The impact of Mesenchymal Stem Cells on host immunity and disease outcome in bacterial lung infection

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Declaration

I hereby declare that the research submitted in this thesis is my own and describes my own work under the supervision of Prof. Rosemary Boyton, in the Department of Medicine, Imperial College London, except where the work / contribution of others has been acknowledged at the point of use. All sources of information have been appropriately cited and accompanied with a full list of references.

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Abstract

Mesenchymal stem cells (MSCs) regulate lung inflammation and enhance bacterial clearance and survival in preclinical models of pneumonia and sepsis, making them an attractive novel treatment for bacterial lung infection resistant to antibiotic therapy. Regulatory T cells (T_{regs}) are a subset of $CD4^+$ T cells acknowledged to be important in maintaining the fine balance of immune responses during bacterial infection. Their precise role in streptococcal and pseudomonal lung infection is undefined and their interaction with MSCs in the context of bacterial infection is undetermined. This thesis explores the impact of MSC on host immunity in bacterial lung infection. The work described in this thesis involves an investigation of MSC antimicrobial effect in established experimental mouse models of Gram positive and Gram negative bacterial lung infection and exploration of the potential role of T_{regs} contributing to the observed antimicrobial effects.

Systemic treatment with MSCs reduced bacterial burden in lung and bronchoalveolar lavage (BAL) following intranasal infection with S. *pneumoniae* and *P. aeruginosa.* Levels of pro-inflammatory cytokines in lung (TNF-α, IL-6, IFN-γ and IL-17F) and BAL (IL-6) were also significantly reduced. Immunity shifted away from a T_H1/T_H17 towards a T_H2 response in mice infected with *S. pneumoniae* and then treated with MSCs. Depletion of T_{regs} in an inducible Foxp3.DTR knockout mouse experimental model prior to infection with *S. pneumoniae* resulted in a significant increase in lung bacterial burden, suggesting that T_{regs} are beneficial to the host response to this bacterium. In contrast, T_{reg} depletion during *P. aeruginosa* lung infection did not impact on bacterial burden or inflammatory response, suggesting no role for T_{regs} in the host response to this bacterium. Following infection with S. *pneumoniae*, bacterial burden and inflammatory response was greater in MSC treated Treg-deplete mice compared with the Treg-replete cohort. This suggests that the MSC protective effect for this lung pathogen may be partially T_{reg} dependent. In contrast, in the *P. aeruginosa* lung infection model, the MSC protective effect was preserved following T_{reg} depletion, suggesting that MSC protective effect is independent of T_{regs}.

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Abbreviations

AAV	Adeno-Associated Virus
ADP	Adenosine Diphosphate
AKI	Acute kidney injury
ALI	Acute lung injury
AM	Alveolar macrophage
AMP	Antimicrobial peptides/proteins
Ang-1	Angiopoiten-1
AP-1	Activator protein 1
APCs	Antigen Presenting Cells
ATP	Adenosine Triphosphate
BAFF	B cell activating factor
BATF	Basic leucine zipper transcription factor ATF-like
BCR	B cell receptor
BIM	Bcl-2 interacting mediator
BLIMP1	B lymphocyte-induced maturation protein 1
BM	Bone marrow
BM-MSC	Bone marrow-derived mesenchymal stem cell
BTK	Bruton tyrosine kinase
cAMP	cyclic adenosine monophosphate
CAP	Community acquired pneumonia
CBP	choline-binding protein
cDNA	complementary DNA

CEC	Corneal epithelial cell
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CFU	Colony Forming Unit
CFU-F	Colony Forming Unit fibroblastic
CLP	Caecal Ligation and Puncture
CLRs	C-type Lectin Receptors
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclo-oxygenase
CSP	Competence-Stimulating-Peptide
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
CXCR4	C-X-C chemokine Receptor type 4
Cx43	Connexin 43
DAMPs	Damage Associated Molecular Patterns
DCs	Dendritic Cells
ddH20	double distilled water
DKO	Double Knock Out
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
ds	double stranded
DT	Diphtheria Toxin

EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Erythropoietin
EV	Extracellular vesicle
FCS	Fetal Calf Serum
f-MLP	N-formyl-methionyl-leucyl-phenylalanine
FMO	Fluorescence Minus One
FO	Follicular
Foxp3	Forkhead box P3
FSK	Foreskin
GAGs	Glycosaminoglycans
GATA-3	GATA binding protein 3
G-CSF	Granulocyte Colony-Stimulating Factor
GFP	Green Fluorescent Protein
GITR	Glucocorticoid-Induced TNFR-Related protein
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GvHD	Graft versus host disease
Heme oxygenase-1	HO-1
hCAP	Human cathelicidin antimicrobial peptide
Hct	Haematocrit
HDP	Host defense peptides/proteins

HGF	Hepatocyte growth factor
HLA	Human Leukocyte Antigen
HLA-G	Human Leukocyte Antigen class 1 molecule
hMSC	human mesenchymal stem cell
HOBIT	Homologue of BLIMP1 in T-cells
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplant
HSPs	Heat Shock Proteins
HSV	Herpes Simplex Virus
hUCESC	human uterine cervical stem cells
i.a	Intra arterial
IBD	Inflammatory Bowel Disease
ICOS-L	ICOS-Ligand
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
i.n	intra-nasal
iNKT cell	invariant Natural Killer T cell
iNOS	inducible nitric oxide synthase

i.p	intra-peritoneal
IPEX	X-linked polyendocrinopathy and enteropathy
IRAK	Interleukin-1 Receptor-Associated Kinase
IRF	Interferon Regulatory Factor
ISCT	International Society for Cellular Therapy
i.v	Intravenous
IVIG	Intravenous Immunoglobulin
IVIS	In Vivo Imaging System
Kb	Kilo-base pair
KGF	Keratinocyte growth factor
KIRs	Killer-cell Immunoglobulin-like Receptors
KO	Knockout
L-Glut	L-Glutamine
LIF	Leukaemia inhibitory factor
LTA	Lipoteichoic Acid
LVS	Live vaccine strain
mAb	monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
M-CSF	macrophage colony-stimulating factor
mDCs	Myeloid DCs
MFI	Median Fluorescence Intensities
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction

Miro 1	Mitochondrial Rho-GTPase-1
MLR	Mixed lymphocyte reaction
MRSA	Methicillin Resistant Staphylococcus Aureus
MSC	Mesenchymal stem cell
mMSC	Murine mesenchymal stem cell
MyD88	Myeloid Differentiation primary response gene 88
MZ	Marginal zone
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHDF	Normal Human Dermal Fibroblasts
NK	Natural Killer
NLRs	NOD-Like Receptors
NO	Nitric oxide
NOD	Nucleotide-binding Oligomerisation Domain
NOTCH2	Neurogenic locus notch homologue protein 2
OPZ	Opsonized Zymosan
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBP	Penicillin binding protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed death 1
pDCs	Plasmacytoid Dendritic Cells

PDGF	Platelet derived growth factor
PDGFRα	Platelet derived growth factor receptor alpha
PGE2	Prostaglandin E2
PGN	Peptidoglycan
PI3K	Phosphatidyl inositol 3-kinase
PRR	Pattern Recognition Receptor
PVR	Polivirus Receptor
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
RA	Retinoic Acid
RAG	Recombination Activating Genes
RIG	Retinoic acid Inducible Gene
RLRs	RIG-I-Like Receptors
RNA	Ribonucleic Acid
RORγt	RAR-related Orphan Receptor gamma t
ROS	Reactive Oxygen Species
Rot	Rotenone
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
Sca-1	Stem cell antigen-1
SDF-1	Stromal-derived factor-1
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus

SNPs	Single Nucleotide Polymorphisms
SOCS	Suppressor Of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
TCR	T Cell Receptor
T-bet	T-box transcription factor
Teff	T effector cells
TGF-β	Transforming Growth Factor-β
TH	T Helper
TIRAP	TIR-domain-containing adaptor protein
TLRs	Toll-Like Receptors
TNF	Tumor Necrosis Factor
TRAF	TNF Receptor Associated Factor
TRAM	Trif-related adaptor molecule
TRIF	Toll/IL-1R domain-containing adaptor-inducing IFNβ
T_{reg}	Regulatory T cell
T3SS	Type 3 secretion system
TSG-6	TNF-α-induced protein 6
ULBP	Unique-long 16 binding protein
VAP	Ventilator-associated pneumonia
VAT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor
WJ	Wharton's Jelly
WT	Wild Type

Chapter 1

1 Introduction

1.1 The immune response to lung bacterial infection

The immune system functions primarily to prevent invasion and consequent tissue damage caused by pathogenic microorganisms, which requires complex coordination of signal recognition and effector cell response. This relies on an array of capabilities, the most important of which are: discriminating between harmful and non-harmful, effectively eliminating pathogens but with preservation of tissue integrity and the establishment of immunological memory to provide long term protection against re-infection.

