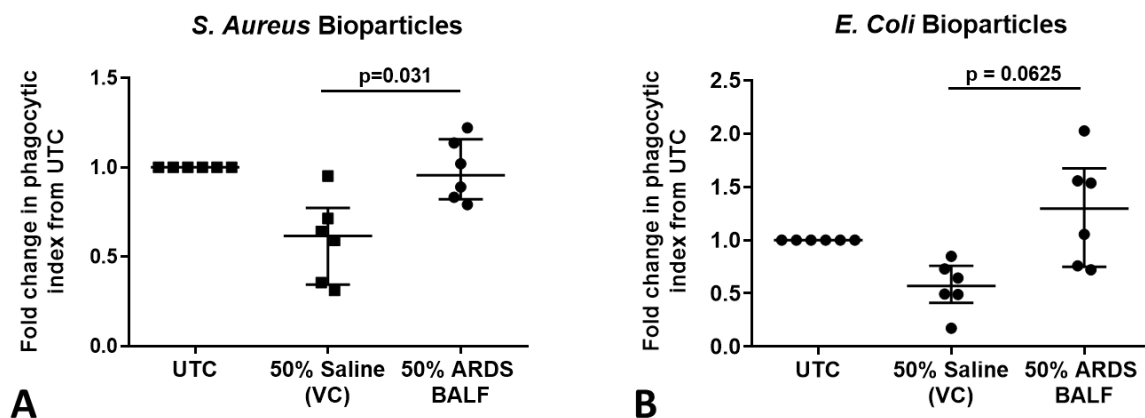


Figure 7.7: Effect of ARDS BALF on alveolar macrophage phagocytosis

UTC = Untreated control (cultured in RPMI + 10% FBS). VC = Vehicle control (50% Saline). Data corrected to fold change in phagocytic index from UTC. Statistical analysis by Wilcoxon matched-pairs signed rank test, n=6 for all groups, *p<0.05. VC values are non-identical in graphs **A** and **B**. Data shown as median and interquartile range. **A:** Treatment with 50% ARDS BALF caused a significant increase in AM phagocytosis of *S. Aureus* bioparticles compared to VC (median of differences 0.324, p=0.031). **B:** Treatment with 50% ARDS BALF caused an increase in AM phagocytosis of *E. coli* bioparticles compared to VC, however this did not reach statistical significance (median of differences 0.589, p=0.063).

AM treatment with 50% saline vehicle control or 50% ARDS BALF has no significant effect on HSD-1 reductase activity (figure 7.8, Friedman ANOVA p=0.383).

IFN γ + LPS treatment significantly increased AM HSD-1 reductase activity compared to vehicle control (figure 7.9, p=0.0087). HMGB-1 treatment and IL-4 + IL-13 treatment had no significant effect on AM HSD-1 activity compared to vehicle control (figure 7.9, p>0.05).

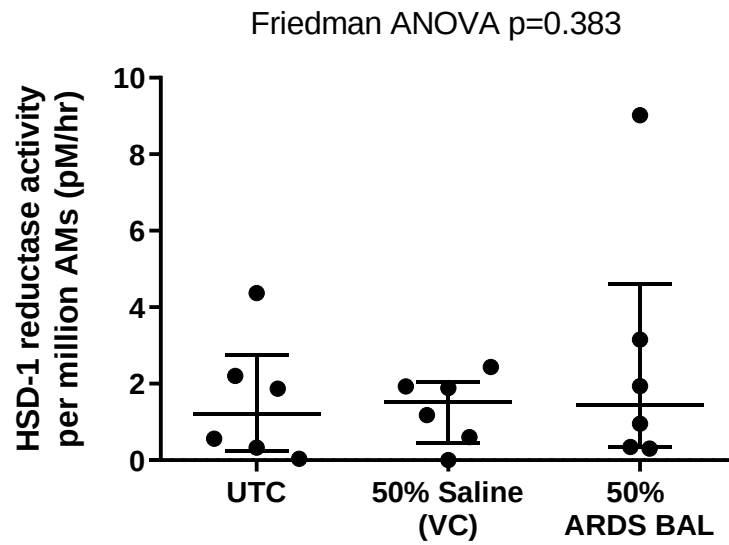


Figure 7.8: Effect of ARDS BALF treatment on alveolar macrophage HSD-1 reductase activity

AM = Alveolar macrophage. HSD-1 = 11 β hydroxysteroid dehydrogenase type 1. UTC = Untreated Control (RPMI +10% FBS). VC = Vehicle control (50% Saline). Data shown as median and interquartile range n=6 for all groups. VC and 50% ARDS BAL treatments have no significant effect on AM HSD-1 reductase activity (Friedman ANOVA p=0.383)

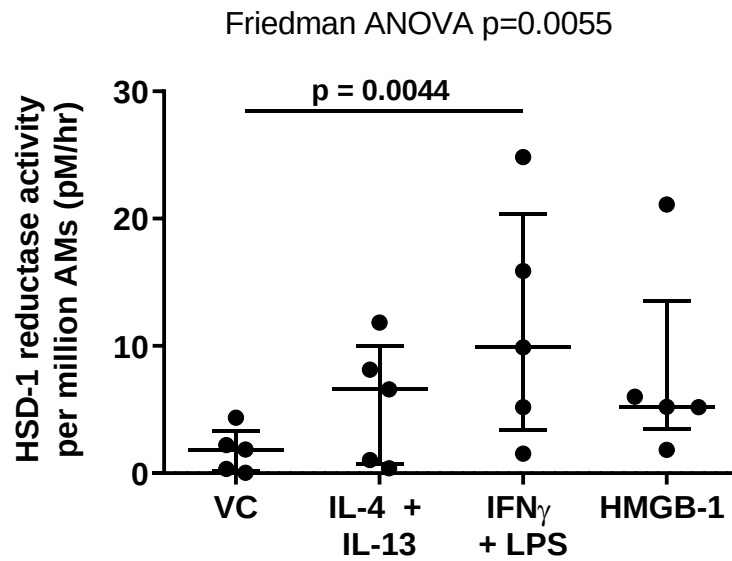


Figure 7.9: Effect of cytokines and HMGB-1 treatment on alveolar macrophage HSD-1 reductase activity

UTC = Untreated Control (RPMI +10% FBS). VC = Vehicle Control (distilled water added at 1:500 to RPMI + 10% FBS). IL-4 + IL-13 = interleukins 4 & 13 used at 40ng/ml each. IFN γ = 50ng/ml interferon γ . LPS = 1 μ g/ml lipopolysaccharide. HMGB-1 = 500ng/ml recombinant human high motility group box-1. Data shown as median and interquartile range, $n=5$ for each group. Friedman ANOVA $p=0.0055$. HMGB-1 treatment significantly increases AM HSD-1 activity compared to VC treatment (Dunn's multiple comparisons test $p=0.0044$). IFN γ + LPS treatment and HMGB-1 treatment have no significant effect on AM HSD-1 activity compared to VC ($p>0.05$).

7.3.2 Impact of ARDS BALF and cytokine treatments on alveolar macrophage phenotype

AM treatment with 50% ARDS BALF increased CD163 surface expression compared to treatment with 50% saline (vehicle control), however this does not reach statistical significance (figure 7.10A, mean of differences 0.441, $p=0.061$). AM treatment with 50% ARDS BALF significantly increased CD206 surface expression compared to treatment with 50% saline (figure 7.10B, mean of differences 0.329, $n=9$, $p=0.042$). AM treatment with 50% ARDS BALF did not significantly change CD80 surface expression compared to treatment with 50% saline (figure 7.10C, mean of differences 0.187, $p=0.122$). AM treatment with 50% ARDS BALF significantly increased Mer surface expression compared to treatment with 50% saline (figure 7.10D, mean of differences 0.296, $p=0.028$). AM treatment with 50% ARDS BALF significantly decreased SIRP α surface expression compared to treatment with 50% saline (figure 7.10E, mean of differences 0.262, $p=0.0063$).

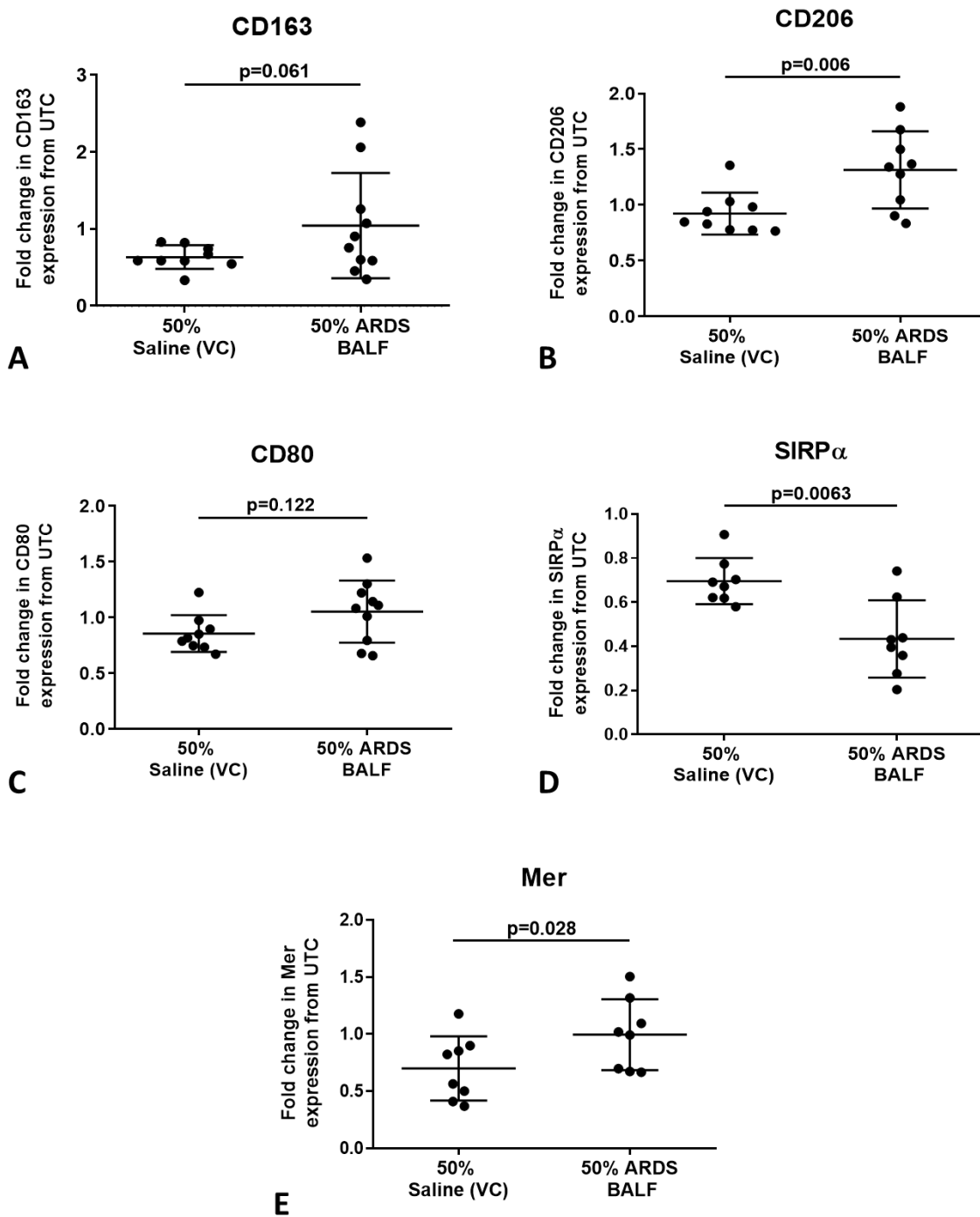


Figure 7.10: Effect of ARDS BALF on alveolar macrophage phenotype and surface receptor expression

UTC = Untreated control (RPMI + 10% FBS). VC = Vehicle control (50% Saline). Mer = Mer receptor tyrosine kinase. SIRP α = Signal regulatory protein alpha. Statistical analysis by paired t-test, $n \geq 8$ for all groups. **A:** 50% ARDS BALF treatment significantly increased CD206 expression on AMs compared to VC treatment (mean of differences 0.391, $p=0.006$). **B:** 50% ARDS BALF treatment increased CD163 expression on AMs compared to VC treatment, however this difference did not reach

statistical significance (mean of differences 0.441, $p=0.061$). **C:** 50% ARDS BALF treatment did not significantly change CD80 expression on AMs compared to VC treatment (mean of differences 0.187, $p=0.122$). **D:** 50% ARDS BALF treatment significantly decreased SIRP α expression on AMs compared to VC treatment (mean of differences 0.262, $p=0.0063$). **E:** 50% ARDS BALF treatment significantly increased Mer expression on AMs compared to VC treatment (mean of differences 0.296, $p=0.028$).

AM surface expression of CD163 significantly decreased following treatment with IFN γ + LPS (figure 7.11A, mean of differences -0.546, $p=0.0054$), and IL-4 + IL-13 (figure 7.11A, mean of differences -0.447, $p=0.002$) compared to VC. AM surface expression of CD206 significantly increased following treatment with IL-4 + IL-13 (figure 7.11B, mean of differences 0.923, $p=0.013$) compared to VC. Cytokine treatments did not significantly affect AM surface expression of CD80 (figure 7.11C, repeated measures ANOVA $p=0.098$). AM surface expression of SIRP α significantly increased following treatment with IFN γ + LPS (figure 7.11D, median of differences 2.48, $p=0.036$) compared to VC. AM surface expression of SIRP α significantly decreased following treatment with IL-4 + IL-13 (figure 7.11D, median of differences -0.59, $p<0.0001$) compared to VC. AM surface expression of Mer significantly decreased following treatment with, IFN γ + LPS (figure 7.11E, mean of differences -0.576, $p=0.015$), and IL-4 + IL-13 (figure 7.11E, mean of differences -0.471, $p=0.0039$) compared to VC.

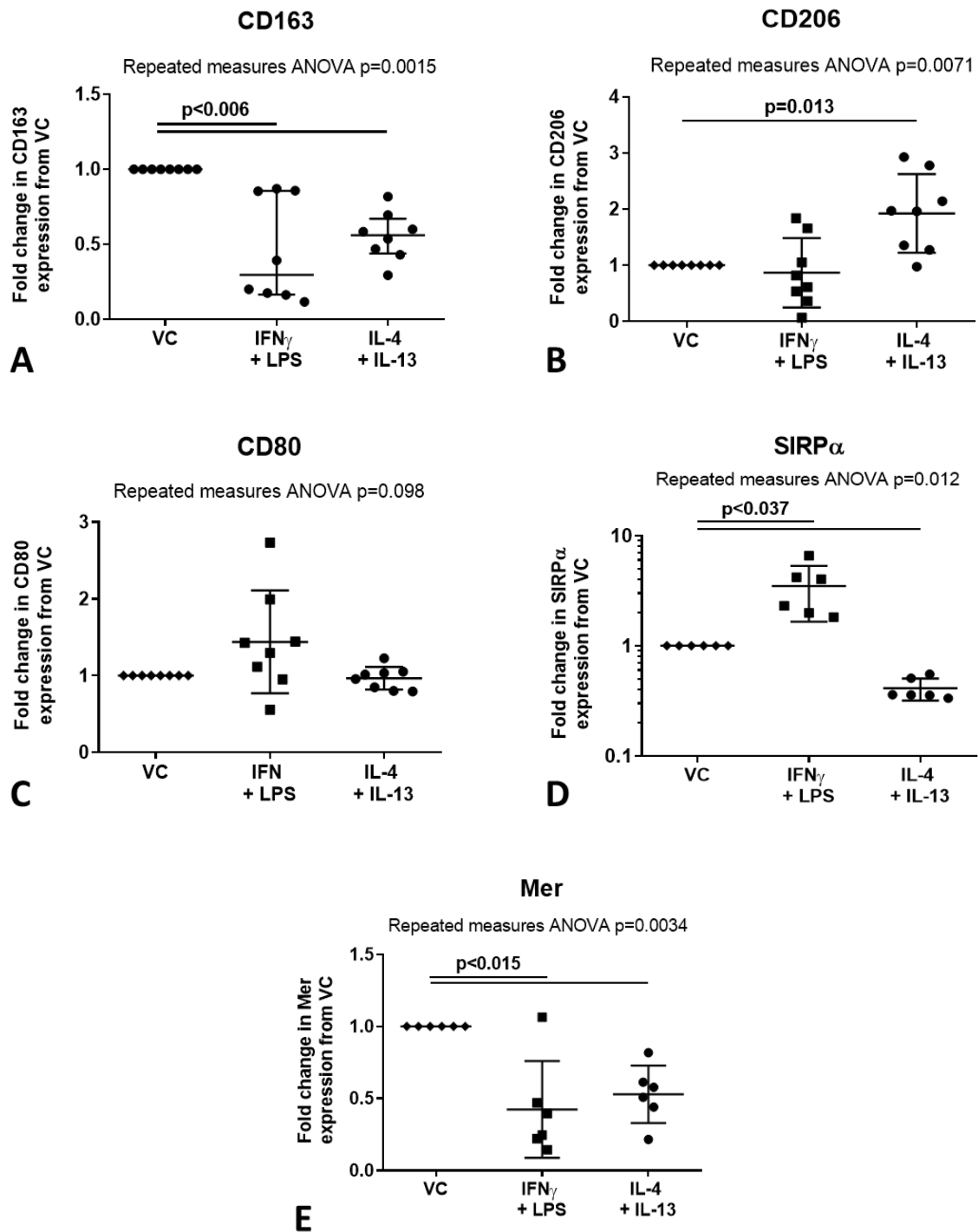


Figure 7.11: Effect of cytokines on alveolar macrophage phenotype and surface receptor expression

VC = Vehicle Control (distilled water added at 1:500 to RPMI + 10% FBS). Statistical analysis by repeated measures ANOVA with Dunnett's multiple comparison test, $n=6-8$. Linear y-axis used for all graphs, except SIRP α (D) for which a log scale y-axis was used. **A:** Cytokine treatments significantly affected AM surface expression of CD163 (repeated measures ANOVA $p=0.0015$). Compared to treatment with VC,

AM surface expression of CD163 was significantly decreased following treatment with IFN γ + LPS (mean of differences -0.546, $p=0.0054$), and IL-4 + IL-13 (mean of differences -0.447, $p=0.002$). **B:** Cytokine treatments significantly affected AM surface expression of CD206 (repeated measures ANOVA $p=0.0071$). Compared to treatment with VC, AM surface expression of CD206 was significantly increased following treatment with IL-4 + IL-13 (mean of differences 0.923, $p=0.013$). There were no significant changes in CD206 expression following treatment with IFN γ + LPS (mean of differences -0.133, $p=0.777$). **C:** Cytokine treatments did not significantly affect AM surface expression of CD80 (repeated measures ANOVA $p=0.098$). **D:** Cytokine treatments significantly affected AM surface expression of SIRP α (repeated measures ANOVA $p=0.012$). Compared to treatment with VC, AM surface expression of SIRP α was significantly increased following treatment with IFN γ + LPS (median of differences 2.48, $p=0.036$). Compared to treatment with VC, AM surface expression of SIRP α was significantly decreased following treatment with IL-4 + IL-13 (median of differences -0.59, $p<0.0001$). **E:** Cytokine treatments significantly affected AM surface expression of Mer (repeated measures ANOVA $p=0.012$). Compared to treatment with VC, AM surface expression of Mer was significantly decreased following treatment with IFN γ + LPS (mean of differences -0.576, $p=0.015$), and IL-4 + IL-13 (mean of differences -0.471, $p=0.0039$).

HMGB-1 treatment significantly affected expression of AM surface markers (figure 7.12, repeated measures ANOVA $p=0.0012$). HMGB-1 treatment significantly decreased AM surface expression of CD163 (figure 7.12, mean of differences -0.567, $p=0.003$). HMGB-1 treatment significantly increased AM surface expression of CD80 (figure 7.12, mean of differences 0.621, $p=0.0166$). HMGB-1 treatment had no significant effect on AM surface expression of CD206, SIRP α and Mer (figure 7.12, $p>0.05$ for all).

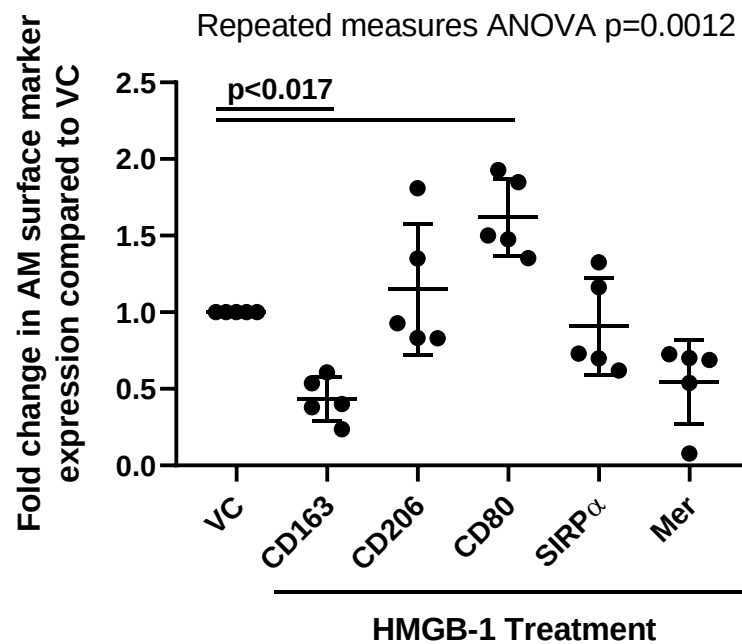


Figure 7.12: Effect of HMGB-1 on alveolar macrophage phenotype and surface receptor expression

VC = Vehicle Control (distilled water added at 1:500 to RPMI + 10% FBS). Statistical analysis by repeated measures ANOVA with Dunnett's multiple comparison test, $n=5$. HMGB-1 treatment significantly affected expression of AM surface markers (repeated measures ANOVA $p=0.0012$). HMGB-1 treatment significantly decreased AM surface expression of CD163 (mean of differences -0.567 , $p=0.003$). HMGB-1 treatment significantly increased AM surface expression of CD80 (mean of differences 0.621 , $p=0.0166$). HMGB-1 treatment had no significant effect on AM surface expression of CD206, SIRP α and Mer ($p>0.05$ for all).

7.3.3 Impact of intracellular pathway inhibitors and HSD-1 tMSCs on alveolar macrophage function

Addition of DMSO (vehicle control for ROCK and PTEN inhibitors) to 50% ARDS BALF mixture had no significant effect on AM efferocytosis (figure 7.13A, mean of differences 0.047, $p=0.918$). Addition of ROCK-inhibitor to 50% ARDS BALF treatment significantly increased AM efferocytosis compared to treatment with DMSO + ARDS BALF (figure 7.13A, mean of differences 0.17, $p=0.0087$). Addition of PTEN-inhibitor to 50% ARDS BALF treatment had no significant effect on efferocytosis compared to treatment with VC + ARDS BALF (figure 7.13B, mean of differences 0.030, $p=0.924$). Transwell co-culture of inactive tMSCs (mean of differences 0.173, $p=0.244$) or activated tMSCs (figure 7.13C, mean of differences 0.095, $p=0.416$) with 50% ARDS BALF-treated AMs had no significant effect on efferocytosis compared to ARDS BALF treatment alone. There is no significant difference in AM efferocytosis following inactive or active tMSC treatment (figure 7.13C, mean of differences 0.078, $p=0.50$).

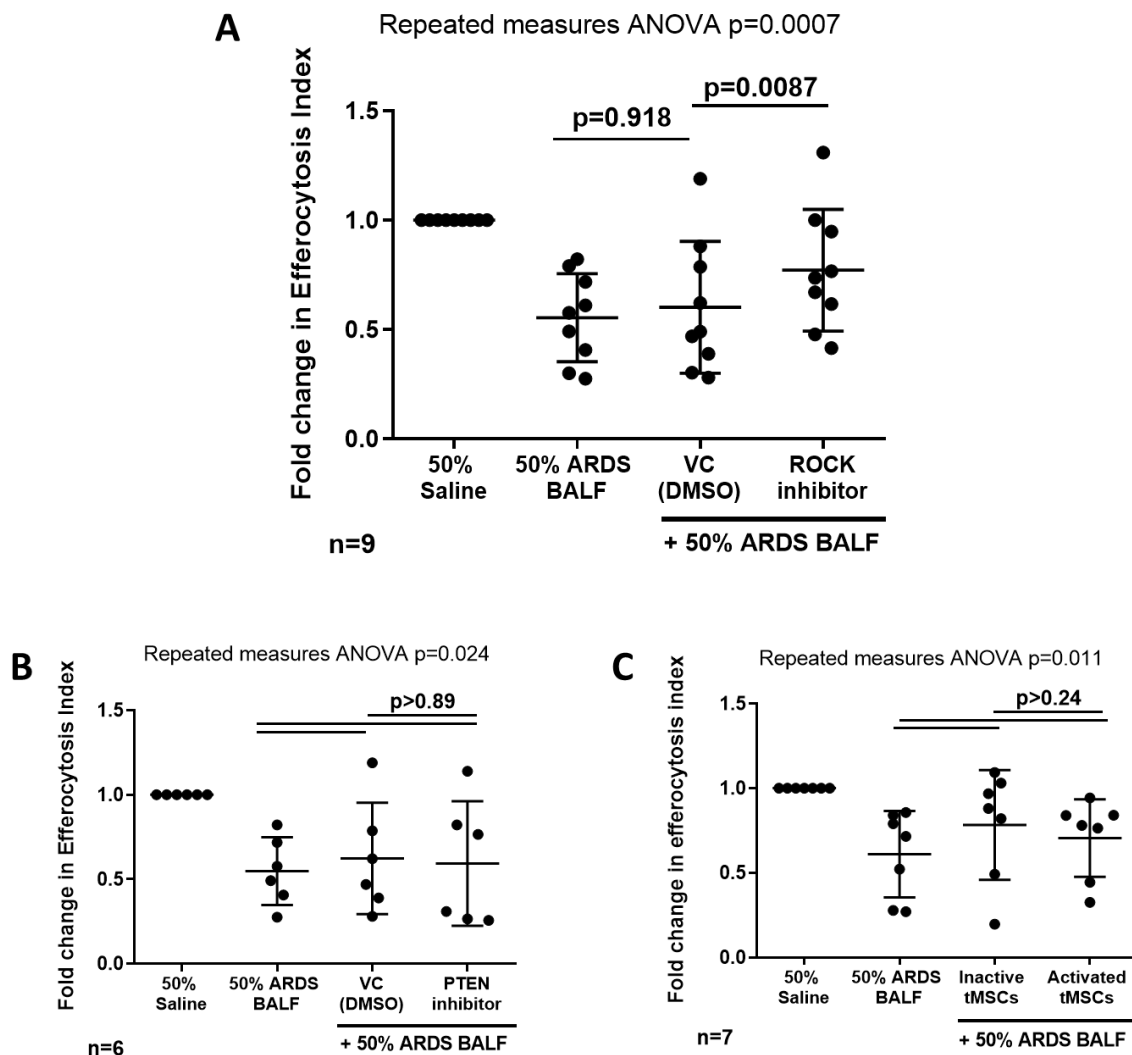


Figure 7.13: Effect of pathway inhibitors and HSD-1 tMSCs on alveolar macrophage efferocytosis

Data shown as fold change in AM efferocytosis index from 50% saline treatment. VC = Vehicle Control (Dimethyl Sulfoxide [DMSO] at 1:50,000 dilution). ROCK-inhibitor = 200nM Y-27632 dihydrochloride; Rho-associated protein kinase inhibitor. PTEN-inhibitor = 2 μ M SF1670; Phosphatase and tensin homolog inhibitor. Statistical analysis by repeated measures ANOVA with Dunnett's multiple comparison test. **A:** Addition of DMSO to 50% ARDS BALF mixture had no significant effect on AM efferocytosis (mean of differences 0.047, $p=0.918$). Addition of ROCK-inhibitor to 50% ARDS BALF treatment significantly increased efferocytosis compared to treatment with VC + ARDS BALF (mean of differences 0.17, $p=0.0087$). **B:** Addition of PTEN-inhibitor to 50% ARDS BALF treatment had no significant effect on efferocytosis compared to treatment with VC + ARDS BALF (mean of differences 0.030, $p=0.924$). **C:** Transwell co-culture of inactive tMSCs (mean of differences 0.173, $p=0.244$) or activated tMSCs (mean of differences 0.095, $p=0.416$) with 50% ARDS BALF-treated AMs had no significant effect on efferocytosis compared to ARDS BALF treatment alone. There is no significant difference in AM efferocytosis following inactive or active tMSC treatment (mean of differences 0.078, $p=0.50$).

Addition of DMSO or ROCK-inhibitor to 50% ARDS BALF mixture had no significant effect on AM phagocytosis of *S. Aureus* pHrodo® bioparticles (figure 7.14A, repeated measures ANOVA $p=0.641$). Addition of DMSO or ROCK-inhibitor to 50% ARDS BALF mixture had no significant effect on AM phagocytosis of *E. coli* pHrodo® bioparticles (figure 7.14B, repeated measures ANOVA $p=0.390$).