1.1.1 Pulmonary Innate Immunity

The first line of defense against microbial infections is the physical barrier provided by epithelial or mucosal surfaces. The lungs represent the largest epithelial surface area to come into contact with environmental agents and potential pathogens (Staub and Albertine, 1988). Respiratory epithelial cells protect the underlying pulmonary parenchyma by secreting microbicidal substances such as lysozyme and lactoferrin, which destabilize the bacterial cell wall and promote bacterial cell degradation, and mucins which disrupt microbial binding to the epithelial cell surface and promote mucociliary clearance (Jacquot *et al*., 1987; Ellison and Giehl., 1991). This is facilitated by the beating of the epithelial cell's cilia and the viscoelastic nature of the fluid overlying the epithelium (Hoegger *et al*., 2014). A pathogen which is able to breach these defense mechanisms and penetrate into the lung parenchyma will encounter type II epithelial cells in the alveoli (Staub and Albertine, 1988). These cells produce pulmonary surfactant (Clements, 1997), components of which have been shown to enhance alveolar macrophage opsonisation, which further promotes pathogen clearance (Hartshorn, 2010).

1.1.2 Pathogen Recognition

The fast, efficient innate response is driven by molecules that are broadly shared by a variety of pathogens and distinguishable from host molecules. They are collectively referred to as pathogen-associated molecular patterns (PAMPS). PAMPs are recognized by pattern recognition receptors (PRRs) which can bind specifically to conserved portions of microbe-derived PAMPs (Akira *et al*., 2006). PRRs have been shown to be present throughout the lung, on cells of the conducting airways and alveolar type II epithelial cells (Kawai and Akira, 2008). In addition to recognizing PAMPs, PRRs can also recognise host-derived danger-associated molecular patterns (DAMPS) following tissue injury (Seong and Matzinger, 2004). DAMPS are nuclear or cytoplasmic proteins released into the extracellular space during necrosis; key examples include heat shock proteins (HSPs) such as HSP 60 and HSP 70 and alarmins such as high mobility group box 1 (HMGB1) (Oashi *et al*., 2000; Asea *et al*., 2000; Chang, 2010). There are four main classes of PRRs: the membrane-bound Toll-like receptors (TLRs) (Medzhitov *et al*., 1997), the C-type lectin receptors (CLRs) (Santis *et al*., 1994), the intracellular retinoic acid-inducible gene (RIG)-1-like receptors (RLRs) (Rothenfusser *et al*., 2005) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Harton *et al.*, 2002).

The TLRs are the most extensively studied PRRs. They reside on the cell surface and within endosomes and can recognise an array of microbial and self-ligands: peptidoglycan, lipopolysaccharide (LPS), Ribo Nucleic Acid (RNA), and the unmethylated CpG Deoxyribo Nuclei Acid (DNA) of bacteria and viruses (Iwasaki and Medzhitov, 2004). CLRs, characterized by the presence of a conserved carbohydrate-binding domain, recognise carbohydrate ligands derived from bacteria, viruses and fungi (Figdor *et al*., 2002). Cytoplasmic NLRs and RLRs work in parallel with TLRs and CLRs to recognise infectious agents that have already entered the cell. RLRs are particularly adept at detection of viral RNA (Kawai and Akira, 2008). The expression of RLRs is greatly enhanced by type I IFN stimulation or virus infection (Raicevic *et al*., 2017).

TLR activation initiates intracellular signaling pathways via the recruitment of adaptor proteins within the cell. Two key adaptor proteins which have been extensively studied are

myeloid differentiation primary-response protein 88 (MyD88), employed by all TLRs except TLR3, and Toll/IL-1R domain-containing adaptor-inducing IFNβ (Trif), utilised by TLR3 and TLR4 (O'Neill and Bowie, 2007). MyD88 functions to activate mitogen-activated protein-kinases (MAPKs) and stimulates nuclear translocation of the transcription factor nuclear factor-κB (NF- κB) (Meylan *et al*., 2006). Trif orchestrates an alternative pathway, which is MyD88 independent, but also leads to activation of MAPKs and NF- κB, as well as interferon-responsive factors (IRFs) which induce type 1 interferons (Stetson and Medzhitov, 2006). Two additional adaptor proteins which contribute to TLR signaling are TIR-domain-containing adaptor protein (TIRAP) which is involved in the MyD88-dependent signaling pathway, and Trif-related adaptor molecule (TRAM) which is needed for Trif-dependent signaling (O'Neil and Bowie, 2007).

Signaling through PRRs activates intracellular signaling cascades that swiftly induce the expression of genes involved in the inflammatory and immune response (Akira *et al*., 2006). This stimulates the production of pro-inflammatory cytokines, anti-microbial proteins, peptides and chemokines as well as the upregulation of adhesion molecules required for the influx of inflammatory cells such as neutrophils (Kawai and Akira, 2008; de Jong *et al*., 2010). Recruitment of individual adaptor proteins in combination with cell type-specific signaling pathways drives a range of sequelae, from inflammatory responses to cell proliferation, differentiation or, in some circumstances, apoptosis (Takeda and Akira, 2005). An additional important role of PRR signaling, which builds on the initial innate response to the threat of pathogen invasion, is the activation of dendritic cells (DCs) and the subsequent initiation and polarization of adaptive immunity (Iwasaki and Medzhitov, 2004).

1.2 Cells of the Innate Immune system

Several cell types contribute to innate immunity: macrophages, neutrophils, monocytes, Dendritic cells (DCs), Natural Killer (NK) cells, invariant natural killer T (iNKT cells) and innate lymphocyte cells (ILCs). These cell types initiate the first response to pathogen invasion, seeking to actively destroy invading microorganisms and recruit/activate adaptive immune cell subsets which assist in limiting the spread of infection. Whilst these immune cells are crucial to the prevention of pathogen invasion, the nonspecific nature of the innate immune

effectors can result in significant immunopathology if not appropriately regulated (Iwasaki and Medzhitov, 2010). Tissue resident lymphocytes occupying the innate-adaptive interface: mucosa-associated invariant T (MAIT) cells, gamma delta (γδ) T cells and tissue resident memory ($T_RM_)$ T cells strengthen the innate response by their abilities to detect a host of factors (microbial products, cytokines, alarmins, stress ligands) (Brennan *et al*., 2013; Godfrey *et al*., 2015; Corbett *et al*., 2014) which can promote an antimicrobial and tissue-protective response (Hung *et al*, 2013; Fan and Rudensky, 2016).

1.2.1 Macrophages

Macrophages are an important component of the innate immune system. Macrophage phagocytosis of foreign particles is pivotal to the initial stages of immune defense. In addition, these phagocytic cells play a fundamental role in later phases of tissue homeostasis, engulfing apoptotic cells and cellular debris, thus assisting tissue repair (Pollard, 2009). They are particularly important in the clearance of apoptotic neutrophils "efferocytosis"; failure of this process has been linked to autoimmune disease (Vandivier *et al*., 2006).

Macrophages have been functionally subdivided into two main groups: classically activated macrophages (M1) and alternatively activated macrophages (M2) (Stout *et al*., 2005; Sicca and Mantovani, 2012). Polarisation of macrophages to an M1 or M2 phenotype is influenced by the microenvironment they encounter, specifically the surrounding cytokine milieu (Stout et *al*., 2005). M1 macrophages, induced by pro-inflammatory molecules such as interferon gamma (IFN-γ) and LPS, exhibit potent antimicrobial properties and produce pro-inflammatory cytokines: Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6), Interleukin 12 (IL-12) and tumour necrosis factor alpha (TNF-α) (Trinchieri, 1997). M2 macrophages secrete less pro-inflammatory cytokines but tend to have enhanced phagocytic activity; they facilitate resolution of inflammation by secreting trophic factors and anti-inflammatory cytokines: interleukin 4 (IL-4), interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) (Bastos *et al* 2002; Mosser and Edwards, 2008). However, M2 macrophages can also promote allergic inflammation, support tumour growth and act as cellular reservoirs for some pathogens (Sica and Mantovani, 2014). The M1/M2 subdivision may be regarded as

an oversimplification; it is now recognised that there are distinct subclasses of M2 macrophages, for example M2a, M2b, M2c and M2d, based on specific patterns of cytokine production (Mosser *et al*., 2003; Murray *et al*., 2014).

The pro-inflammatory capabilities of macrophages in part depend on activation of the inflammasome, a multiprotein complex which resides in the cytoplasm of cells of myeloid lineage, including DCs and macrophages (Schroder and Tschopp, 2010). In response to cellular danger signals or DAMPS the inflammasome activates caspase-1, which cleaves pro-inflammatory cytokines such as pro-interleukin (IL)-1β into their biologically active forms (Schroder and Tschopp, 2010; Strowig *et al*., 2012). These cytokines go on to interact with other immune and non-immune effector cells to coordinate an inflammatory response. Given the strong pro-inflammatory activity of cytokines such as IL-1β it is of critical importance that the activity of the inflammasome is carefully regulated (Gross *et al*., 2011).

1.2.2 Neutrophils

Neutrophils are the most abundant immune cell population in human blood and are indispensable for initial defense against invading microorganisms. Mature neutrophils are characterised by their segmented nucleus and granules that are filled with numerous proteins, at least 700 have been identified (Rorvig *et al.,* 2013). Classical murine neutrophils can be identified by Ly6G+ expression, while human neutrophils express CD14, CD15, and CD16 (Pillay *et al*., 2013). They recognise microbes through the expression of many different PRRs such as NODs and TLRs (Mantovani *et al*., 2011). Granulocyte colony-stimulating factor (G-CSF) is the predominant factor regulating the life cycle of the neutrophil by stimulating cell proliferation and differentiation, enhancing survival and promoting mobilisation from the bone marrow niche (Basu *et al*., 2002). Interleukin (IL)-17 also promotes granulopoiesis and stimulates neutrophil recruitment by up-regulating G-CSF and chemokines (Ye *et al*., 2001) and down-regulating inhibitors of the leukocyte adhesion cascade (Eskan *et al*., 2012).The mechanism of neutrophil recruitment into the lung following exposure to pathogen is a multistep process involving extravasation across the pulmonary epithelium, trafficking across the lung interstitium and migration across the alveolar epithelium into the air spaces (Mizgerd *et al*., 1996; Vestweber, 2015; de Oliveira *et al*., 2016). A host of

chemotactic factors are involved in this process, including cytokines IL-8 and IL-17 and endogenous pro-inflammatory DAMPS produced following tissue injury and necrosis (McDonald *et al*., 2011; Wilhelmsen *et al*., 2012; Eskan *et al*., 2012). Some of these signals have been shown to inhibit neutrophil apoptosis and prolong the cell life span (Geering *et al*., 2013).

On arrival at the site of infection neutrophils release an array of antimicrobial factors: antimicrobial peptides, reactive oxygen species (ROS) and proteinases. Some of these proteinases (neutrophil elastase and matrix metalloproteinases) help degrade the extracellular matrix during migration (Gaggar *et al*., 2008); others, such as the serine proteases (elastase, proteinase-3 and cathepsin G) directly kill microbes and can inactivate bacterial toxins (Pham, 2006). Excessive inflammation leading to potential host tissue damage as a consequence of unregulated activity of these proteases is offset by serine protease inhibitors ("serpins") (Korkmaz B *et al*., 2010) which may also have a role in regulating neutrophil survival (Benarafa *et al*., 2011). In addition to the release of granules containing enzymes and anti-microbial peptides, neutrophils are potent phagocytes, directly targeting and engulfing invading pathogens (Lee *et al*., 2003). Beyond phagocytosis, neutrophils employ Neutrophil Extracellular Traps (NETS) to capture microbes extracellularly by ensnaring them through the release of a framework of DNA and proteases (Brinkmann *et al*., 2004), and autophagy to digest them intracellularly (Mitroulis *et al*., 2010; Mitsios *et al.,* 2016).

The traditional view that neutrophils are predominantly anti-microbial effectors in acute infection or injury has evolved in recent years. It is now recognised that neutrophils are functionally versatile and can induce anti-inflammatory responses which may be of benefit to the host (Pillay *et al*., 2012; Perobelli *et al*., 2015; Silvestre-Roig *et al*., 2016; Hajishengallis *et al.,* 2016). An example of this suppressor neutrophil subtype is regulatory neutrophil (RN) IL-10+ which is capable of IL-10 production and has been shown to coordinate with Tregs to reduce graft-versus-host-disease post haematopoietic stem-cell-transplantation (HSCT) (Perobelli *et al*., 2016). Neutrophil-derived transforming growth factor β1 (TGFβ1) has been demonstrated to downregulate inflammatory responses by macrophages (Gasser and Schifferli, 2004). The ability to influence other immune cells is made possible by the lifespan of the neutrophil being longer than first thought. Pillay et al demonstrated that human

neutrophils in homeostatic conditions have an average half-life of 5.4 days, not the 24 hours that was previously believed to be the case (Pillay *et al.*, 2010).

There is currently no consensus on a specific combination of cell surface markers to identify 'regulatory neutrophils'; classification is based on their functional characteristics (Perobelli *et al.,* 2017). In murine models, studies have shown that a subset of neutrophils produce IL-10 in response to infection with Gram positive bacteria such as *S. aureus* (Tsuda *et a*l., 2004), and in inflammatory conditions, such as post burn (Noel *et al*., 2011) and following G-CSF treatment (Perobelli *et al*., 2016). A separate neutrophil subset producing IL-22 (RN IL-22+) has been described in barrier tissues such as the intestinal mucosa, skin and lung where these cells appear to work alongside other IL-22 secreting immune cells (Th17, Th22, NK cells, γδ, and innate-like lymphocytes (ILCs)) in maintaining tissue homeostasis, ready to respond to target invading pathogen by inducing gene expression of antimicrobial peptides, chemokines and cytokines (Ouyang and Valdez, 2008; Jenq *et al*., 2012).

1.2.3 Dendritic Cells (DCs)

Dendritic cells (DCs), derived from monocytes through differentiation with GM-CSF and IL-4, serve as a major link between innate and adaptive immunity because of their ability to efficiently process and present antigens to naïve T cells (Abbas and Sharpe, 2005). Immature DCs exhibit high endocytic activity but have low T cell activation potential; they survey and sample their environment for pathogens through their PRRs (Kadowaki *et al*., 2001). DCs can be activated by the pathogen, either directly or indirectly, via the pathogen-induced nonspecific tissue response (Banchereau and Steinman, 1998). Activated DCs phagocytose pathogens and degrade their proteins for presentation at their cell surface using MHC molecules (Cella *et al*., 1997). Upon maturation and activation by TLR ligands and cytokines, these specialised antigen presenting cells (APCs) upregulate co-stimulatory molecules (CD40, CD80 and CD86) and produce cytokines such as IFN-γ, IL-12 or IL-10 (Dalod *et al*., 2002) which rapidly activate other innate protective cells such as NK and NKT cells (Fernandez *et al*., 1999; Fujii *et al*., 2002) and induce the differentiation and polarization of T helper subsets (Sallusto and Lanzavecchia, 2002; Abbas and Sharpe, 2005). However, DCs can be immunogenic or tolerogenic depending on the context; they are capable of shutting

lymphocytes off (Probst *et al*., 2003). DCs which are not activated, displaying antigens such as self-proteins, induce tolerance. This is thought to be critical for maintaining unresponsiveness to self (Probst *et al*., 2003; Steinman *et al*., 2003).

Previously, DC have been characterized as universal 'all purpose' APCs, however it is now recognised that there are several different types of DC, each able to respond to particular pathogens and to interact with specific subsets of T cells. DCs have previously been functionally subdivided into two main groups: $CD11c^{+}CD123^{-}$ myeloid DCs (mDCs) and $CD11c^{-}CD123^{+}$ plasmacytoid DCs (pDCs). These two groups differ in cytokine profile, mDCs secreting IL-10 and IL-12, as well as IFN-γ (secreted by pDCs), in response to microbial stimuli (Ito *et al*., 1999). Another key difference between pDCs and mDCs is their differential expression of TLRs. PDCs express mainly TLR7 and TLR9, however, low levels of TLR1, TLR6, and TLR10 mRNAs have also been detected. MDCs show a broader TLR expression pattern; these cells express all TLRs, except TLR7 and TLR9 (Hornung *et al.,* 2002; Liu *et al*., 2005). In recent years DC classification has been revised to three major DC subsets: plasmacytoid DC (pDC), myeloid/conventional DC1 (cDC1) and myeloid/conventional DC2 (cDC2). These subsets are independently controlled by specific transcription factors (Guilliams *et al*., 2014) and can be identified by these differential markers: cDC1s - $CD8\alpha^{+}$ (lymphoid) and $CD103^{+}$ (tissue), BATF3 and IRF8 dependent; cDC2s – $CD11b^{+}$ and $CD172a^{+}$, IRF4 dependent; and pDCs – IFNα-secreting, E2-2 dependent (Hansen and Anderson, 2017).

1.2.4 Natural Killer (NK) cells

Natural killer (NK) cells are non-T, non-B lymphocytes that function at the interface between innate and adaptive immunity. Originally characterized by their rapid cell-mediated cytotoxicity, specifically the ability to lyse virally infected or transformed cells (Greenberg *et al*., 1975; Glimcher *et al*., 1977), it is now recognised that NK cells have a more complex role in immune defense (Vivier *et al*., 2008, 2011). They employ an array of innate receptors to sense their environment and respond to alterations caused by infections and cellular stress by inducing apoptosis, providing contact-dependent co-stimulation and efficiently producing soluble mediators including cytokines: IFN-γ, TNF-α, IL-10 and GM-CSF (Scharton and Scott, 1993; Vankayalapati *et al*., 2005; Lee *et al*., 2007). They also contribute indirectly

by regulating APC and T cell responses (Degli-Esposti and Smyth, 2005; Orange, 2008). NK cells employ a combination of these mechanisms in host defense against pathogen invasion, regulation of immune responses and surveillance of stressed and cancerous cells (Degli-Esposti and Smyth, 2005; Vivier *et al*., 2008; Sivori *et al*., 2010).

NK cells are characterized phenotypically by the presence of CD56 and the lack of CD3 (Robertson and Ritz, 1990). In humans, two main subsets are described, based on the cell surface density of two key markers, CD56 and CD16 (Nagler *et al*., 1989; Cooper *et al*., 2001). NK cells expressing predominantly CD16 ($CD56^{dim}$ $CD16^{+}$) constitute the majority of peripheral blood NK cells (>90%) and exhibit potent cytotoxic activity through the release of granules containing granzymes and perforin (Nagler *et al*., 1989). Cytotoxic granules are preformed within NK cells, ready for deployment upon activation from cytokine signals generated by other immune effector cells (Trapani and Smyth, 2002; Orange, 2008). CD56-expressing NK cells ($CD56^{bright}$ $CD16^{-}$) are less cytotoxic but are more prolific producers of cytokines in response to stimulation by monocyte-derived cytokines (monokines) in the initial stages of the innate immune response (Cooper *et al*., 2001).