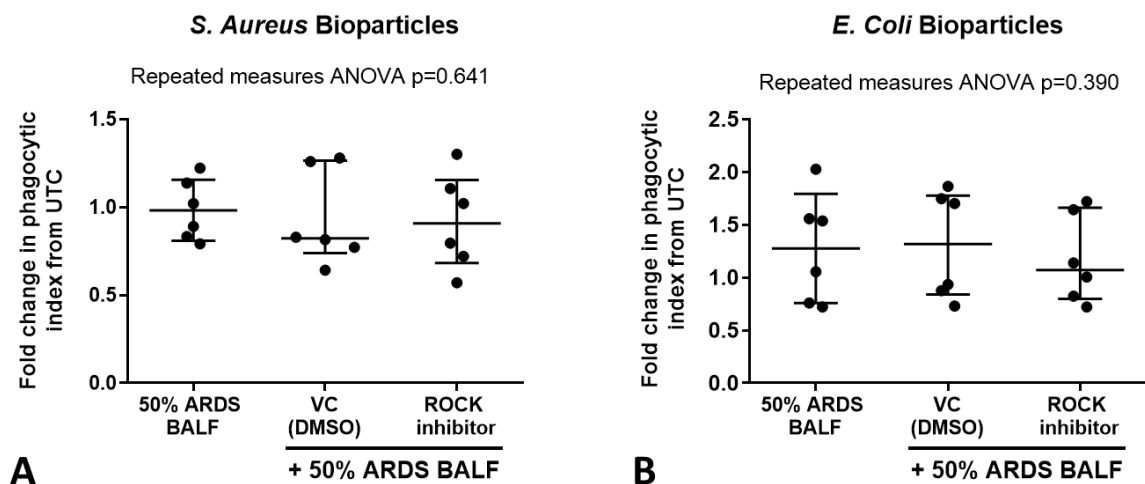


Figure 7.14: Effect of ARDS BALF and ROCK-inhibitor treatment on alveolar macrophage phagocytosis

UTC = Untreated control. VC = Vehicle Control for ROCK-inhibitor (Dimethyl Sulfoxide [DMSO] at 1:50,000 dilution). ROCK-inhibitor = 200nM Y-27632 dihydrochloride; Rho-associated protein kinase inhibitor. 50% Saline acted as vehicle control for 50% ARDS BALF treatment. Data shown as mean and standard deviation, corrected to fold change in phagocytic index from UTC. Statistical analysis by repeated measures ANOVA. **A:** Addition of VC or ROCK-inhibitor to 50% ARDS BALF mixture had no significant effect on AM phagocytosis of *S. Aureus* pHrodo® bioparticles (repeated measures ANOVA $p=0.641$). **B:** Addition of VC or ROCK-inhibitor to 50% ARDS BALF mixture had no significant effect on AM phagocytosis of *E. coli* pHrodo® bioparticles (repeated measures ANOVA $p=0.390$).

7.3.4 Impact of intracellular pathway inhibitors and HSD-1 tMSCs on alveolar macrophage phenotype

Addition of ROCK-inhibitor to 50% ARDS-BALF treatment had no significant effect on AM surface expression of CD163, CD206, CD80, SIRP α and MerTK, compared to treatment with DMSO + ARDS BALF or ARDS BALF alone (figure 7.15A-E, n=7, repeated measures ANOVA $p>0.45$ for all).

Transwell co-culture of inactive tMSCs or activated tMSCs with 50% ARDS BALF-treated AMs had no significant effect on AM surface expression of CD163, CD206, and CD80 compared to ARDS BALF treatment alone (figure 7.16A-C, n=5, repeated measures ANOVA $p>0.45$ for all graphs). Transwell co-culture of inactive tMSCs or activated tMSCs with 50% ARDS BALF-treated AMs had no significant effect on AM surface expression of functional receptors SIRP α and Mer compared to ARDS BALF treatment alone (figure 7.16D-E, n=4, repeated measures ANOVA $p>0.45$ for all graphs).

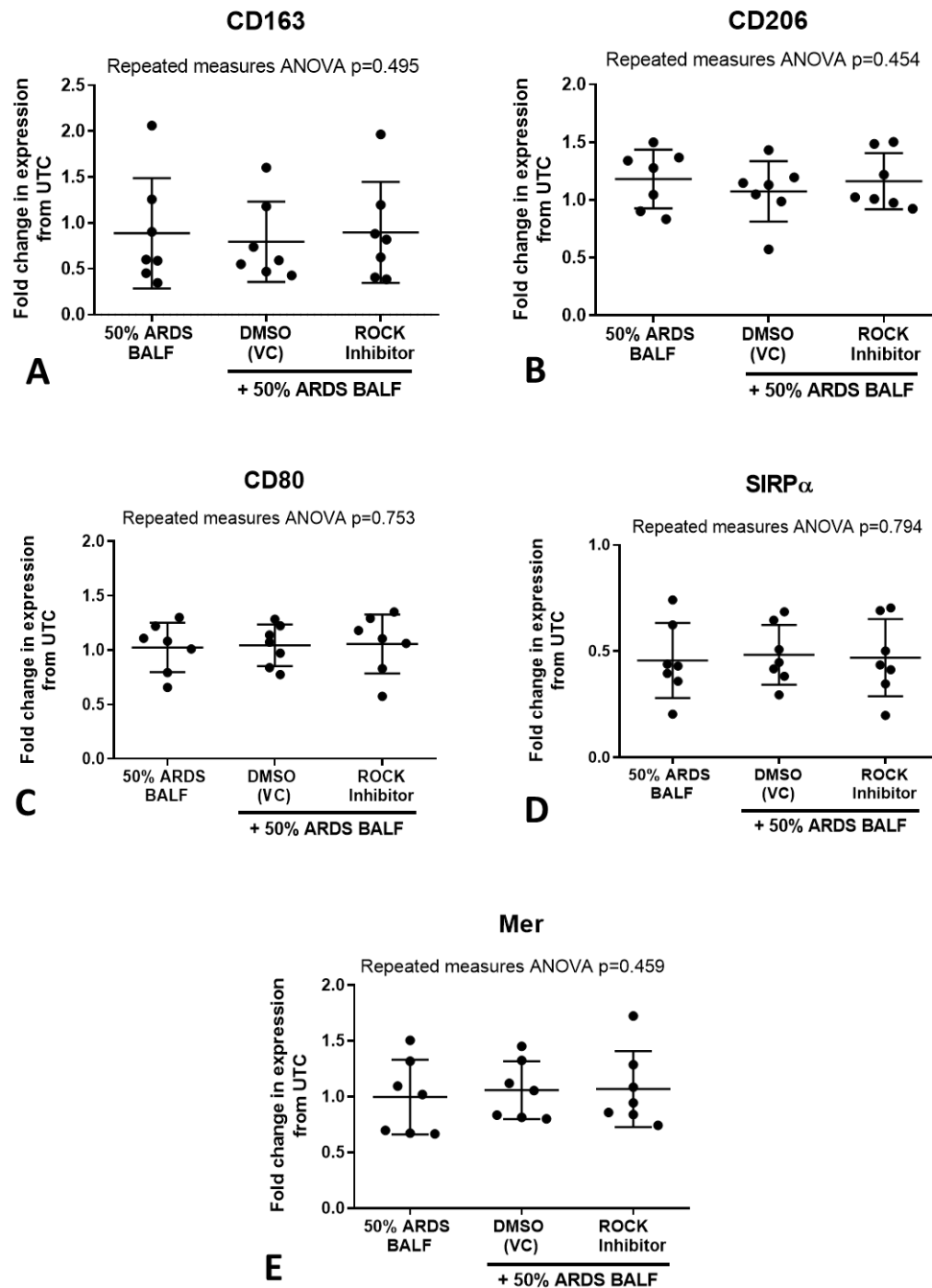


Figure 7.15: Effect of ROCK-inhibitor on alveolar macrophage phenotype and surface receptor expression

VC = Vehicle Control (Dimethyl Sulfoxide [DMSO] at 1:50,000 dilution). ROCK-inhibitor = 200nM Y-27632 dihydrochloride; Rho-associated protein kinase inhibitor. Mer = Mer receptor tyrosine kinase. SIRP α = Signal regulatory protein alpha. Statistical analysis by paired t-test, $n=7$ for all groups. **A-E**: Addition of ROCK-inhibitor to 50% ARDS-BALF treatment had no significant effect on AM surface expression of CD163, CD206, CD80, SIRP α and MerTK, compared to treatment with VC + ARDS BALF ($p>0.05$ for all) or ARDS BALF alone (repeated measures ANOVA $p>0.45$ for all).

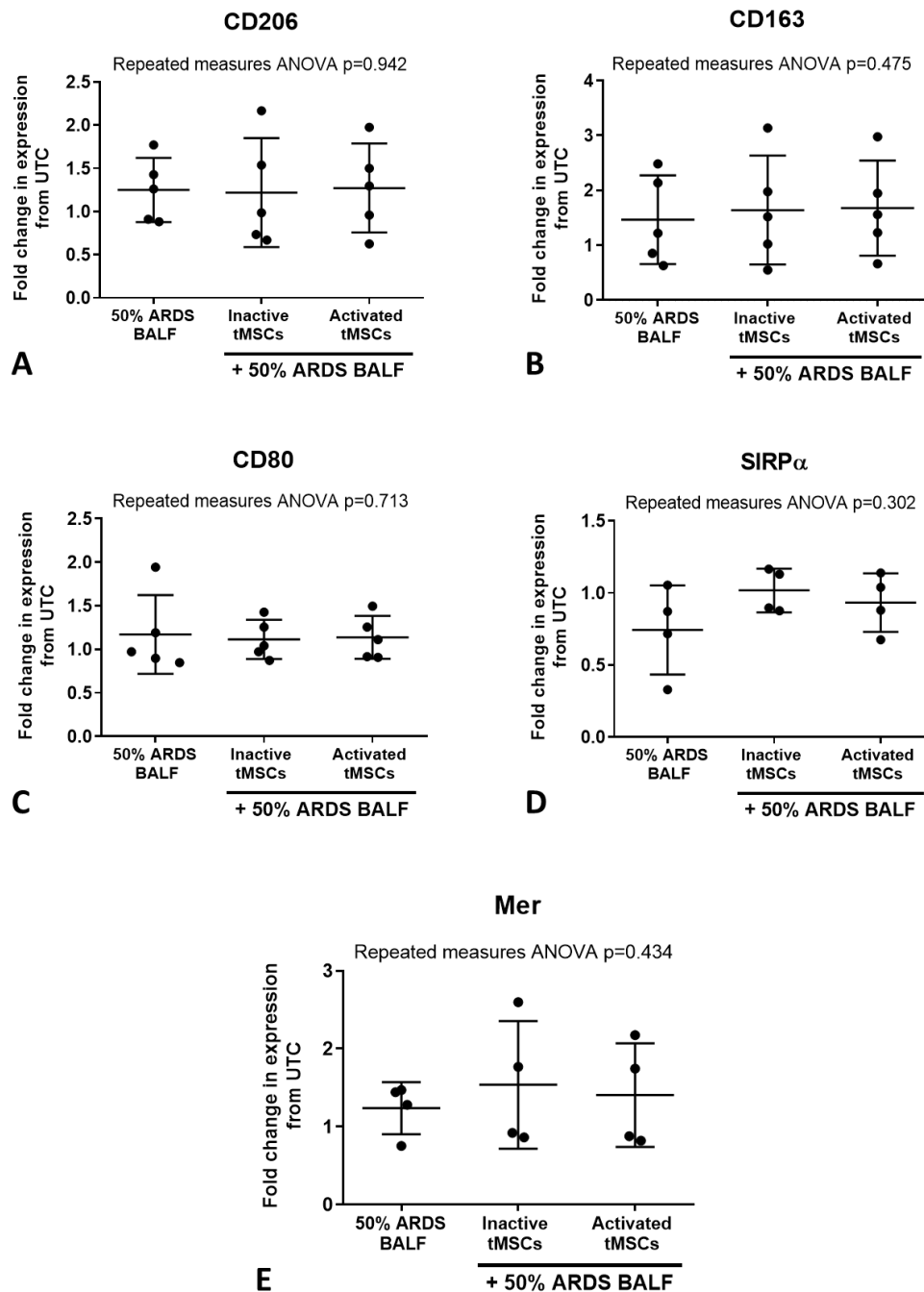


Figure 7.16: Effect of HSD-1 tMSC transwell co-culture on alveolar macrophage phenotype and surface receptor expression

AMs were exposed to 50% ARDS BALF for 48 hours, then media was changed to serum-free RPMI containing 10^{-7} M cortisone and a 6 hour transwell co-culture was performed with inactive HSD-1 tMSCs, activated HSD-1 tMSCs, or no cells. Following co-culture, surface marker expression was assessed. Statistical analysis repeated measures ANOVA, $n=4-5$. **A-F**: Transwell co-culture of inactive or activated tMSCs with 50% ARDS BALF-treated AMs had no significant effect on surface expression of CD163, CD206, CD80, SIRP α or Mer compared to ARDS BALF treatment alone (repeated measures ANOVA $p>0.30$ for all).

7.4 DISCUSSION

The main findings in this chapter are that *in vitro* treatment of AMs with pooled ARDS BALF induced the same impairment in efferocytosis observed in patients with ARDS. Impaired efferocytosis was also observed when AMs were treated with pro-inflammatory mediators: HMGB-1, IFN γ and LPS. AM treatment with ARDS BALF also downregulated mRNA transcription of Rac1, an effector GTPase which causes actin re-arrangement and engulfment of apoptotic cells, thereby supporting findings of decreased efferocytosis. Activation of Rac1 is the final common goal of many intracellular efferocytosis pathways. Unexpectedly, ARDS BAL increased AM phagocytosis of bacterial bioparticles. The pro-phagocytic effect was greater with gram-positive (*S. Aureus*) bioparticles, than gram-negative (*E. coli*) bioparticles. This shows that ARDS BALF treatment has a differential effect on AM phagocytosis and efferocytosis. ARDS BALF treatment has no significant effect on AM viability or apoptosis, indicating that the functional and phenotypic changes observed are unrelated to cell death or apoptosis.

The effect of ARDS BALF on AM phenotype and surface marker expression was unexpected and contrary to what had been hypothesised. Treatment with ARDS BALF increases AM expression of M2 markers CD206 and CD163. No change was seen in expression of M1 marker CD80. Similarly, ARDS BALF increased AM surface expression of Mer (pro-efferocytic) and decreased SIRP α (anti-efferocytic). Taken in isolation, these surface receptor changes would normally be associated with increased efferocytosis, however the opposite is true following ARDS BALF treatment.

Pro-inflammatory mediators (HMGB-1, IFN γ and LPS) were found to decrease expression of CD163 and Mer, whilst increasing expression of CD80 and SIRP α . The changes in surface marker expression elicited by these components correlated with efferocytic function: decreased efferocytosis was associated with phenotypic shift towards M1, decreased Mer, and increased SIRP α expression. Intriguingly, although ARDS BALF treatment and pro-inflammatory mediator (HMGB-1, IFN γ and LPS) treatments both elicit impaired AM efferocytosis, the treatments have completely **opposite** effects on AM phenotype and efferocytic receptor expression.

One interpretation of these results is that ARDS BALF decreases efferocytosis by an alternate mechanism to M1 phenotypic shift, and these surface receptor changes are compensatory measures taken by the AMs in an unsuccessful attempt to restore normal efferocytic function. Further analysis of ARDS BALF to identify other mediators which affect AM phenotype and function is required. A similar correlation has been observed in cigarette smokers, between decreased AM efferocytosis(318, 319), increased Mer receptor expression on AMs(133), and increased transcription of genes associated with M2 polarisation in AMs(104). COPD patients also have impaired AM efferocytosis(320) and overexpression of M2 surface markers CD206 and CD163 on AMs(321). These changes in COPD AMs are also likely to be compensatory, in an unsuccessful attempt to restore efferocytosis.

ARDS BALF treatment of AMs had no significant effect on HSD-1 reductase activity, which supports the findings in chapter 6 that there are HSD-1 independent mechanisms of impaired AM efferocytosis in ARDS. The inability of ARDS BALF to decrease AM HSD-1 activity indicates that in patient with ARDS, either AM HSD-1 deficiency occurs during sepsis and then leads to the development of ARDS, or that

the impaired HSD-1 activity observed in ARDS patients is due to pro-inflammatory infiltrating monocytes / immature macrophages in the alveolar space which are known to have reduced HSD-1 activity. Co-culture of tMSCs with ARDS BALF-treated AMs also had no significant effect on efferocytosis or phenotype. Since ARDS BALF had no effect on AM HSD-1 reductase activity, upregulating HSD-1 activity likely had a minimal impact on AM function. Also, as mentioned in chapter 6, the duration of co-culture may have been too brief prior to AM functional assessment.

Addition of ROCK-inhibitor to ARDS BALF treatment was able to partially restore AM efferocytic function, however addition of PTEN-inhibitor had no significant effect. It is not clear why inhibition of different factor within the same Rho-ROCK-PTEN pathway elicited different effects, however it may be because the role of PTEN is less important than ROCK in antagonising the PI3K pathway. Addition of ROCK-inhibitor to ARDS BALF treatment had no significant effect on AM phagocytosis of gram positive or negative bioparticles. Therefore, if ROCK-inhibitor does not impair bacterial phagocytosis, which is important if considering its potential therapeutic use in sepsis-related ARDS. Addition of ROCK-inhibitor to ARDS BALF treatment had no significant effect on AM phenotype as assessed by expression of surface receptors CD206, CD163, CD80, Mer and SIRP α .

A limitation of the *in vitro* model of ARDS is that the effect of ARDS BALF treatment is only being assessed on one cell type (AMs), whereas in the alveolar space other cells types (e.g. alveolar epithelial cells) are also present, with interactions occurring between different cells types. For future work, the model could be improved by assessing the effect of ARDS BALF treatment on precision-cut slices of lung tissue. Another limitation of this study is that ROCK-inhibitor was given simultaneously with,

instead of after, ARDS BALF treatment. Therefore, to establish if ROCK-inhibitor could have a “treatment effect” in ARDS, future experiments need to investigate whether ROCK-inhibitor can still restore AM efferocytic function if given 24 hours after AM exposure to ARDS BALF.

ROCK inhibition promotes PI3K signalling, which has a multitude of effects on cellular function beyond upregulation of Rac1, including control of the cell cycle, proliferation, polarity, chemotaxis, and migration(290, 291). Therefore, ROCK inhibition would have pleiotropic off-target effects in the context of upregulating AM function in ARDS. However, ROCK-inhibitors are currently used as clinical therapy: Netarsudil is a ROCK-inhibitor licenced for the treatment of glaucoma in the USA(322, 323). Pre-clinical studies identifying the ability of Y-27632 to enhance outflow facility in glaucoma patients led to the development of Netarsudil, which was found to have a higher potency of ROCK inhibition than Y-27632(324, 325). If Netarsudil can be safely administered via a topical route in glaucoma, could it also have potential as a nebulised therapy to restore AM function in ARDS? Future studies will determine whether Netarsudil can restore AM efferocytosis in an *in vitro* model of ARDS, in a similar way to Y-27632. Other existing medications could also be tested using the *in vitro* model of ARDS, to determine if they can restore AM efferocytosis and thus offer a potential therapeutic strategy, e.g. N-acetylcysteine(313), macrolide antibiotics(314, 315), and statins(163).

As mentioned in chapter 6, advances in immunometabolism have found that macrophage phenotype is associated with metabolic changes, e.g. switch to a pro-inflammatory M1 phenotype is associated with decreased mitochondrial respiration and increased glycolysis(307). AMs from COPD patients have dysregulated

mitochondrial oxidative phosphorylation, leading to a defective phenotype with decreased intracellular bacterial killing (309). Future studies using the *in vitro* model of ARDS to assess AM metabolic function would help to reveal the mechanism by which ARDS BALF impairs AM function. The *in vitro* model of ARDS could also be used to block other components of ARDS BALF (e.g. TNF- α , IL-6) in order to determine the cause of the inverse correlation elicited between AM phenotype and function.

In summary, ARDS BALF treatment of AMs *in vitro* can decrease efferocytosis, causing the same functional impairment observed in ARDS patients. ARDS BALF treatment also increases AM bacterial phagocytosis, indicating a differential effect on AM function. Unexpectedly, ARDS BALF treatment increases expression of pro-efferocytic surface receptors (CD206 and MerTK), whilst decreasing expression of the anti-efferocytic receptor SIRP α . These data therefore suggest that the AM efferocytosis defect that occurs in response to ARDS BALF is not mediated by receptor changes, which if taken alone would be expected to increase efferocytosis. ROCK-inhibitor treatment can partially restore AM efferocytic function following ARDS BALF exposure; which suggests that modulation of the ROCK-PTEN-Rac pathway is the mechanism by which ARDS BALF suppresses AM function. Inhibition of the ROCK intracellular signalling pathway may offer a therapeutic strategy to restore AM function in ARDS patients. Further studies involving ARDS patient AMs, the *in vitro* model of ARDS, and murine models are required to investigate the role of the ROCK-PTEN-Rac pathway in ARDS AM dysfunction.

CHAPTER 8 : HSD-1 TRANSGENIC MESENCHYMAL STEM CELL THERAPY IN MURINE MODELS OF LUNG INJURY

8.1 INTRODUCTION

Multiple studies have shown that sepsis is the underlying aetiological factor for developing ARDS in ~80% of patients(1, 14, 16, 17). Sepsis can cause lung injury directly (e.g. pneumonia) or indirectly (e.g. peritonitis and bacteraemia). In ARDS patients, there are complex interactions between co-morbidities, pre-disposing factors, the immune response, and infective organisms. In the preceding chapter, a reductionist model was employed to assess alveolar macrophage (AM) function in ARDS. However, the limitations of this *in vitro* model were recognised, and therefore *in vivo* murine lung injury studies were also undertaken. These would allow me to assess the integrated responses of the host, which takes into account contributions from other cell types, giving a model which is closer to human ARDS. No single animal model can replicate the complexity of human ARDS, since multiple events or 'hits' are involved in disease pathogenesis; therefore animal models of both direct and indirect lung injury are required(221). Pre-clinical murine models of direct and indirect sepsis-related lung injury have shown that mesenchymal stem cell (MSC) administration can reduce mortality, lung injury, pulmonary oedema, inflammatory cytokine release, alveolar cellular infiltration, and bacterial load(202, 203, 222-225). However, as shown in chapter 4 and in the literature(238), the global ability of MSCs to attenuate lung injury decreases with *in vitro* expansion.

Data presented in chapter 6 shows that BALF cortisol: cortisone ratios are significantly lower than serum ratios in ARDS patients (see figure 6.12), which would suggest dysregulated alveolar steroid metabolism.

The enzyme 11 β -hydroxysteroid dehydrogenase type-1 (HSD-1) acts as a reductase to convert inactive cortisone to active cortisol within specific tissues(84). Within the lung, HSD-1 is predominantly expressed within alveolar macrophages in the alveolar space and bronchial epithelial cells(85). Data presented in chapter 6 shows that AMs from sepsis patients with ARDS have impaired HSD-1 reductase activity compared to sepsis patients without ARDS (see figure 6.6A). Correlating with mortality data, AM HSD-1 activity was significantly higher in sepsis patients who survived hospital admission compared to those who died (see figure 6.6B). These data suggested that impaired alveolar HSD-1 activity and a lower cortisol: cortisone ratio were associated with pathogenesis of sepsis-related ARDS and increased severity of lung injury.

Results from this clinical study were supported by findings from animal models. Using the intra-tracheal lipopolysaccharide (IT-LPS) direct lung injury murine model of ARDS, Dr Sian Lax (University of Birmingham) found that HSD-1 knockout (KO) mice have exaggerated and prolonged inflammation, with increased alveolar permeability & apoptotic neutrophil accumulation compared to wild type (WT) mice (see figure 1.2). Cortisol is known to upregulate macrophage efferocytosis(176), therefore alveolar HSD-1 deficiency may cause apoptotic neutrophil accumulation and prolonged inflammation in murine models of lung injury. Data presented in chapter 6 shows a correlation between AM HSD-1 deficiency, impaired efferocytosis, apoptotic neutrophil accumulation and increased inflammatory cytokines in the alveolar space of ARDS patients. Upregulation of HSD-1 in the alveolar space to increase the cortisol: cortisone ratio may therefore potentially offer a therapeutic strategy for ARDS.

MSCs localise to the lungs following intravenous administration(187, 225), making MSCs an ideal vector for delivering transgene therapy in pulmonary diseases. Use of transgenic MSCs (tMSCs) which express HSD-1 may be a strategy to compensate for the loss of intrinsic anti-inflammatory potential seen in MSCs following successive passage. It was hypothesised that the synergistic anti-inflammatory action of combined MSC and HSD-1 gene therapy will be superior to MSC therapy alone in murine models of sepsis-related lung injury (see figure 8.1). The aim of this chapter is to determine whether HSD-1 tMSCs can attenuate inflammatory injury in a murine model of indirect septic lung injury; caecal ligation and puncture. HSD-1 knockout (KO) mice were used in some of these experiments, to recapitulate as closely as possible the HSD-1 deficient alveolar environment observed in ARDS patients.

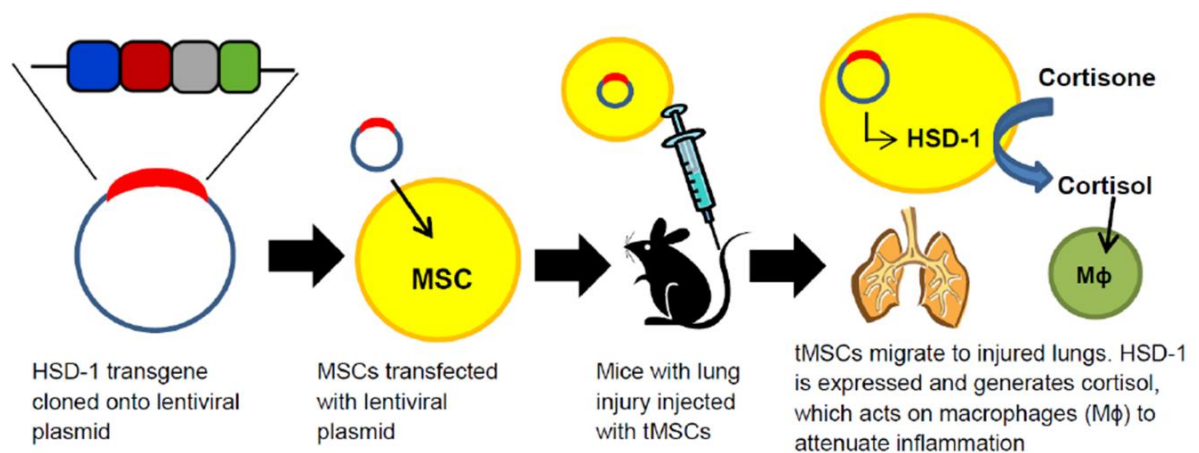


Figure 8.1: Summary of the mechanism by which HSD-1 tMSCs could attenuate inflammation in a murine model of lung injury.

8.2 METHODS

Full methods can be found in section 3.8.

8.2.1 Ethical statement

All procedures were performed in compliance with UK law under the Animal [Scientific Procedures] Act 1986, with approval from the Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO). The project licence code was PAAB1C3B2. The 3Rs principles (Reduction, Replacement and Refinement) guided the design and methodology of our animal studies.

8.2.2 Experimental animals and study design

Male wild-type (WT) C57BL/6 mice (aged 5-7 weeks old) were obtained from Charles River, UK and maintained at the Biomedical Services Unit (BMSU), University of Birmingham, UK. A colony of 11 β -Hydroxysteroid Dehydrogenase Type 1 Knockout (HSD-1 KO) C57BL/6 mice was also maintained at BMSU. This colony was originally a kind gift from Professor Gareth Lavery, University of Birmingham. Caecal ligation and puncture (CLP) surgery was performed on mice aged 8-12 weeks. To ensure that mice were appropriately monitored post-procedure, CLP was performed on no more than 8 mice at any one time. HSD-1 transgenic mesenchymal stem cells (tMSC) were created as described in chapter 5. Mice undergoing CLP were divided into 3 intravenous treatment groups: phosphate buffered saline (PBS), activated HSD-1 tMSCs and inactive HSD-1 tMSCs (n=6 per group). Our experimental methods were designed to minimise the number of animals used and maximise scientific results (e.g. consistent use of the C57BL/6 strain, male gender and age).

This enabled comparisons across WT and HSD-1 KO mice, and measurement of multiple endpoints in the same mouse. Details of animal housing and husbandry are detailed in section 3.9.3.