Murine NK cells differ from human NK cells in a number of ways, including the cell surface markers they express and the receptors through which activation and inhibitory signals are transduced (Choissone *et al*., 2009; Sivori *et al*., 2010). Four subsets have been defined, based on their expression of markers CD11b and CD27. Immature naïve murine NK cells are $CD11b^{dull}$ $CD27^{dull}$ or $CD11b^{dull}$ $CD27^{+}$, differentiated mature NK cells are $CD11b^{+}$ $CD27^{dull}$ and $CD11b^{+}$ $CD27^{+}$ double positive NK cells represent an intermediate population (Choissone *et al*., 2009). It has been suggested that $CD11b^{dull}$ murine NK cells behave similarly to $CD56^{bright}$ human NK cells (Vivier *et al*., 2008).

The activation of NK cells is dependent on a balance between activation and inhibitory signals, designed to constrain killing to appropriate target cells. Signals are communicated through one of three classes of receptor: Killer-cell Immunoglobulin-like Receptors (KIRS), Ly49 lectin-like receptors and NKG2 lectin-like receptors; KIRS are present in human NK cells but not in mice (Sivori *et al*., 2010). NK cell effector function is mediated by interactions between inhibitory receptors and cell surface MHC class I expression; cells expressing 'self' MHC class I molecules are more resistant to NK cell attack than cells lacking MHC I expression (Ljunggren and Karre., 1990). The relative protection conferred by MHC class I

expression results from the interaction between MHC I ligand and NK cell inhibitory receptor, which confers an intrinsic responsiveness to NK cells, so called NK cell "licensing" (Kim *et al*., 2005). Inhibitory KIRs discriminate normal cells from cancerous or virus-infected cells that have lost or decreased their expression of MHC class I (Sivori *et al*., 2010). NK cell killing of target cells is stimulated by an overriding activation signal, transduced via receptors such as NKp46, NKp44, NKp30, NKp80, NKG2D, and 2B4 (Moretta *et al.,* 2001; Betser-Cohen *et al*., 2010). It is thought that no single activation receptor is dominant; rather, signals from groups of receptors work synergistically to activate NK cell cytotoxicity and cytokine production (Betser-Cohen *et al*., 2010; Long *et al*., 2013).

1.2.5 Tissue Resident Lymphocytes

The classical view that lymphocytes continuously recirculate through the lymphoid organs and blood has developed to embrace the concept that certain subsets of lymphocytes can establish residency in non-lymphoid tissues (Sojka *et al*., 2014; Gasteiger *et al.,* 2015). This is especially relevant at barrier sites such as skin and lung where they have been demonstrated to serve as defenders of tissue integrity in response to invading pathogens (Shibata *et al*., 2007; Brennan *et al*., 2011; Gray *et al*., 2011). Tissue resident lymphocytes include innate lymphoid cells (ILCs), "unconventional" or "innate-like" T cells (iNKT, MAIT, γδ T cells) and tissue-resident memory (T_{RM}) T cells (Artis and Spits, 2015; Clark, 2015; Eberl *et al*., 2015; Godfrey *et al*., 2015). The T cell receptors (TCRs) of the majority of these T cells recognise antigens independently of MHC, or presented by non-classical, non-polymorphic MHC-like molecules; T_{RM} T cells are classical MHC restricted (Godfrey *et al*., 2015; Mueller and Mackay, 2016).

The shared features or "hallmarks" of this population of lymphocytes have been recognised as: i) capacity for long-term maintenance and self-renewal (Gray *et al.*, 2011), ii) abundance at barrier sites (Sumaria *et al*., 2011) and iii) the ability to detect a host of factors (microbial products, cytokines, alarmins, stress ligands) (Brennan *et al*., 2013; Godfrey *et al*., 2015; Corbett *et al*., 2014) which can promote an antimicrobial and tissue-protective response (Hung *et al*, 2013; Fan and Rudensky, 2016). These specialist immune cells quickly generate effector cytokines, cytolytic molecules and growth factors when tissue homeostasis is under

threat from pathogen invasion (Chiba *et al*, 2008; Shibata *et al*., 2007; Iwasaki and Medzhitov, 2010). This is in contrast to the T cell response, which requires clonal expansion and the development of antigen-specific memory, and therefore takes days (Kamat and Henney, 1976).

This prompt response may be explained by a form of ‘‘pre-programming’’ through selection and expansion during previous encounters with the relevant antigen, resulting in these lymphocytes being able to function like innate cells (Mueller and Mackay, 2016). Thus, tissue resident lymphocytes can be regarded as members of the innate immune system with memory-like properties, which may have their basis in the shared expression of the transcription factor PLZF (promyelocytic leukemia zinc finger) (Constantinides *et al*., 2014, 2015; Lu *et al*., 2015**;** Koay *et al*., 2016; Chen *et al*., 2017). PLZF-deficient mice have a deficiency of iNKT and MAIT cells, and those cells remaining function poorly (Savage *et al*., 2008; Kovalovsky *et al*., 2008; Rahimpour *et al*., 2015). How these lymphocyte populations in lymphoid and non-lymphoid organs are maintained, and whether they recirculate from lymphoid to non-lymphoid tissues, remains controversial. Maintenance in the tissue has been demonstrated to involve transcription factors BLIMP1 (B lymphocyte-induced maturation protein 1; aka PRDM1) and HOBIT (homologue to BLIMP1 in T cells; aka ZFP683). (Mackay *et al*., 2016).

1.2.6 Innate Lymphocyte Cells (ILCs)

ILCs reside in lymphoid and non-lymphoid tissues across multiple species and have been shown to communicate with both haematopoietic and non-haematopoietic cells to coordinate immunity, inflammation and homeostasis (Iwasaki and Medzhitov 2010; Spits and Di Santo, 2011; Spits *et al*., 2013). ILCs have lymphoid cell morphology, but lack the expression of myeloid or dendritic cell-surface markers (Moro, K. *et al*. 2010; Spits *et al*., 2013). They express subunits of cytokine receptors: interleukin IL-2 receptor-α (CD25) and IL-7 receptor-α (CD127), but, distinct from T and B cells, they do not express antigen receptors so do not exhibit antigen specificity (Spits *et al*., 2013). The ILC family includes NK cells and lymphoid tissue inducer (LTi), as well as the more recently described non-cytotoxic ILC populations (Artis and Spits, 2015). Initially, three groups of non-cytotoxic ILCs were

acknowledged, based on their requirements for specific transcription factors and the cytokines they express: group 1 ILCs (NK cells and ILC1s), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s and LTi cells) (Spits *et al*., 2013). In the last 5 years, the use of single-cell RNA sequencing has led to the identification of more than 50 distinct ILC clusters in different tissues (Gury-BenAri *et al*., 2016). So within the three initially proposed ILC groups there has been a reclassification into five distinct subsets: NK cells, ILC1s, ILC2s, ILC3s, and LTi cells (Vivier *et al*., 2018).

It is now evident that the observed similarities between ILCs and T-helper-cell subsets in cytokine expression and effector function permits ILCs to be regarded as the innate counterpart of T cell effector subsets (Artis and Spits 2015; Eberl *et al*., 2015; Fan and Rudensky, 2016). ILC1s produce IFN-γ and GM-CSF and are active against certain bacteria and viruses (Bernink *et al*., 2013; Klose *et al*., 2014). ILC2s have a cytokine profile similar to Th2 cells, secreting IL-4, IL-5, IL-9 and IL-13 plus the epidermal growth factor receptor (EGFR) ligand amphiregulin; they promote type 2 inflammation required for immunity to helminths, but also contribute to allergic inflammation and tissue repair (Moro *et al*. 2010; Neill *et al*. 2010; Monticelli *et al*., 2011; Mjosberg *et al*., 2011). ILC3s, like Th17 cells, are capable of producing IL-17A, IL-17F, IL-22, GM-CSF and TNF and have diverse effector functions from antibacterial immunity to chronic inflammation or tissue repair (Cella *et al*., 2009; Buonocore *et al*., 2010; Sonnenberg *et al*., 2011). ILC3s are particularly important in the maintenance of intestinal homeostasis and containment of intestinal commensal organisms; this is achieved predominantly through the secretion of IL-22 (Sonnenberg *et al*., 2011). ILC3s have also been demonstrated to regulate adaptive Th17 cell responses (Hepworth *et al*., 2013). LTi cells are crucial to embryonic development, inducing the growth of secondary lymph nodes and Peyer's patches. They do this by stimulating mesenchymal stromal cells (MSC) to produce chemoattractant factors for hematopoietic cells (HSCs), which recruits HSCs to the lymphoid structure and retains them there (Mebius *et al*., 1997). Like ILC3s, LTi cells are dependent on transcription factor RORγt, but have been shown to follow a different developmental path (Vivier *et al*., 2018).

ILCs are derived from a common lymphoid progenitor (CLP) via precursor cells which express the α4β7 integrin, so-called α-lymphoid precursor (αLP) cells (Klose *et al*., 2014). Downstream of this is an Id2-expressing precursor that can give rise to all ILCs, including LTi

cells, but not conventional NK cells, and an $Id2^{+}PLZF^{+}$ precursor that is restricted to ILCs but is unable to develop into LTi cells (Constantinides *et al*., 2014; Klose *et al.*, 2014). Two transcription factors that affect early differentiation of ILCs and NK cells (but not T and B cells) are TOX and NFIL3 (Aliahmad *et al*., 2010; Constantinides *et al*., 2014; Geiger *et al*., 2014). TCF-1 and GATA3 have also been identified as key transcription factors in the development of ILCs; HSCs post genetic ablation of GATA 3 could not develop into ILCs or T cells, but could become NK cells and B cells (Yagi *et al*., 2014). ILCs, allowed to mature before GATA 3 deletion, exhibited inhibition of ILC2 but not ILC3 populations, suggesting that GATA3 is pivotal for survival of ILC2s (Mjosberg *et al*., 2012). Master transcription factor of Th1 cells, T-bet, is important for the development and IFNγ-secretion profile of ILC1s (Klose *et al*., 2014). RORγt is fundamental for the development of ILC3s, as it is for Th17 cells (Eberl *et al*., 2004; Ivanov *et al*., 2006).