8.2.3 Caecal ligation and puncture (CLP) surgery

One hour prior to CLP, mice were given intravenous treatments (via the tail vein) of either 50µl phosphate buffered saline (PBS, Sigma-Aldrich) alone, activated HSD-1 tMSCs suspended in 50µl PBS, or inactive HSD-1 tMSCs suspended in 50µl PBS. A range of tMSC doses were used between 50,000 to 250,000. Analgesia was given 30 minutes prior to CLP: subcutaneous buprenorphine at 0.1mg/kg body weight (Temgesic®, Reckitt Benckiser, Switzerland). Mice were anaesthetised with isoflurane gas (5% in oxygen delivered at 1.5L/min) for induction and maintenance anaesthesia (1-3% in oxygen delivered at 1.5L/min). All CLP surgeries were undertaken in animal surgery theatres within the BMSU, using aseptic technique, i.e. use of sterile gown, gloves, drapes, and surgical equipment. The CLP experimental design was adapted from previously published protocols(277, 326). CLP surgery was performed on tables heated to 37.5°C. Following induction of anaesthesia, fur was shaved from the abdomen and 2% chlorhexidine (Avagard™, Pymble, Australia) was then used to sterilise the skin. A midline laparotomy incision was performed followed by exposure of the caecum, ligation of the lower 30% with 2.0 Ethilon™ nylon suture (Ethicon, USA) and a single puncture of the ligated caecum with a 19G microlance needle (Becton Dickinson, UK). A small amount of faeces was then expressed by gently squeezing the ligated caecum with forceps, before being placed back into the abdominal cavity. The abdominal wall was sutured with 6.0 Vicryl® (Ethicon, USA)

followed by skin suture with 4.0 Prolene® (Ethicon, USA). All mice were recovered in heat boxes and recovery incubators until euthanised. Postoperative resuscitation was with 500µl of Hartman's solution (Aquapharm 11®, Animalcare Ltd, UK) subcutaneously and a dose of buprenorphine as described before. Sham CLP surgery involved laparotomy and handling of caecum (without ligation and puncture) prior to closure. Two different time courses of CLP were used: 4 hours and 24 hours. During the 4 hour time course, animals were monitored continuously. During the 24 hour time course, mice were monitored every 2-4 hours, and given a further 500µl of subcutaneous Hartmann's solution at 6 hours post-CLP. Mice exposed to either time course were also given additional boluses of buprenorphine to maintain an appropriate level of analgesia.

8.2.4 Sample collection, processing and analysis

On completion of the CLP time course (4 or 24 hours post-CLP), mice were deeply anaesthetised with 5% isoflurane in oxygen delivered at 1.5L/min. Cardiac puncture was performed and death confirmed. Cardiac blood was aliquoted for processing later. Immediately post-mortem, peritoneal lavage fluid (PLF) was collected by instilling 2 mls of PBS including 1% EDTA into the lower quadrants of the abdomen, gently compressing the abdomen several times, then aspirating back from the lower quadrants. The chest cavity and neck were then opened, and a small incision was made in the upper trachea. A fine bore polythene tube was inserted into the trachea until the tip was slightly above the carina. Broncho-alveolar lavage fluid (BALF) was collected by lavaging the lungs twice with 600µl of PBS including 1% EDTA via the tracheal tube. The liver and caecum were examined for colour change and signs of

necrosis. Lung tissue was extracted for use in HSD-1 activity assays as described in section 8.2.6. All samples were immediately transferred on ice to the laboratory for processing and analysis.

Cardiac blood was centrifuged at 13,500 r.p.m. in a micro-centrifuge (Stuart SCF2, Bibby Scientific Ltd, UK) at for 10 minutes. The sera layer was aspirated and stored at -40°C for future analysis. Prior to centrifugation, 20µl of neat PLF and BALF from each mouse was taken for bacterial culture, as discussed in section 3.9.7. PLF and BALF were centrifuged at 400g for 10minutes; supernatants were then aspirated and stored at -40°C for future analysis. The cell pellets were re-suspended in 1ml PBS including 2% BSA and 10% murine serum (Sigma-Aldrich), and incubated on ice for 15 minutes to allow blocking of non-specific Fc receptor binding prior to antibody labelling as discussed in section 3.9.6. During this incubation period, a cell count and cytospin of BALF and PLF cells were performed as described in sections 3.3.5 and 3.3.6. Cells were then labelled for flow cytometric cell identification, as described in section 3.9.6. Quantitative bacterial culture from PLF was performed as described in section 3.9.7. Protein and cytokine assays were performed using BAL, PLF and serum as described in sections 3.8.1 and 3.8.4.

8.2.5 11β-Hydroxysteroid Dehydrogenase Type-1 Activity Assay of Murine Lung Tissue (Thin Layer Chromatography)

This assay was developed and has previously been validated by the Institute for Metabolism and Systems Research at the University of Birmingham(327, 328).

Thin layer chromatography (TLC) was used to measure the HSD-1 reductase activity of murine lung tissue, i.e. the ability to convert 11-dehydrocorticosterone (11-DHC,

murine homolog of cortisone) to corticosterone (murine homolog of cortisol). Murine lung tissue was collected as described in section 8.2.5. Lung tissue was dissected into pieces ~50mg in weight. All tissue pieces were weighed. Pieces of lung tissue were added to glass tubes containing 500µl of serum-free RPMI 1640 supplemented with 10^{-7} M 'cold' non-radioactive 11-DHC (Sigma-Aldrich) and 2.5µl of 'hot' radioactive tritiated 11-DHC (stock concentration 1.5 pM/ml, stock radioactivity 1.4 MBq/ml, a gift from Dr Rowan Hardy, University of Birmingham). A control glass tube without lung tissue was also prepared. Glass tubes were incubated for 6 hours at 37°C and 5% CO₂. Lung tissue was then removed from the tubes and the culture media was either analysed immediately or sealed and frozen at -20°C for future analysis. Frozen samples were thawed at room temperature prior to analysis by thin layer chromatography, which was performed as described in section 3.5. The percentage of steroid conversion was determined as described in section 3.5, then corrected for lung tissue weight.

8.2.6 Statistical Analysis

Data were analysed using Prism 8 software (GraphPad, USA). Normality of data was assessed using the D'Agostino & Pearson test. Differences between continuously distributed non-parametric data were assessed using Mann-Whitney tests. Differences between three or more non-parametric data sets were assessed using the Kruskal-Wallis one-way analysis of variance (ANOVA) and Dunn's multiple comparison tests. Two-tailed p-values of ≤ 0.05 were considered as significant. Results from parametric data are shown as mean and standard deviation. Results from non-parametric data are shown as median and interquartile range.

8.3 RESULTS

8.3.1 Pilot and safety studies for HSD-1 tMSC administration in the CLP model

The first CLP pilot study used HSD-1 KO mice with a 24 hour time course. Two mice were in each intravenous treatment group: PBS, 250,000 inactive HSD-1 tMSCs, or 250,000 active HSD-1 tMSCs. The dose of tMSCs had been chosen based on previous studies administering similar doses of MSCs in murine models of lung injury (see tables 1.5 and 1.6) and from clinical trials of MSCs in ARDS patients(239, 240) in which the dose was based on weight (10 million MSCs per kg in man equates to 250,000 MSCs for a 25g mouse). These HSD-1 KO mice in the active tMSC treatment group showed greatly accelerated decline based on our clinical scoring system (see section 3.9.3). Unfortunately, one mouse unexpectedly died after 12 hours (condition 18, reported to local home office inspector as protocol violation, breaching severity limits). The second mouse gained a clinical score of 6 (maximal score) at 24 hours and was humanely culled by schedule 1 method. Post mortem revealed significant necrosis of lung and liver tissue in the active tMSC treated mice. In contrast, the saline and inactive tMSC treated mice had normal appearances of lung tissue on post mortem. PLF total cell count and neutrophil count were similar between treatment groups (figure 8.2A-B). A trend towards increased PLF neutrophil apoptosis was observed in mice receiving inactive or active tMSC treatment (figure 8.2C). Quantitative culture revealed PLF bacterial overgrowth in active tMSC treated mice, compared to PBS or inactive tMSC treated mice (figure 8.2D). In response to this condition 18 notice, a safety study of active tMSCs in uninjured mice was

performed to better characterise the activity of tMSC after administration, before any further CLP experiments could be performed.

A dose response was carried out using active tMSCs (50,000, 75,000, 100,000 and 125,000) administered intravenously to HSD-1 KO mice (n=2). These mice were observed closely over 24 hours. No adverse effects were observed at any dose, and post mortem reveal normal organ appearance in all mice. A lower dose of 125,000 tMSCs was determined safe to administer in future CLP experiments.

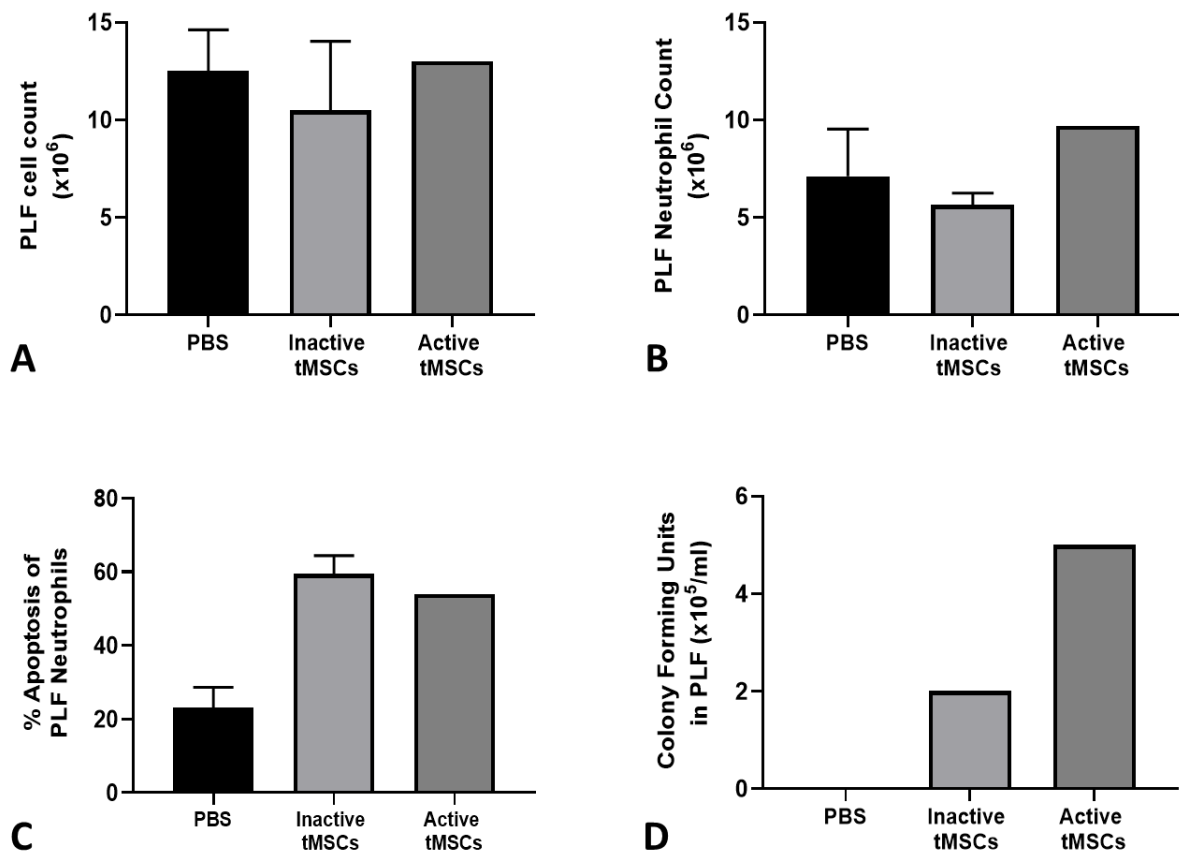


Figure 8.2: PLF cellular recruitment and bacterial growth following a 24 hour time course of HSD-1 KO mice undergoing CLP

HSD-1 KO mice received PBS, 250,000 inactive tMSCs or 250,000 active tMSCs intravenously 1 hour prior to CLP; $n=2$ for each group. 1 mouse in the active tMSC group died prior to the 24hr endpoint. Data presented as mean and standard deviation. **A-B:** PLF total cell count and neutrophil count appear similar between treatment groups. **C:** A trend towards increased PLF neutrophil apoptosis is observed in mice receiving inactive or active tMSC treatment. **D:** A trend towards increased bacterial growth is observed in mice receiving active tMSCs.

Using this validated tMSC dose (125,000) a CLP pilot study was carried out to investigate the effect of administering active tMSCs in WT mice, in combination with septic insult, over 24 hours. No significant difference in PLF cell count was observed between PBS and active tMSC treatment (figure 8.3A, medians 10.6 vs 8×10^6 ,

p=0.571). No significant difference in PLF neutrophil count was observed between PBS and active tMSC treatment (figure 8.3B, medians 8 vs 6.1×10^6 , p=0.343). No significant difference in the proportion of neutrophils in PLF was observed between PBS and active tMSC treatment (figure 8.3C, medians 76.3% vs 78.6%, p=0.99). No significant difference in PLF neutrophil apoptosis was observed between PBS and active tMSC treatment (figure 8.3D, medians 16% vs 9.5%, p=0.686). Bacterial growth in PLF was significantly higher following active tMSC treatment compared to PBS (figure 8.3E, medians 7.5 vs 67.5×10^7 CFU/ml, p=0.0286). No significant difference in BAL cell count observed between PBS and active tMSC treatment (figure 8.3F, medians 5 vs 4.25×10^4 , p=0.40). Differential BALF cell count from both groups showed >96% alveolar macrophages (data not shown). BALF quantitative culture from both groups resulted in no bacterial growth (data not shown). No significant difference in weight loss was observed between PBS and active tMSC treated mice (figure 8.3G, medians 9.96 vs 8.51%, p=0.229). Active tMSC treatment showed a trend towards decreased inflammatory cytokines (KC, IL-6, MIP-1 α , and MIP-2) in BAL and PLF compared to PBS treatment, however none of these results reached statistical significance.

CLP experiments lasting 24 hours did not result in significant lung injury, as there was negligible neutrophilic infiltration following PBS treatment. It is likely that insufficient time had elapsed following induction of CLP. Due to Home Office restrictions on this severe sepsis model, we were unable to prolong the experiment longer than 24 hours.