ILCs have been demonstrated as having diverse functions, including promoting host defense against pathogen invasion, particularly at barrier surfaces where they are well placed for rapid activation by host-derived cytokines and growth factors (Spits and Di Santo, 2011; Spits *et al*., 2013). Broadly speaking, ILC1s react to intracellular pathogens, such as viruses and intracellular bacteria; ILC2s respond to large extracellular parasites such as helminths; ILC3s target extracellular microbes, such as extracellular bacteria and fungi (Zheng *et al*., 2008; Sonnenberg *et al*., 2011; Pulendran *et al*., 2012; Gladiator *et al*., 2013; Van Maele *et al*., 2014) ILC1s have been demonstrated to be active against *Salmonella enterica* and *Toxoplasma gondii* infection in the intestine (Klose *et al*., 2014). ILC3-derived IL-22 has been shown to contribute to the neutralisation of Gram-negative bacterium *Citrobacter rodentium* by promoting the expression of specific antimicrobial peptides. Mice deficient in IL-22 could not clear the pathogen and exhibited marked intestinal inflammation and loss of the epithelial barrier (Zheng *et al*., 2008; Sonnenberg *et al*., 2011). In the lungs, IL-22 from ILC3s has been demonstrated as important for pathogen clearance following infection with the fungus *Candida albicans* (Gladiator *et al*., 2013) and bacterium *Streptococcus pneumoniae* (Van Maele *et al*., 2014). ILC2 production of cytokines IL-4, IL-5, IL-9 and IL-13 has been shown to enhance macrophage phagocytosis and smooth muscle activity which promotes parasite expulsion and facilitates tissue repair (Spits and Di Santo, 2011; Pulendran *et al*., 2012). If left unchecked, however, ILC2s can fuel T-cell responses that

contribute to allergic airway inflammation (Drake *et al*., 2014; Halim *et al*., 2014). Indeed, ILC2s have been noted to be increased in the peripheral blood of patients with asthma relative to healthy controls (Bartemes *et al*., 2014).

ILCs are capable of expressing MHC class II molecules and processing antigens, so can influence the adaptive immune system by regulating T cell activity; T cell derived IL-2 then stimulates ILC activities, creating a positive feedback loop. However, ILCs and T cells are in competition for survival factors and stimulatory cytokines such that this positive feedback is self-limiting, regulating immune response (Oliphant *et al*., 2014).

1.2.7 Invariant natural killer T (iNKT) cells

iNKT cells, also known as type I or classical NKT cells, are found in the thymus, secondary lymphoid organs and blood and were initially identified as sharing some markers of both NK and T cells (Bendelac *et al*., 1995), however it is now known that some NKT subsets do not express NK cell-related molecules (Coquet *et al*., 2008). iNKT cells express an invariant TCRα chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) and a TCRβ chain of limited diversity (Brennan *et al*., 2013; Salio *et al*., 2014). They recognize glycolipid antigens presented by the non-classical MHC class I-like molecule CD1d (Bendelac *et al*., 1994, 1995) and exhibit a robust reaction to the glycosphingolipid alpha-galactosylceramide (α-GalCer) (Kawano *et al*., 1997).

CD1d-restricted T cells can be further segregated into three groups called type I (or "invariant") NKT, type II (or "variant") NKT, and "others" dictated by their Ag specificity and TCR usage (Godfrey *et al*., 2015). iNKT cells are the best characterised of these groups, they comprise less than 1% of peripheral blood mononuclear cells (PBMCs) in humans but are relatively abundant in mice (Godfrey *et al*., 2015). They are important in the initial stages of host defense against infection because they can be activated by specific bacterial ligands, for example sphingolipids in the cells walls of *Streptococcus* (Kinjo *et al*., 2006, 2011). Once activated, iNKT cells can rapidly produce chemokines (CCL2, CCL3, CCL4, CCL5, CCL11, and CXCL8) and cytokines (TNF-α, IFN-γ, IL-4, IL-5, IL-10, IL-13, IL-17, IL-21, IL-22, and GM-CSF) (Matsuda *et al*., 2008). The actual cocktail produced is governed by the nature of the stimuli and the cell subset triggered. The diversity of secreted molecules is such that iNKT cells can

influence the functions of a wide variety of immune populations from neutrophils and macrophages to NK cells, γδ T cells, B and conventional T cells (Galli *et al*., 2007; De Santo *et al*., 2010; Cohen *et al*., 2011; Nieuwenhuis *et al*., 2012). Furthermore, iNKT cells have been demonstrated to exhibit targeted killing activity towards transformed and virally infected cells (Metelitsa *et al*., 2001).

In addition to reacting to pathogens and signals generated by non-infectious insults, iNKT cells can survey the condition of the tissue in which they reside and initiate a reparative process if required. This has been demonstrated to involve iNKT recognition of low levels of often transiently expressed endogenous lipids (Brennan *et al*., 2011, 2014; Facciotti *et al*., 2012; Kain *et al*., 2015). This facilitates iNKT cell detection of any disruption to tissue integrity which may be caused by infection with viruses, fungi or bacteria which lack the potent cell wall ligands to activate them directly (Brennan *et al*., 2011; Cohen et al., 2011).

Like ILCs, NKT cell subsets expressing cytokines and transcriptional regulators comparable to their conventional T-helper analogues (T_H1, T_H2, and T_H17) have been identified: NKT1 is IFN-γ-producing, $PLZF_{low}$/T-bet$^+$, NKT2 is IL-4-producing, $PLZF_{high}$/T-bet$^-$ and NKT17 is IL-17-producing and RORγt$^+$ (Watarai *et al*., 2012; Engel *et al*., 2016). In mice, the lungs are particularly enriched for NKT17 cells compared to other tissues (Michel *et al*., 2007). They appear to be dominant within the lung parenchyma in contrast to their NKT 1 and NKT2 counterparts which reside in the vasculature; they are thought to have a patrolling role in the steady state, acting as “lung-resident sentinels” (Lee *et al*., 2015). Interestingly, lung MAIT cells also display a phenotype of IL-17-producing cells defined by high expression of IL-7Rα and IL-18R1 and the lack of NK1.1 expression (Rahimpour *et al*., 2015). They have been demonstrated as capable of early recognition and response to extracellular bacteria, including *S. pneumoniae*, infecting airway epithelial cells (Hartmann *et al*., 2018).

1.2.8 Mucosa-associated invariant T (MAIT) cells

MAIT cells, like iNKT cells, express a semi-invariant TCR consisting of a unique TCRα chain (Vα19-Jα33 in mice and Vα7.2-Jα33 in humans) combined with a restricted set of TCRβ chains; they localise to the liver, and to a lesser extent the intestine, lungs and skin (Dusseaux *et al*., 2011; Meierovics *et al*., 2013; Salio *et al*., 2014). In contrast to iNKT cells,

MAIT cells are abundant in humans, but rare in mice (Treiner and Lantz, 2006; Chandra and Kronenburg, 2015). MAIT cells have a memory phenotype and may express transcription factors PLZF, RORγt, and T-bet (Koay *et al*., 2016; Chen *et al*., 2017). They also express several cell surface proteins that can be used for characterization; these include a variety of homing receptors: CXCR6, CCR9, α4β7, CD103, which may assist their migration to the skin, liver, lung, and gut mucosa, and cytokine receptors: IL-7Rα, IL-12R, and IL-18Rα (Leeansyah *et al*., 2014; Rahimpour *et al*., 2015).

MAIT TCRs are activated by microbial vitamin B metabolites presented by the MHC class I-like molecule MR1 (Kjer-Nielsen *et al*., 2012; Corbett *et al*., 2014). Sensing of MR1-bound microbial products activates MAIT cells to produce pro-inflammatory cytokines, perforins and granzymes which efficiently lyse bacterially-infected epithelial cells (Chua *et al*., 2012; Le Bourhis *et al., 2013;* Meierovics *et al*., 2013; Kurioka *et al*., 2015). Variation in riboflavin metabolism among clinical isolates of *S. pneumoniae* has been demonstrated to stimulate differential activation of MAIT cells, which impacted on the level of MAIT cell-mediated control over bacterial growth (Hartmann *et al*., 2018). How lung MAIT cells interact with commensal bacteria is not yet fully understood; however, there is a severe impairment in MAIT cells in the thymus, spleen, and gut of germ-free mice (Kaoy *et al*., 2016). In a murine model of pulmonary tularemia MAIT cells were found to accumulate in the lungs in the acute phase of infection. They responded to both MR1 and IL-12 40 kDa subunit (IL-12p40) signals from infected APCs, producing cytokines IFN-γ, TNF-α, and IL-17A, which served to limit *F. tularensis* live vaccine strain (LVS) intracellular growth. Importantly, pulmonary *F. tularensis* LVS infection of MR1-deficient (MR1−/−) mice, which lack MAIT cells, resulted in a reduction in levels of these critical cytokines, blunting of $CD4^+$ and $CD8^+$ T cell recruitment to the lungs and poor control of pulmonary *F. tularensis* LVS growth (Meierovics *et al*., 2013).