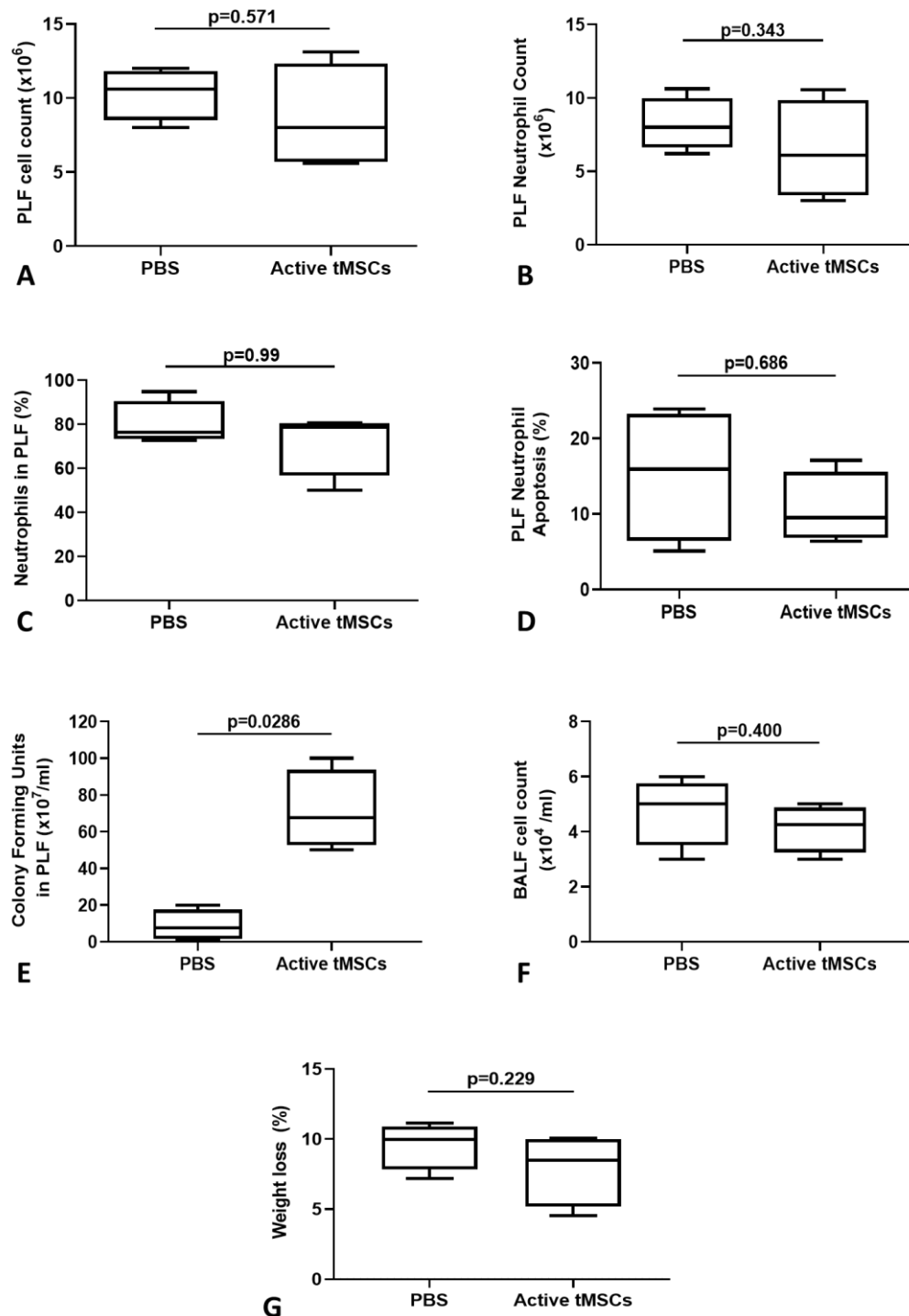


Figure 8.3: Cellular recruitment and bacterial growth following a 24 hour time course of WT mice undergoing CLP

WT mice received PBS or 125,000 active tMSCs intravenously 1 hour prior to CLP. Data presented as Tukey box plots. Statistical analysis by Mann-Whitney test, $n=4$ for both groups. **A:** No significant difference in PLF cell count observed between PBS and active tMSC treatment (medians 10.6 vs 8×10^6 , $p=0.571$). **B:** No significant difference in PLF neutrophil count observed between PBS and active tMSC

treatment (medians 8 vs 6.1×10^6 , $p=0.343$). **C:** No significant difference in the proportion of neutrophils in PLF observed between PBS and active tMSC treatment (medians 76.3% vs 78.6%, $p=0.99$). **D:** No significant difference in PLF neutrophil apoptosis observed between PBS and active tMSC treatment (medians 16% vs 9.5%, $p=0.686$). **E:** Bacterial growth in PLF was significantly higher following active tMSC treatment compared to PBS (medians 7.5 vs 67.5×10^7 CFU/ml, $p=0.0286$). **F:** No significant difference in BALF cell count observed between PBS and active tMSC treatment (medians 5 vs 4.25×10^4 , $p=0.40$). Differential BAL cell count from both groups showed >96% alveolar macrophages (data not shown). **G:** No significant difference in weight loss observed between PBS and active tMSC treatment (medians 9.96 vs 8.51%, $p=0.229$).

8.3.2 Murine lung tissue HSD-1 activity following tMSC administration

To further characterise the progression of active tMSCs following administration and assess actual HSD-1 activity within the lung following after administration, a time course was performed using uninjured HSD-1 KO mice (see figure 8.4). This allowed allow me to determine the time between intravenous administration of tMSCs and maximal lung HSD-1 activity. Lung HSD-1 reductase activity peaked at 4 hours after intravenous administration of active tMSCs (figure 8.4, 0.0027 vs 0.0104 pM/mg/hr, $p=0.029$). After 4 hours, lung HSD-1 reductase activity in HSD-1 KO mice was elevated to a level corresponding to approximately one third of the activity seen in WT mice (figure 8.4, medians 0.0104 vs 0.0309 pM/mg/hr). Lung HSD-1 reductase activity 24 hours after active tMSC administration was significantly reduced compared to activity seen at 4 hours (figure 8.4, 0.0104 vs 0.0051 pM/mg/hr, $p=0.029$), and was not significantly different to lung HSD-1 activity in untreated HSD-1 KO mice (figure 8.4, 0.0027 vs 0.0051 pM/mg/hr, $p=0.20$). Therefore, shortening the duration of the CLP model to 3 hours (4 hours post-tMSC administration) would allow us to assess the effect of peak HSD-1 transgene expression.

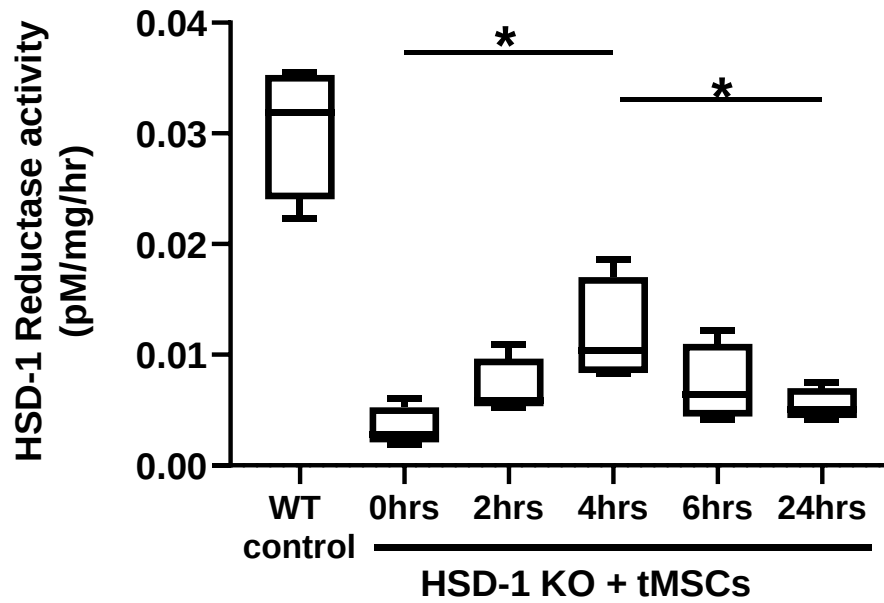


Figure 8.4: Time course of lung HSD-1 reductase activity following tMSC administration in HSD-1 KO mice

Statistical analysis by Mann Whitney test, n=4 for all groups. 75,000 active HSD-1 tMSCs were administered intravenously to HSD-1 KO mice. '0 hours' refers to HSD-KO mice which did not receive tMSCs. Untreated WT mouse lungs were also analysed for a positive control. Lung HSD-1 reductase activity peaked at 4 hours after intravenous administration of active tMSCs (0.0027 vs 0.0104 pM/mg/hr, $p=0.029$). Lung HSD-1 reductase activity 24 hours after active tMSC administration was significantly reduced compared to activity seen at 4 hours (0.0104 vs 0.0051 pM/mg/hr, $p=0.029$). Lung HSD-1 reductase activity 24 hours after active tMSC administration was not significantly different to lung HSD-1 activity in untreated HSD-1 KO mice (0.0027 vs 0.0051 pM/mg/hr, $p=0.20$).

8.3.3 Impact of HSD-1 tMSC treatment in a 3 hour CLP model using WT mice.

WT mice received PBS, 125,000 inactive tMSCs, or 125,000 active tMSCs intravenously 1 hour prior to CLP (figure 8.5). The endpoint was 3 hours after CLP, and 4 hours after tMSC administration, at the time when lung HSD-1 activity would be maximal (figure 8.4). The three treatment groups caused significant differences in the PLF cell count (figure 8.5A, $p=0.0071$). Treatment with active tMSCs significantly reduced PLF cell count compared to PBS (figure 8.5A, mean rank difference 7.75, $p=0.024$) but inactive tMSC treatment did not (mean rank difference 7.13, $p=0.059$). Treatment with inactive tMSCs did not significantly affect PLF cell count compared to PBS treatment (figure 8.5A, $p=0.99$). The treatment groups caused significant differences in the PLF neutrophil count (figure 8.5B, $p=0.0025$). Treatment with active tMSCs significantly reduced PLF neutrophil count compared to PBS (figure 8.5B, mean rank difference 8.17, $p=0.015$) and inactive tMSC treatment (mean rank difference 7.77, $p=0.033$). Treatment with inactive tMSCs did not significantly affect PLF neutrophil count compared to PBS treatment (figure 8.5B, $p=0.99$). The treatment groups caused significant differences in the PLF neutrophil proportion (figure 8.5C $p=0.0125$). Treatment with active tMSCs significantly reduced PLF neutrophil proportion compared to PBS (figure 8.5C, mean rank difference 8.60, $p=0.018$) but inactive tMSC treatment did not (mean rank difference 6.33, $p=0.154$). Treatment with inactive tMSCs did not affect PLF neutrophil proportion compared to PBS treatment (figure 8.5C, $p=0.99$). The treatment groups caused no significant difference in PLF neutrophil apoptosis (figure 8.5D, $p=0.253$). The treatment groups caused no significant difference in PLF bacterial count (figure 8.5E, $p=0.580$).

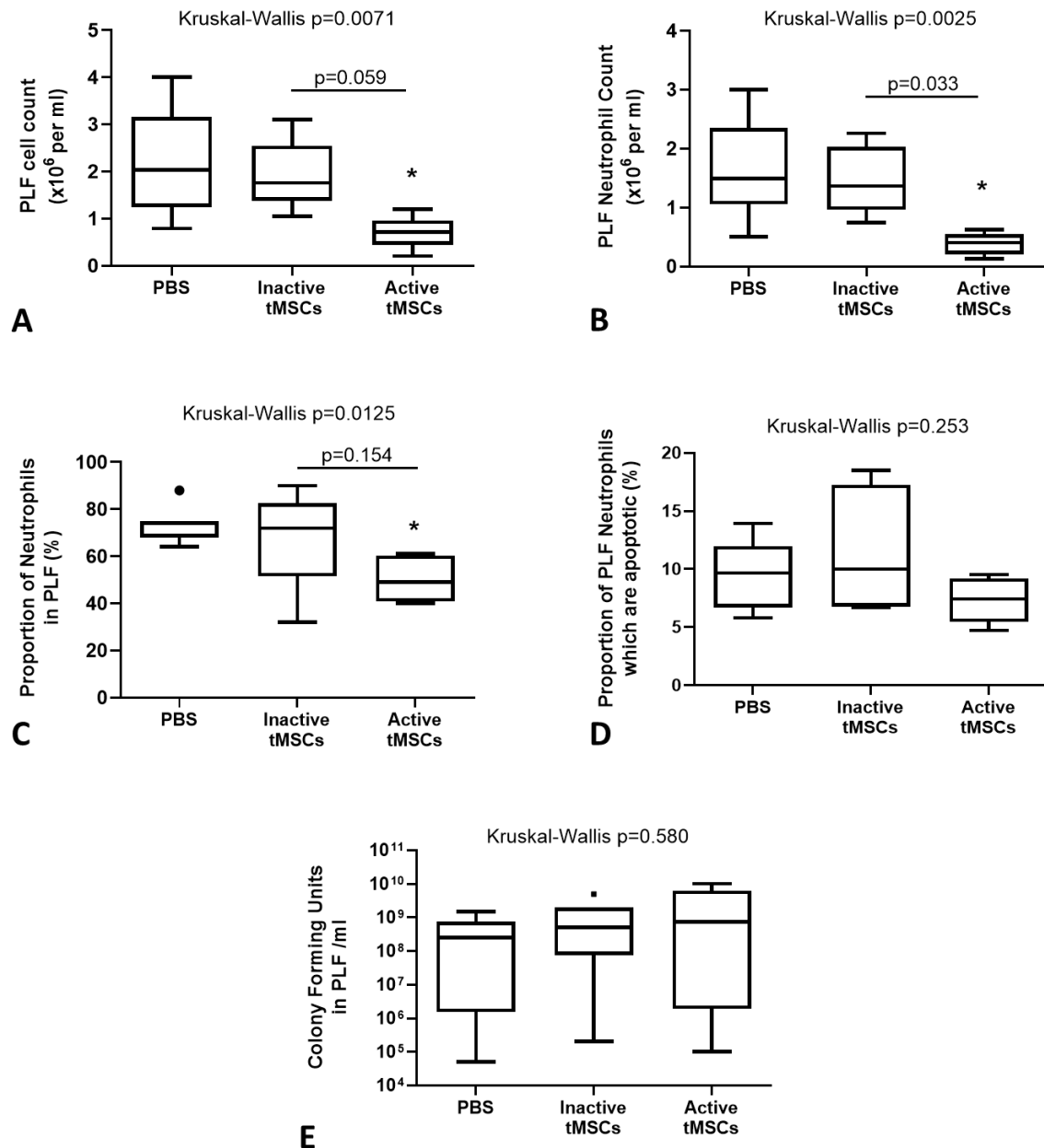


Figure 8.5: PLF cellular recruitment and bacterial growth following a 3 hour time course of WT mice undergoing CLP

WT mice received PBS, 125,000 inactive tMSCs, or 125,000 active tMSCs intravenously 1 hour prior to CLP. Data presented as Tukey box and whisker plots. Statistical analysis by Kruskal-Wallis tests and Dunn's multiple comparison test, $n=6$ for both groups. * $p < 0.05$ compared to untreated control. **A:** The treatment groups caused significant differences in the PLF cell count ($p=0.0071$). Treatment with active tMSCs significantly reduced PLF cell count compared to PBS ($p=0.024$) but not inactive tMSC treatment ($p=0.059$). Treatment with inactive tMSCs did not significantly affect PLF cell count compared to PBS treatment ($p=0.99$). **B:** The treatment groups caused significant differences in the PLF neutrophil count ($p=0.0025$). Treatment with active tMSCs significantly reduced PLF neutrophil count

compared to PBS ($p=0.015$) and inactive tMSC treatment ($p=0.033$). Treatment with inactive tMSCs did not significantly affect PLF neutrophil count compared to PBS treatment ($p=0.99$). **C:** The treatment groups caused significant differences in the PLF neutrophil proportion ($p=0.0125$). Treatment with active tMSCs significantly reduced PLF neutrophil proportion compared to PBS ($p=0.018$) but not inactive tMSC treatment ($p=0.154$). Treatment with inactive tMSCs did not significantly affect PLF neutrophil proportion compared to PBS treatment ($p=0.99$). **D:** The treatment groups caused no significant differences in PLF neutrophil apoptosis ($p=0.253$). **E:** The treatment groups caused no significant differences in PLF bacterial count ($p=0.580$).

Following this 3 hour CLP time course in WT mice, no significant differences were observed in the concentrations of PLF or serum cytokines between the three treatment groups: PBS, inactive tMSCs and active tMSCs (see tables 8.1 and 8.2). Cytokines measured included monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , MIP-2, KC (murine homolog of chemokine C-X-C motif ligand 1), interleukin (IL)-1 β , IL-6, IL-10, tumour necrosis factor- α (TNF α) and vascular endothelial growth factor (VEGF).

	PBS	Inactive tMSCs	Active tMSCs	Kruskal-Wallis test
KC (ng/ml)	96.48 53.02 – 196.08	59.88 32.77 – 125.75	101.68 68.03 – 146.21	p = 0.432
MCP-1 (ng/ml)	25.02 12.89 – 41.03	13.05 10.12 – 20.98	16.60 11.02 – 30.23	p = 0.360
MIP-1α (ng/ml)	157.24 87.20 – 279.02	71.61 46.81 – 97.04	109.79 56.18 – 261.49	p = 0.151
MIP-1β (ng/ml)	2.74 1.93 – 6.65	2.20 1.39 – 3.34	3.21 2.29 – 5.12	p = 0.484
MIP-2 (ng/ml)	27.24 14.69 – 147.07	133.92 41.34 – 226.16	122.58 26.63 – 190.90	p = 0.336
IL-6 (ng/ml)	83.58 10.83 – 191.08	165.94 102.97 – 194.01	203.59 54.67 – 291.26	p = 0.360
TNFα (pg/ml)	1294.0 51.0 – 1733.0	440.1 53.4 – 753.6	696.4 425.8 – 1185.0	p = 0.351
IL-1β (pg/ml)	529.8 57.2 – 642.1	57.6 57.2 – 57.6	57.6 57.6 – 1113	p = 0.207
VEGF (ng/ml)	13.77 8.50 – 19.42	9.74 8.08 – 12.25	11.73 9.07 – 12.79	p = 0.360
IL-10 (ng/ml)	2.91 0.92 – 5.65	1.38 0.82 – 2.47	1.86 1.07 – 2.96	p = 0.466
Pooled cytokines (ng/ml)	546.76 401.57 – 613.66	416.11 331.0 – 614.35	634.65 452.15 – 768.35	p = 0.360

Table 8.1: PLF cytokines in WT mice following a 3 hour CLP time course

All data presented as median and interquartile range. Statistical analysis by Kruskal-Wallis test, n=6 for all groups. KC = murine homolog of chemokine C-X-C motif ligand 1 (CXCL1). MCP-1 = Monocyte chemoattractant protein-1. MIP = Macrophage Inflammatory Protein. IL = Interleukin. TNF α = Tumour Necrosis Factor- α . VEGF = Vascular Endothelial Growth Factor.

	Saline Treated	Inactive tMSC Treated	Active tMSC Treated	Kruskal- Wallis test
KC (ng/ml)	151.37 115.71 – 281.86	145.64 95.68 – 190.02	240.16 74.18 – 262.38	p = 0.664
MCP-1 (ng/ml)	7.50 3.16 – 20.59	3.80 1.91 – 9.43	6.17 3.74 – 26.99	p = 0.602
MIP-1α (ng/ml)	21.20 6.78 – 39.74	10.50 5.30 – 52.69	46.13 9.92 – 104.88	p = 0.489
MIP-1β (ng/ml)	2.48 1.12 – 7.42	1.65 1.31 – 5.76	3.06 1.43 – 8.56	p = 0.833
MIP-2 (ng/ml)	109.43 30.34 – 202.86	71.05 32.87 – 113.34	39.0 18.34 – 180.76	p = 0.511
IL-6 (ng/ml)	45.27 24.15 – 77.03	62.24 51.03 – 131.85	25.79 14.21 – 113.53	p = 0.388
TNFα (pg/ml)	1088 51 – 1667	544 53 – 1421	1182 315 – 3099	p = 0.598
IL-1β (pg/ml)	57.18 57.18 – 57.61	57.61 57.18 – 57.61	57.61 57.18 – 57.61	p = 0.588
VEGF (ng/ml)	3.70 2.75 – 9.21	4.20 3.39 – 7.68	5.35 2.06 – 9.71	p = 0.988
IL-10 (ng/ml)	3.10 0.06 – 7.48	1.48 1.15 – 4.37	2.26 1.14 – 6.70	p = 0.786
Pooled cytokines (ng/ml)	405.94 281.54 – 552.03	329.70 201.26 – 511.12	476.63 167.30 – 621.01	p = 0.798

Table 8.2: Serum cytokines in WT mice following a 3 hour CLP time course

All data presented as median and interquartile range. Statistical analysis by Kruskal-Wallis test, n=6 for all groups. KC = murine homolog of chemokine C-X-C motif ligand 1 (CXCL1). MCP-1 = Monocyte chemoattractant protein-1. MIP = Macrophage Inflammatory Protein. IL = Interleukin. TNF α = Tumour Necrosis Factor- α . VEGF = Vascular Endothelial Growth Factor.

The next aim was to administer HSD-1 tMSCs to HSD-1 KO mice and perform 3 hour CLP experiments. However, the HSD-1 KO mouse colony collapsed due to lack of breeding, and the colony could not be re-derived within the timescale of this project.

8.4 DISCUSSION

The main findings from this chapter are that active HSD-1 tMSC treatment can reduce cellular inflammation in a murine model of peritoneal sepsis, but can also lead to bacterial overgrowth and tissue necrosis. The ability of activated tMSCs to reduce neutrophilic infiltration in CLP compared to PBS and inactive tMSCs was observed at 3 hours post-injury (4 hours post-treatment), but not at 24 hours post-injury. This is likely due to transgenic HSD-1 expression in the lungs peaking at 4 hours, then decreasing significantly between 4 and 24 hours post tMSC administration. It can be inferred that expression of the HSD-1 transgene is responsible for the decrease in cellular inflammation observed between inactive and active tMSC treated WT mice. Although inactivated tMSCs decrease neutrophilic infiltration compared to PBS, this did not reach statistical significance at 3 hours post injury.

The pilot studies showed that activated tMSC treatment was associated with increased bacterial load at 24 hours post-injury. The first pilot study using 250,000 MSCs showed increased severity scores, tissue necrosis and unexpected mortality in the active tMSC treated group. Subsequent safety studies showed that tMSC treatment in uninjured mice caused no adverse effects or organ damage. Therefore, it can be concluded that the increased severity observed in the first pilot study was due to the dose of active HSD-1 tMSCs being too high, causing immunosuppression due to excessively elevated cortisol levels, leading to uncontrolled bacterial growth and tissue necrosis. Previous studies have found that intravenous administration of non-transgenic MSC in murine CLP results in increased survival, organ function, IL-10 secretion, bacterial clearance, monocyte phagocytosis and reduced inflammatory cytokine release(187, 224, 231). In this study, the intrinsic tissue repair, anti-bacterial

and pro-phagocytic characteristics of the MSC vector(186, 208, 214, 215, 217, 228, 329) were unable to counteract the immunosuppression caused by overexpression of the HSD-1 transgene. Increased HSD-1 expression within murine lung tissue is likely to have led to increased conversion of cortisone to cortisol. Cortisol is known to inhibit macrophage bactericidal function(179), which is the likely cause of the bacterial overgrowth seen in mice treated with HSD-1 tMSCs. The majority of tMSCs appear to deposit within the lungs following administration, which is in keeping with the findings of previous studies investigating MSC administration in CLP(187, 224, 231).

Previous experiments using CLP as a model of sepsis had shown some evidence of lung injury after 24 hours, with neutrophilic infiltration and elevated cytokine levels(330). Unfortunately, due to the low n-numbers I had at the 24 hour CLP time point, I was unable to demonstrate this. As part of safety studies in uninjured mice, HSD-1 functional assays showed that maximal lung HSD-1 activity occurs 4 hours after intravenous administration of HSD-1 tMSCs. The duration of the CLP model was then reduced to 3 hours due to safety concerns; 3 hour CLP experiments with tMSCs were required before I could extend the time course back to 24 hours. I had planned to deliver multiple low doses of tMSCs, every 6 hours in a 24hr CLP time course to determine the effect on lung injury, however my amendment to the protocol was not approved within the timeframe of this project. Even with use of an indirect model of lung injury such as CLP, I would also need to undertake experiments with a direct model of lung injury (e.g. pneumococcal pneumonia as described below) since CLP alone is insufficient to model ARDS.

Maximal lung HSD-1 activity occurs 4 hours after intravenous administration of HSD-1 tMSCs. This correlates with the findings of previous studies on MSC pharmacodynamics, which showed that MSC initially accumulate in the lungs following intravenous administration, however after a few hours their numbers in the lungs start to decrease(236, 298, 331, 332). Despite the majority of HSD-1 tMSCs localising to the lungs after administration, peritoneal neutrophil infiltration was reduced, which indicates that the cortisol produced by increased HSD-1 expression in the lungs, and release of MSC paracrine factors from the lungs, are having a systemic effect. These results could also indicate that some tMSCs had migrated to the peritoneum.

Whilst HSD-1 tMSCs may be able to attenuate cellular inflammation in a murine model of remote lung injury, the immunosuppression caused in the context of sepsis-related ARDS results in bacterial overgrowth, tissue necrosis, and worse disease severity. Therefore, HSD-1 tMSCs are unlikely to offer a therapy for human sepsis-related ARDS. However, HSD-1 tMSCs may yet have utility in pulmonary inflammatory diseases such as sarcoidosis, the aetiology of which is not associated with sepsis. Wang et al(245) found that intravenous administration of IL-10 over-expressing tMSCs could attenuate inflammation and reduce mortality in an intra-tracheal lipopolysaccharide (IT-LPS) murine model of direct lung injury. This model causes aseptic inflammatory lung injury, and it was postulated that the HSD-1 tMSCs would also similarly improve outcomes in this type of lung injury model.

There are several limitations to this study. Firstly, HSD-1 tMSC therapy was only assessed in an indirect sepsis-related lung injury model (CLP). In order to fully model ARDS, the intervention would need to be assessed in a direct sepsis-related lung

injury model (e.g. pneumonia). I tried to set up a pneumococcal pneumonia murine model, however pilot studies were still ongoing at the time of writing this thesis. Future studies will aim to complete these pilot studies, and assess the impact of inactive and active HSD-1 tMSCs on direct lung injury using this model. Secondly, the CLP model was unable to induce lung injury after 24 hours, which is the maximum time we were allowed to run the CLP model according to Home Office guidelines. Previous studies from other countries using murine CLP required the model to continue for 2-3 days before lung injury developed (224, 333). Thirdly, this study was unable to determine the effect of HSD-1 tMSC administration in HSD-1 KO mice since the colony collapsed and could not be re-derived in the timescale of this project. However, undertaking these experiments in HSD-1 KO mice would form part of the planned future work. Fourthly, administration of tMSCs prior to CLP injury does not reflect the clinical scenario of administering treatment post-injury. Future studies would require tMSCs administration post-injury to assess treatment efficacy. Although administration in WT mice has been unsuccessful, use of the lower dose (125,000 tMSCs) may show a beneficial effect in HSD-1 KO mice, since these mice have an alveolar HSD-1 deficiency similar to that observed in ARDS patients. Since HSD-1 transgene activity peaks at 4 hours post tMSC administration, multiple doses of HSD-1 tMSCs may be required to maintain a constant HSD-1 transgene activity in future murine studies.

During this project I attempted to set up a pneumococcal pneumonia murine model of direct lung injury. However, this proved more challenging than expected due to multiple problems. First, the C57BL/6 strain used for the HSD-1 KO and WT mice was resistant to the D39 strain of *streptococcus pneumoniae* that was originally

planned to be used(334). The invasive TIGR4 (serotype 4) strain of pneumococcus was then chosen for future experiments and is known to be infective in C57BL/6 mice (335). Both strains of pneumococcus used were kindly provided by Professor Tim Mitchell (University of Birmingham). Secondly, I discovered that pneumococcus loses its virulence following repeated *in vitro* passage, so some of the initial pilot experiments did not result in lung injury due to use of non-virulent pneumococcus. To counteract this, virulent strains were isolated using *in vivo* murine peritoneal passage of pneumococcus, and subsequent isolation from blood. Also, limitations regarding the severity limit for the model were encountered as per the project licence (moderate severity limit). Since this was a new model being established at my institution, numerous pilot studies were required to optimise the model, e.g. to determine the ideal dose, model duration, etc. Unfortunately, it was not possible to progress past the pilot study stages during the timescale of this project.

In summary, transgene-active HSD-1 tMSC treatment can reduce cellular inflammation in a WT murine model of peritoneal sepsis, but can also lead to bacterial overgrowth and tissue necrosis. Based on these findings alone, it is unlikely that HSD-1 tMSCs will offer a viable therapeutic strategy for human ARDS. However, further *in vivo* studies using HSD-1 KO mice in models of indirect and direct sepsis-related lung injury will more closely reflect the alveolar environment in human ARDS, and are required to fully assess the therapeutic potential of HSD-1 tMSCs.