Additionally, MAIT cells are capable of efficiently killing bacteria- infected cells (Le Bourhis *et al*., 2010; Shaler *et al*., 2017). MAIT cells have been shown to be activated by bacteria and yeasts, in an MR1- dependent manner, but not by viruses, (Gold *et al*., 2010; Le Bourhis *et al*, 2010). However, recent work suggests that in some viral diseases MAIT cells can be activated without MR1 involvement (van Wilgenburg *et al*., 2016). MAIT cells are also activated in some non-infectious, inflammatory diseases, such as systemic lupus erythematosus (SLE). Activation appears to be cytokine driven (IFN-α, IL-15, IL-12, IL-18) in

the absence of exogenous antigens (Chiba *et al*., 2017). A third mechanism for MAIT cell activation, exposure to super antigens (SAgs) produced by *Staphylococcus aureus* and *S. pyogenes,* has recently been identified (Shaler *et al*., 2017). MAIT cells activated in this way rapidly produced potent pro-inflammatory cytokines but then became anergised to subsequent bacterial challenge through upregulation of specific inhibitory receptors. The investigators concluded that whilst MAIT cells have an established role in antibacterial immunity they may also contribute to pathogenesis in certain bacterial infections (Shaler *et al*., 2017; Sandberg *et al*., 2017).

1.2.9 Gammadelta (γδ) T cells

T cells expressing the γδ TCR, γδ T cells, are another important T cell subset at the interface between innate and adaptive subsets that resides in barrier tissues and is involved in the stress response to injured, infected or transformed tissues (Hayday *et al*., 2009; Bonneville *et al*., 2010). The γδ TCR differs from the αβ TCR by possessing a longer, immunoglobulin-like CDR3 structure, which facilitates a variety of mechanisms of antigen recognition. This in contrast to the stringent MHC restriction of conventional αβ T cells (Rock *et al*., 1994). Ligand recognition by the γδ TCR includes lipids presented by CD1 family members, peptides bound to stress-inducible MHC class I-like molecules (MICA and MICB) and conventional MHC molecules and even some soluble ligands lacking presenting molecules (Hayday *et al*., 2009; Bonneville *et al*., 2010; Chien *et al*., 2014; Sandstrom *et al*., 2014).

In common with iNKT cells and MAIT cells, γδ T cells have been shown to express NK activating receptors such as NKG2D and NKG2A, which suggests that these cell subsets may have overlapping functions with NK cells (Kong *et al*., 2009). For example, increased expression of stress-induced MHC class I-related molecules MICA/B on infected or damaged cells enhanced interactions with the activating NKG2D receptor on γδ T cells resulting in target cell death (Nedellec *et al.*, 2010). Such activity facilitates surveillance of tissue integrity, even in the absence of microbial invasion, and can promote regeneration and repair post non-infectious insult (Sumaria *et al.*, 2011).

γδ T cell activation can also be mediated by antibody-opsonized cells through the binding of IgG Fc by the Fcγ receptor III CD16, so-called antibody-dependent cell-mediated cytotoxicity

(Bonneville *et al*., 2010). Activated γδ T cells become cytotoxic and secrete pro-inflammatory cytokines such as IFN-γ and TNF-α and IL-17 (Hayday *et al*., 2009; Bonneville *et al*., 2010). Akin to MAIT cells, IFN-γ and IL-17 are produced by distinct γδ T cell subsets in response to a variety of stimuli (Jensen *et al*., 2008). γδ T cells have been observed as the major producers of IL-17 in the first few hours following murine intraperitoneal infection with *E. coli,* which was the main driver for neutrophil recruitment; depletion of both γδ T cells and IL-17 significantly impaired neutrophil response (Shibata *et al*., 2007). A particular subset of human γδ T cells characterized as Vγ9Vδ2+ also exhibits antigen cross presenting abilities similar to antigen presenting cells (APCs). This is achieved via the expression of adhesion receptors, co-stimulatory molecules and MHC class I and II molecules and culminates in the activation of conventional T cell subsets which augment the immune response (Meuter *et al.*, 2010).

In summary, the different components of the innate immune system offer prompt recognition of invading microorganisms and orchestrate the initial approach to pathogen elimination. This includes neutrophil and phagocyte activation and recruitment for direct bacterial killing, cytokine production to activate APCs and consequent antigen processing and presentation to stimulate the next branch of host defense; adaptive immunity. The discovery of immune cells displaying properties which span the innate-adaptive border of immunity suggests that the distinction between innate and adaptive systems is not clear cut.

1.3 Cells of the adaptive immune system

Adaptive immunity is mounted as a slower, secondary response following pathogen invasion and activation of the innate immune response. Antigen-mediated activation of naïve T and B cells drives clonal expansion of these lymphocyte subsets, which in turn provides differentiated effector cells to assist in pathogen eradication (Kamat and Henney, 1976). T helper (Th) cells and cytotoxic T cells (CTL) produce cytokines which function to coordinate the immune response and B cells produce antibodies to neutralise targets. Once the primary infection has been eliminated by a combination of innate and adaptive immune cell subsets, adaptive immune cells have a further role in establishing immunological memory (Nossal and Makela, 1962; Miller and Sprent, 1971; Vischer *et al*., 1967). This ensures that subsequent encounters with the same pathogen are dealt with more efficiently due to quick re-activation and expansion of memory cells (Nossel *et al*., 1965).

1.3.1 T_H1 and T_H2 cells

The distinction between T_H1 and T_H2 cells was made in the mid 1980s (Mossmann *et al*., 1986). In a seminal study, Mossmann and Coffman proposed that naïve $CD4^+$ T cells could differentiate into one of two T cell subsets with distinct cytokine profiles. T_H1 cells produce pro-inflammatory cytokines such as IFN-γ, considered to play an important role in the eradication of intracellular pathogens; T_H2 cells secrete interleukin (IL)-4, IL-5 and IL-13 which activate responses to allergens, parasitic helminths and other extracellular microbes (Mossmann *et al*., 1986). These cytokines activate mast cells, stimulate the recruitment of eosinophils and enhance Immunoglobulin (Ig)-E antibody responses, which can lead to a more chronic inflammatory response (Locksley, 2010).

The cytokine environment to which naïve $CD4^+$ T-cells are exposed is a central influence on T helper cell subset differentiation; an example of this is DC and macrophage secretion of IFN-γ and IL-12 stimulating T_H1 cell development (Hsieh *et al*., 1993). The key cytokine required for naïve T cell polarization to T_H2 cells is IL-4. Interaction of IL-4 with its cognate receptor stimulates the recruitment and phosphorylation of STAT6, which induces the expression of GATA-3 and other T_H2 cell specific genes (Kurata *et al*., 1999). GATA-3 is acknowledged to be the master regulator of T_H2 cells (Zheng and Flavell, 1997), a role played by T-bet for T_H1

cells (Szabo *et al*., 2000). These transcription factors play a pivotal role in determining the cytokine profile of each T helper cell subset (Zheng and Flavell, 1997; Szabo *et al*., 2000). T-bet expression correlates with IFN-γ expression in T_H1 cells, and retroviral gene transduction of T-bet into polarized T_H2 cells redirects them to a T_H1 cytokine profile: induction of IFN-γ and repression of IL-4 and IL-5 (Szabo *et al*., 2000). Not only is T-bet a crucial factor in suppressing *il-4* gene expression in T_H1 cells (Djuretic *et al*., 2007), its continuous expression is required for T_H1 cells to silence their IL-4-producing potential (Zhuang *et al*, 2009).

1.3.2 T_H17 cells

The T_H1/T_H2 paradigm outlined by Mossman and Coffman was revised when it was discovered that naïve $CD4^+$ T cells could in fact differentiate into additional subsets with distinct cytokine profiles and functions, examples being T_H17 and regulatory T cells (Harrington *et al*., 2005; Park *et al*, 2005; Bettelli *et al*., 2006). The existence of the T cell subset, now denoted T_H17 cells, was first suggested when human IL-17A was identified in cDNA libraries of T cells, and later confirmed to be secreted as a homodimer by activated memory T cells (Yao *et al*., 1995; Fossiez *et al*., 1996). This discovery saw the beginnings of a rapid expansion in the study of T cell responses in autoimmunity and infection. Until this time, T_H1 cells had been heavily implicated as the effectors in a variety of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE) (Merrill *et al*., 1992) and Diabetes Mellitus (DM) (Shehadeh *et al*., 1993). However, in a murine model of EAE, Cua and colleagues determined that it was IL-23 and not the T_H1 cell-associated cytokine IL-12 that was critical for initiating autoimmune inflammation in the brain (Cua *et al*., 2003). Appreciation that the IL-17–IL-23 inflammatory axis was in fact responsible for several models of autoimmune disease, originally attributed to dysregulated T_H1-mediated responses, caused a shift in thinking, which ultimately resulted in the acknowledgment of the T_H17 subset of T cells (Harrington *et al*., 2005; Park *et al*, 2005). This subset is now appreciated to play a key role in the effector response to infection (Zhang *et al*., 2009; Liu *et al*., 2010; Warfel *et al*., 2013; Xu *et al*., 2014; Bagheri *et al*., 2015).