CHAPTER 9 : THESIS SUMMARY

9.1 OVERVIEW

Despite 50 years of research in ARDS, a pharmacological therapy remains elusive. While advances in supportive management such as lung protective ventilation have improved outcomes, ARDS remains a major cause of morbidity and mortality in critically ill patients(3).

It has previously been shown that ARDS patients have a relative alveolar cortisol deficiency due to reduced AM HSD-1 reductase activity(91). It is known that increased HSD-1 reductase activity and cortisol promote monocyte-derived-macrophage efferocytosis(147). However, no study has yet investigated AM efferocytosis in ARDS patients. Additionally, there are limited studies investigating *in vivo* neutrophil apoptosis in ARDS patients, with contradictory results. This thesis investigated the following questions: 1) Do the immunomodulatory and pro-repair ability of MSCs change with prolonged *in vitro* expansion? 2) Is there a pathophysiological link between reduced AM HSD-1 activity, defective AM efferocytosis, and apoptotic neutrophil accumulation in ARDS? 3) Can an *in vitro* model of ARDS replicate AM functional defects, and be used to test potential therapies? 4) Can use of HSD-1 tMSCs restore *ex vivo* ARDS AM efferocytosis and attenuate inflammation in murine models of lung injury?

9.1.1 Prolonged *in vitro* mesenchymal stem cell expansion

Chapter 4 shows that prolonged expansion of MSCs in the presence of inflammatory cytokines did not affect the ability of MSC-CM to promote epithelial wound healing.

This is reflected by the fact that prolonged expansion of MSCs either increases (Ang-1) or has no effect (VEGF, TGF- β 1) on secretion of pro-repair mediators. However, prolonged MSC expansion significantly decreased the ability of MSCs to induce IL-10 release by macrophages, following direct co-culture in the presence of LPS. This effect may occur independently of PGE₂ release by MSCs. These findings indicate that some of the immuno-modulatory, anti-inflammatory effects of MSCs may decline with prolonged expansion. Therefore, the anti-inflammatory effects of a combined cellular and gene based therapy may be superior to cellular therapy alone.

9.1.2 Creation and characterisation of HSD-1 tMSCs

Chapter 5 describes the successful creation of a recombinant lentivirus which contains the HSD-1 and GFP transgenes under the control of a tetracycline promoter. Subsequent transfection of human MSCs with this recombinant lentivirus created HSD-1 tMSCs. These tMSCs expressed functional HSD-1 enzyme in the presence of a transcriptional activator (doxycycline). The HSD-1 enzyme predominantly functioned as a reductase, and expression was maintained for 72 hours following removal of the transcriptional activator. The tMSCs also maintained a stem cell phenotype following transfection. To determine if these HSD-1 tMSCs could attenuate inflammation in ARDS, they were used in co-culture experiments with AMs from ARDS patients, and administered in murine models of lung injury.

9.1.3 The AM-ARDS clinical study

As shown in chapter 6, the AM-ARDS clinical study found that development of sepsis-related ARDS was associated with decreased AM HSD-1 reductase activity, decreased AM efferocytosis, and increased alveolar neutrophil apoptosis. Secondary necrosis of these neutrophils, due to reduced AM efferocytosis, likely contributes to the increased alveolar inflammatory cytokines, protein permeability and mortality observed in ARDS patients. This is the first study to identify impaired AM efferocytosis in ARDS patients, and a potential association between impaired AM function and ARDS pathogenesis. Strategies to restore normal AM function may therefore have therapeutic utility in ARDS patients. However, transwell co-culture of HSD-1 tMSCs had no significant effect on *ex vivo* ARDS AM efferocytosis, indicating there are likely to be HSD1-independent mechanisms of impaired efferocytosis in ARDS. Isolating AMs from ARDS patients proved difficult and had a low yield, thus limiting the experiments that could be performed. Therefore, an *in vitro* model of ARDS was set up to further assess AM function.

9.1.4 Alveolar macrophage function in an *in vitro* model of ARDS

Chapter 7 shows that ARDS BALF treatment of AMs *in vitro* can decrease efferocytosis, causing the same functional impairment observed in ARDS patients. AM treatment with ARDS BALF also downregulated mRNA transcription of Rac1, an effector GTPase which causes actin re-arrangement and engulfment of apoptotic cells, thereby supporting findings of decreased efferocytosis. ARDS BALF treatment also increased AM bacterial phagocytosis, indicating a differential effect on AM

function. Unexpectedly, ARDS BALF treatment increased expression of the pro-efferocytic surface receptors (CD206 and MerTK), whilst decreasing expression of the anti-efferocytic receptor SIRP α . These changes in surface receptor expression may be compensatory mechanisms, whereby AMs unsuccessfully attempt to restore normal efferocytic function following ARDS BALF treatment. ROCK-inhibitor treatment can partially restore AM efferocytic function following ARDS BALF exposure; inhibition of the ROCK intracellular signalling pathway may offer a therapeutic strategy to restore AM function in ARDS patients. However, HSD-1 tMSC co-culture was unsuccessful at restoring AM efferocytosis in this *in vitro* model of ARDS. *In vitro* work has limitations, therefore in order to assess the integrated responses of the host and take into account interactions between other cell types, HSD-1 tMSCs were administered in murine models of lung injury.

9.1.5 HSD-1 tMSC therapy in murine models of lung injury

As shown in chapter 8, transgene-active HSD-1 tMSC treatment reduced cellular inflammation in a WT murine model of peritoneal sepsis, but also lead to bacterial overgrowth and tissue necrosis. Based on these findings alone, it is unlikely that HSD-1 tMSCs will offer a viable therapeutic strategy for human ARDS. However, further *in vivo* studies using HSD-1 KO mice in models of indirect and direct sepsis-related lung injury will more closely reflect the alveolar environment in human ARDS, and are required to fully assess the therapeutic potential of HSD-1 tMSCs.

9.2 LIMITATIONS

- 1) By only investigating MSCs from 4 donors, the experiments may have lacked sufficient power to detect a difference in secreted factors caused by prolonged expansion. The Institute of Regenerative Medicine at Texas A&M, from where the MSCs were purchased, had a limited number of donors, and not all MSCs available were at the passage required (P2). Another limitation was the use of cell lines, such as A549 cells for wound healing experiments, and THP-1 cells to generate macrophages for co-culture experiments. Use of primary cells would have provided a closer model for human cellular interactions, as they behave in a more physiological manner.
- 2) Unexpectedly, HSD-1 reductase activity was observed in tMSCs which had been cultured in media containing tetracycline-free FBS, and never exposed to exogenous doxycycline. Minimal HSD-1 transgene expression in the absence of the transcriptional activator was resulting in detectable enzymatic activity. Ideally, to control for this effect a null-transfected tMSC would have been created using recombinant lentivirus containing the GFP transgene but not HSD-1. However, due to time constraints this option was unfortunately not possible. Instead, “transgene-inactive” tMSCs were pre-treated with GA to minimise the underlying HSD-1 activity prior to use in transwell co-culture and murine experiments.
- 3) The AM-ARDS study had several limitations. ARDS patient recruitment was challenging because prior steroid use, immunosuppression or immunodeficiency were all exclusion factors, however many patients

diagnosed with ARDS had a transplant background. Even if meeting the inclusion criteria, not all patients could safely receive a bronchoscopy, mainly due to their ventilation status. Following bronchoscopy on ARDS patients, the BALF was often highly neutrophilic, making it difficult to isolate AMs. Even when AMs were isolated, the average number isolated was 1.2 million, meaning that often only one type of functional assay could be performed per patient.

- 4) A limitation of the *in vitro* model of ARDS is that the effect of ARDS BALF treatment is only being assessed on one cell type (AMs), whereas in the alveolar space other cells types (e.g. alveolar epithelial cells) are also present, with interactions occurring between different cells types. Another limitation of this study is that ROCK-inhibitor was given simultaneously with, instead of after, ARDS BALF treatment.
- 5) There are several limitations to the *in vivo* work. Firstly, HSD-1 tMSC therapy was only assessed in an indirect sepsis-related lung injury model (CLP). In order to fully model ARDS, the intervention would need to be assessed in a direct sepsis-related lung injury model (e.g. pneumonia). I attempted to set up a pneumococcal pneumonia murine model, however pilot studies were still ongoing at the time of writing this thesis. Secondly, the CLP model was unable to induce lung injury after 24 hours, which is the maximum time we were allowed to run the CLP model according to Home Office guidelines. Thirdly, this study was unable to determine the effect of HSD-1 tMSC administration in HSD-1 KO mice since the colony collapsed and could not be re-derived in the timescale of this project.

9.3 FUTURE RESEARCH

During the course of this project, additional questions have arisen which require further investigation:

- 1) Prolonged MSC expansion experiments need to be repeated, but MSCs need to be cultured in serum-free media (instead of 5% FBS) prior to CM collection. This would allow isolation of anti-inflammatory and pro-repair extracellular vesicles, and to determine whether prolonged expansion has an effect on their production.
- 2) To establish if ROCK-inhibition could have a “treatment effect” on impaired AM efferocytic function in an *in vitro* model ARDS, the ROCK-inhibitor would need to be administered 24 hours after AM exposure to ARDS BALF. Studies are also required to determine whether Netarsudil (an FDA-approved ROCK-inhibitor) can restore AM efferocytosis in the *in vitro* model of ARDS, in a similar way to Y-27632. Other existing medications with a known pro-efferocytic effect could also be tested using the *in vitro* model of ARDS, to determine if they can restore AM efferocytosis and thus offer a potential therapeutic strategy, e.g. N-acetylcysteine(313), macrolide antibiotics(314, 315), and statins(163). The *in vitro* model of ARDS could be used to block factors in BALF which are known to inhibit efferocytosis (e.g. anti-HMGB1 or anti-TNF α antibodies), in order to determine the cause of the inverse correlation between AM phenotype and function elicited by ARDS BALF. The *in vitro* ARDS model could be improved by treating precision-cut slices of lung tissue with ARDS BALF, which would allow assessment of multiple cell types in concert and bring the model closer to the *in vivo* human disease.

- 3) Recent advances in the field of immunometabolism have discovered that macrophage phenotypic polarisation is associated with metabolic changes, e.g. M1 phenotype is associated with decreased mitochondrial respiration and increased glycolysis(307). Use of the *in vitro* model of ARDS to assess AM metabolic function would allow further elucidation of the role of AMs in ARDS pathogenesis, and potentially reveal alternative methods of restoring AM function.
- 6) Further *in vivo* studies using HSD-1 KO mice in models of indirect and direct sepsis-related lung injury will more closely reflect the alveolar environment in human ARDS, and are required to fully assess the therapeutic potential of HSD-1 tMSCs. Since HSD-1 transgene activity peaks at 4 hours post tMSC administration, multiple doses of HSD-1 tMSCs may be required to maintain a constant HSD-1 transgene activity in future murine studies. Use of the lower dose (125,000) of HSD-1 tMSCs may show a beneficial effect in HSD-1 KO mice, since these mice have an alveolar HSD-1 deficiency similar to that observed in ARDS patients.

9.4 CONCLUSIONS

This thesis has shown that dysregulated AM function contributes to ARDS pathogenesis. Development of sepsis-related ARDS is associated with decreased AM HSD-1 reductase activity and efferocytosis, which likely contributes to the increased alveolar neutrophil apoptosis, alveolar inflammation and mortality observed. ARDS BALF treatment of healthy AMs *in vitro* can also decrease efferocytosis, causing the same functional impairment observed in ARDS patients. HSD-1 tMSC therapy was unsuccessful at restoring AM efferocytosis both *ex vivo* and in an *in vitro* model of ARDS, indicating that HSD-1 independent mechanisms contribute to impaired AM efferocytosis in ARDS. HSD-1 tMSC therapy was able to reduce cellular inflammation in a model of murine peritonitis, but also caused bacterial overgrowth, indicating that HSD-1 tMSC therapy may not have utility in ARDS patients. However, further studies using multiple doses of HSD-1 tMSCs in HSD-1 KO mice (as described above) are required to fully assess the therapeutic potential of HSD-1 tMSCs in ARDS. ROCK-inhibitor treatment partially restored AM efferocytosis in an *in vitro* model of ARDS, indicating that this strategy may offer therapeutic potential in ARDS patients.

CHAPTER 10 : ABBREVIATIONS

AM	Alveolar Macrophage
α MEM	α Minimal Essential Media
ANOVA	Analysis of Variance
ARDS	Acute Respiratory Distress Syndrome
APACHE	Acute Physiology and Chronic Health Evaluation Score
APC	Allophycocyanin
BALF	Broncho-Alveolar Lavage Fluid
BSA	Bovine Serum Albumin
CFU	Colony Forming Unit
CO ₂	Carbon Dioxide
CLP	Caecal Ligation and Puncture
COPD	Chronic Obstructive Pulmonary Disease
CPAP	Continuous Positive Airways Pressure
CV	Coefficient of Variation
DAMP	Danger Associated Molecular Pattern
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic Acid
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
FDA	United States of America Food and Drug Administration
FITC	Fluorescein Isothiocyanate

FiO ₂	Fraction of Inspired Oxygen
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
H6PD	Hexose-6-Phosphate Dehydrogenase
HFOV	High Frequency Oscillatory Ventilation
HMGB-1	High Mobility Group Box-1
HRP	Horse radish peroxidase
HSD	11 β Hydroxysteroid Dehydrogenase
ICU	Intensive Care Unit
IFN	Interferon
IL	Interleukin
IQR	Inter-quartile range
LOS	Length of Stay
LPS	Lipopolysaccharide
MAP	Mean Arterial Pressure
MDM	Monocyte Derived Macrophage
MFI	Mean Fluorescent Index
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
NADP	Nicotinamide adenine dinucleotide phosphate
NF- $\kappa\beta$	Nuclear Factor- $\kappa\beta$
PAMP	Pathogen Associated Molecular Pattern
PaO ₂	Partial Pressure of Oxygen
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PE	Phycoerythrin
PEEP	Positive end-expiratory pressure
P/F ratio	Ratio of plasma oxygen pressure to inspired oxygen pressure
PLF	Peritoneal lavage fluid
PPI	Protein permeability index
PS	Phosphatidylserine
ROS	Reactive Oxygen Species
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute cell culture media
RQ	Relative Quantity
SD	Standard Deviation
SOFA	Sequential Organ Failure Assessment
TMB	Tetramethylbenzidine
TLR	Toll-like receptor
TNF- α	Tumour Necrosis Factor- α
UCL	University College London
UTC	Untreated Control
VFD	Ventilator free day
WT	Wild-type

CHAPTER 11 : REFERENCES

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