Differentiation of T_H17 cells has been shown to be independent of the cytokines and transcription factors required for T_H1 or T_H2 cell differentiation; furthermore, both IFN-γ and

IL-4 negatively regulate T helper cell production of IL-17 (Park *et al*., 2005). T_H17 cell differentiation was initially thought to require the presence of IL-23, produced by APCs during antigen stimulation (Aggarwal *et al*., 2003; Harrington *et al*., 2005). IL-23 was also shown to induce expression of the related cytokine IL-17F (Aggarwal *et al*., 2003). Consistent with these observations, mice lacking the p19 subunit of IL-23 ($p19^{-/-}$) failed to produce T_H17 cells and were resistant to EAE (Cua *et al*., 2003). Subsequent studies demonstrated that differentiation of the T_H17 lineage requires the presence of cytokines TGF-β and IL-6 in combination with T cell antigen receptor (TCR) stimulation, and not IL-23 (Bettelli *et al*., 2006). Bettelli and colleagues also showed that TGF-β may induce either anti-inflammatory T_{regs} or pro-inflammatory T_H17 cells, depending on the presence of IL-6 (Bettelli *et al*., 2006). The role of IL-23 was later attributed to stabilisation of the T_H17 cell phenotype and maturation of inflammatory T_H17 cells (McGeachy *et al*., 2007, 2009). Additional cytokines shown to contribute to T_H17 cell development and differentiation include IL-21 (Yang *et al*., 2008) and IL-1β (Acosta-Rodriguez *et al*., 2007; Chung *et al*., 2009; El-Behi *et al*., 2011).

The key transcription factor for T_H17 cells, essential for IL-17 production, is the retinoic acid receptor-related orphan nuclear receptor RORγt (Ivanaov *et al*., 2006). Interferon regulatory factor 4 (IRF4) has also been shown to be an important protein in T_H17 cell differentiation, as it is in T_H2 cell differentiation (Lohoff *et al*., 2002), with IL-1 identified as a key regulator of both IRF4 and RORγt expression (Chung *et al*., 2009). The phosphorylation of STAT 3 as a consequence of IL-6 mediated signaling is also critical for T_H17 cell development (Yang *et al*., 2007). STAT 3 also suppresses TGF-β-induced Foxp3 expression, so is inhibitory to the production of T_{regs} (Bettelli *et al*., 2006). Basic leucine zipper transcription factor ATF-like (BATF), a member of the activator protein 1(AP-1) family of transcription factors, is also essential for T_H17 cell differentiation; mice lacking BATF cannot maintain expression of RORγt and are resistant to EAE (Schraml *et al*., 2009).

RORγt-expressing T_H17 cells can be broadly subdivided into two groups: T_H17 cells activated by TGF-β and IL-6 are involved in dampening down immunopathogenic responses and protecting the host at barrier sites (secreting IL-17 and IL-10) (McGeachy *et al*., 2007); $T_H17$cells activated by IL-23 are more inflammatory, provoking chronic inflammation in the context of infection and driving autoimmunity (Chen *et al*., 2006). Pro-inflammatory effects

generated by T_H17 cells are mediated by 'signature cytokines' IL-17, IL-22 and GM-CSF (Zheng *et al*., 2007; El-Behi et al., 2011). Important for fighting infection, IL-17 induces the expression of chemokines responsible for directing neutrophil chemotaxis, and antimicrobial peptides which target invading pathogens at mucosal surfaces and skin (Shen *et al*., 2005).

IL-17A is the founding member of the IL-17 family of cytokines which numbers 6 in total, designated IL-17A-F. IL-17F is the most homologous to IL-17A (58%) and, like IL-17A, is mainly produced by T_H17 cells, whilst IL-17B, C and D can also be produced by non-T cells (Rathore and Wang, 2016). IL-17-A, and IL-17F play a pivotal role the eradication of extracellular pathogenic bacteria and fungi (Chen *et al*., 2011; Hernandez-Santos *et al*., 2013). IL-17A and F can form heterodimers (IL-17A-IL-17F) or homodimers (IL-17A/IL-17F); IL-17A homodimers have been demonstrated as the most efficient in chemokine induction and subsequent neutrophil recruitment in mouse model of asthma (Liang *et al*., 2007). Like IL-17A, IL-17F employs IL-17RA and IL-17RC as its receptor and utilises Act1 and TRAF6 as its signal transducers to stimulate the expression of pro-inflammatory cytokines and chemokines in a variety of cell types (Chang and Dong 2009). It is interesting to note that in isolation, IL-17A and IL-17F are regarded as modest activators of signaling, but they work in synergy with other pro-inflammatory cytokines (TNFα, IFN-γ, IL22 and IL-1β) to generate a more potent inflammatory response (Shen *et al*. 2005; Onishi and Gaffen, 2010).

1.3.3 Regulatory T cells (T_{regs})

Regulatory T cells (T_{regs}) inhibit the activation, proliferation, differentiation and cytokine production of effector T-cell populations (Sakaguchi, 2004). This regulation of T effector cells and other cell types effectively dampens down the innate and adaptive immune response, redressing the balance between pro and anti-inflammatory states and restoring immune homeostasis (Sojka *et al*., 2008). This is critical if the immune system is to maintain its fine balance between defending the body from microbial invasion whilst minimising collateral inflammatory damage to host cells (Sakaguchi *et al*., 2007). In recent years T_{reg} biology has become a dynamic field of immunological research; once regarded as a homogenous immunosuppressive population, T_{regs} have been demonstrated to be a highly adaptable and

diversified cell type. T_{reg} heterogeneity in origin, localisation, differentiation, plasticity and mechanisms of immunosuppression is now recognised, if not yet fully understood (Josefowicz *et al.,* 2012; Burzyn *et al*., 2013; Grignolio *et al*., 2014).

Another indispensable function of T_{regs} is mediation of peripheral tolerance by targeting autoreactive T cells that arise *de novo* or escape thymic deletion. It has been established for some time that depletion or functional alteration of T_{regs} leads to the development of autoimmune disease in otherwise normal animals (Sugihara *et al*., 1988; Smith *et al*., 1992; Sakaguchi *et al*., 1995). Significantly, the transfer of a $CD4^+$ $CD25^+$ T cell subset, from syngeneic healthy mice, prevented the development of autoimmunity in these lymphopenic animals (Sakaguchi *et al*., 1995), suggesting that there is an important role for a T cell subtype with the capacity to prevent the activation of autoreactive cells. In subsequent work, Sakaguchi's group demonstrated that $CD4^+CD25^+$ T cells appear around day 3 postnatal and increase up to the adult levels by day 10; these T cells were coined "regulatory" (Asano *et al.,* 1996). It is now understood that several mechanisms exist to safeguard against self-directed immunity: clonal deletion, editing, anergy, ignorance and immune deviation (Luo *et al*., 2016; Lu *et al*., 2017). In addition, specialised cells capable of immunosuppressive activities have also been recognised, some of which interact with T_{regs}: tolerogenic DCs (Liu *et al*., 2015), regulatory B cells (Mauri *et al*., 2017) and regulatory ILCs (Wang *et al*., 2017).

1.3.4 T_{reg} subtypes and the importance of Foxp3

T_{regs} are heterogeneous in phenotype, function, and the way of generation. Some are naturally occurring (nT_{regs}); others are induced in the periphery (iT_{regs}) (Bluestone and Abbas, 2003). The two subpopulations differ in their surface phenotype, co-stimulation and cytokine dependence, T cell receptor (TCR) repertoire and homing behavior (Shevach, 2011). nT_{regs} develop in the thymus during the early stages of foetal and neonatal T cell development (Sakaguchi *et al*., 2001). They constitute 5-10% of $CD4^+$ T cells (in humans and mice) and are capable of recognising diverse self-antigens (Belkaid, 2007). Once generated, nT_{regs} migrate into peripheral tissues where they prevent the activation of other, self-reactive T cells that have the potential of developing into effector cells (Salomon *et al*., 2000). They co-express CD4 and CD25, as well as the costimulatory molecule cytotoxic T-

lymphocyte antigen 4 (CTLA4) and the tumour necrosis factor (TNF)-superfamily member GITR (glucocorticoid-induced TNF receptor family related protein) (Salomon *et al*., 2000).

Discovery that T_{regs} express the transcription factor Foxp3 supported the concept that T_{regs} represented a distinct T cell lineage (Fontenot *et al*., 2003). Foxp3 is a member of the forkhead/winged-helix family of transcriptional regulators. It functions to repress some of the downstream effectors of the T cell activation pathway, such as *il-2* transcription, but induces expression of *cd25* and *ctla-4 (*Sadlon *et al.,* 2010*)*. Disruption of Foxp3 function in humans leads to development of an X-linked polyendocrinopathy and enteropathy (IPEX) syndrome characterized by autoimmune disease, inflammatory bowel disease (IBD) and allergy (Bennett *et al*., 2001). In mice, deletion of $Foxp3^+$ T cells leads to multi-organ autoimmune disease in a similar vein, called scurfy (Godfrey *et al*. 1991). Conclusive evidence for Foxp3 as a lineage specific marker for T_{regs} came from Foxp3 eGFP reporter mice (Fontenot *et al*., 2005). These mice expressed GFP in $TCR\beta^+$ T cells only; conditional deletion of *Foxp3* in $CD4^+$ T cells led to a lymphoproliferative disorder mirroring the scurfy phenotype. It is now clear that Foxp3 is a key regulatory gene for the development of T_{regs} and the most optimal marker for this population (Josefowicz and Rudensky, 2009). The autoimmune defect which occurs with Foxp3 ablation can be rescued by the adoptive transfer of wild-type (WT) $Foxp3^+$ T cells (Huter *et al*., 2008). Furthermore, retroviral gene transfer of Foxp3 converts naïve T cells toward a regulatory T cell phenotype similar to that of naturally occurring $CD4^+$ regulatory T cells (Hori *et al*., 2003).

Early evidence of the existence of iT_{regs} came from studies showing the generation of T_{regs} by oral antigen feeding (Mucida *et al*., 2005) and by antigen-specific APCs (Apostolou *et al*., 2002) without the presence of a working thymus. iT_{regs} are conventional $CD4^+CD25^-$ cells that upon exposure to self or foreign antigens transform into Tr1 cells (in the presence of IL-10 or IL-27) or T_H3 cells (in the presence of TGF-β and induction of f*oxp3* expression) (Klunker *et al*., 2009). In contrast to the stable Foxp3 expression observed in nT_{regs}, the generation of iT_{regs} has been associated with a more transient $Foxp3^+$ phenotype (Fontenot *et al*., 2003; Hori *et al*., 2003). Whilst iT_{regs} and nT_{regs} share a similar function it has been suggested that iT_{regs} are an essential non-redundant regulatory subset that supplements nT_{reg} cells, in part by expanding TCR diversity within regulatory responses (Haribhai *et al*.,

2011). T_{regs} have since been classified into two separate populations based on their functionality as opposed to site of induction: central T_{regs} (cT_{regs}) and effector T_{regs} (eT_{regs}) (Cretney *et a*l., 2011; Smigiel *et al*., 2014). cT_{regs} reside in lymphoid tissues and express the lymphoid homing molecules CD62L and CCR7; eT_{regs} are primarily non-lymphoid T_{regs} which express low levels of lymphoid homing molecules and instead upregulate activation-induced markers such as CD44, ICOS, GITR (Smigiel *et al*., 2014).

1.3.5 Mechanisms of T_{reg} suppression

A number of possible mechanisms for the suppressive function of T_{regs} *in vivo* have been proposed: inhibition of effector T cell priming and expansion (Morlacchi *et al*., 2011), blocking of migration of these cells from the site of immunization (Davidson and Schevach, 2009) and inhibition of the differentiation of naive precursors into pathogenic effector T cells (Oldenhove *et al*., 2003; DiPaolo *et al*., 2005). Each of these 'mechanisms' have a number of facets to them, such as the release of immunosuppressive cytokines (IL-10, IL-35 and TGF-β), induced cytolysis of target cells, disruption of metabolic processes in target cells and modulation of DC maturation (Shevach 2009; Vignali 2012).

Upon activation, T_{regs} produce an array of immunomodulatory cytokines, the most important of which are IL-10 and TGF-β. IL-10 can limit the production of IFN-γ, IL-2, IL-4 and IL-5 from $CD4^+$ T cells and can inhibit the expression of MHC class II and co-stimulatory molecules CD80 and CD86 on monocytes and macrophages (Couper *et al*., 2008). TGF-β acts in a similarly immunosuppressive manner, inhibiting the secretion of immunoglobulins (Corthay, 2009). There can be little doubt that TGF- β plays a key role in lymphocyte homeostasis at many levels as demonstrated by the dramatic immune pathology evident in mice that are deficient in TGF-β or TGF-β signaling pathways (Li *et al*., 2006). T_{regs} can also inhibit the effects of pro-inflammatory cytokines such as TNF-α by releasing soluble TNFRII into the extracellular space to bind this cytokine and mitigate its inflammatory effects (van Mierlo *et al*., 2008). Activated T_{regs} are able to express granzymes (A and B) and perforin which directly induce apoptosis of adaptive immune cells (Vignali *et al*., 2008). The Vignali group also highlighted a novel cytokine, Ebi3-IL-12alpha heterodimeric cytokine, designated IL-35 that appears to contribute to T_{reg} suppression. Ectopic expression of IL-35 confers

regulatory activity on naive T cells, whereas recombinant IL-35 suppresses T cell proliferation (Collison *et al*., 2007).

Direct cell-to-cell contact has traditionally been regarded as important in T_{reg} suppression of effector T cells, in part through the induction of metabolic disruption or competition for growth factors, specifically repression of *il-2* transcription in the T effector cell, which causes apoptosis (Pandiyan *et al*., 2007). Interestingly, the apoptotic death induced in the targets was shown to be Bcl-2 interacting mediator of cell death (BIM)-dependent and perforin/Fas-independent (Pandiyan *et al*., 2007). An alternative inhibitory pathway promoted by cell-cell contact is the modulation of cyclic adenosine monophosphate (cAMP) levels in target cells. Elevation of cAMP has long been associated with inhibition of cellular proliferation and differentiation, and in lymphocytes drives selective inhibition of cytokine gene expression, including *il-2* and *ifn-γ* (Minguet *et al*., 2005). Pandiyan's cytokine deprivation study challenges the popular concept from transwell experiments that cell-cell contact is required for T_{reg} suppressive activities. It seems plausible that mechanisms of T_{reg} suppression require local cytokine delivery or uptake in a finely balanced microenvironment (Sojka *et al*., 2008). Lastly, T_{regs} can indirectly prevent T cell activation by their interactions with DCs (Belkaid and Rouse, 2005). T_{reg}-secreted cytokines can induce DCs to produce indoleamine 2, 3 dioxygenase (IDO) which prevents effector T cells from responding to antigen (Mellor and Munn, 2004).

1.3.6 T_{reg}-mediated control of effector T cell responses

A recent development in our understanding of T_{reg} biology is the recognition of the significant heterogeneity which exists within the $CD4^+Foxp3^+$ compartment (Feurer *et al*., 2009). The discovery of T_{reg} functional diversity, that is the ability of the T_{reg} to adapt its suppression programme to match the type of response being reined in, has opened up new ways of thinking about how T_{regs} might be manipulated for therapeutic engagement (Feurer *et al*., 2009; Koch *et al*., 2009). A subset of T_{regs}, specifically geared to regulating the activities of T_H1 cells (T_H1-type T_{regs}) has been demonstrated to be dependent on the transcription factor T-bet, which is fundamental to the differentiation and function of the 'targeted' T_H1 cells (Koch *et al*., 2009). A similar phenomenon has been shown to exist for

T_H2 cells, regulated by a distinct subset of T_{regs} which are dependent, like the T_H2 cells themselves, on the expression of IRF4, which interacts with Foxp3 (T_H2-type T_{regs}) (Zheng *et al*., 2007b, 2009). Conditional ablation of IRF4 in T_{regs} has been shown to provoke dysregulation of autoreactive T_H2 responses in an otherwise healthy animal, involving increased production of T_H2 cytokines, IL-4 dependent immunoglobulin isotype class switching and an increase in plasma cell proliferation (Zheng *et al*., 2009). This potential sharing of capabilities between regulatory and effector cells could be problematic if not carefully controlled, but there appear to be mechanisms in place for this. For T_H1-type T_{regs}, stimulation with IFN-γ and induction of STAT-1 is followed by ineffective upregulation of IL-12 receptor β2 such that differentiation to T_H1 effector cells does not result (Koch *et al*., 2012).

1.3.7 Regulatory: effector cell balance in preservation of tissue integrity

Infectious challenge to the host promotes a complex network of cellular and humoral responses. In the absence of regulatory mechanisms to contain 'overzealous' responses, collateral tissue damage can result, aiding pathogen dissemination, which is of further detriment to the host (Belkaid and Rouse, 2005). Several types of regulatory cells exist, some of which are induced in the periphery in response to infectious challenge (iT_{regs} or pT_{regs}) and some that are thymic in origin and considered natural regulators (nT_{regs}) (Bluestone and Abbas, 2003). Increased frequency and numbers of T_{regs} have been reported in animal models of sepsis and in septic patients (Monneret G *et al*., 2003; Wisnoski *et al*., 2007; Okeke *et al*., 2013). The question remains as to whether T_{regs} play a protective role by downregulating inflammation, or in fact contribute to immune dysfunction by suppressing the effector T cell response in any given setting?

A chief function of nT_{regs} is to respond to signals associated with tissue damage and then to minimise this damage by suppressing the inflammatory response (Powrie *et al*., 2003). There is good evidence of this nT_{reg}-mediated regulatory process being important in gastrointestinal homeostasis. In mouse models of colitis, adoptive transfer of naïve T cell populations lacking nT_{reg} cells into T cell-deficient mice caused massive gut inflammation. In contrast, transfer of $CD4^+CD25^+CD45RB^{lo}$ T cells together with those naïve T cells suppressed

disease development, an effect mediated by IL-10, TGF-β and CTLA-4 (Maloy *et al*., 2003). In T cell-deficient mice infected with *Helicobacter pylori*, transfer of $CD4^+CD25^-$ T cells promoted better bacterial clearance, but at the expense of increased gastric inflammation, resulting in an overall worsening of immunopathology (Raghavan *et al*., 2003). Similar nT_{reg} immunoregulatory activity has been shown to be important in the handling of infection by other organs such as the lung. Mice deficient in recombination-activating gene 2 (*rag2*) infected with *Pneumocystis jiroveci* had no detectable pathology, however, the transfer of $CD4^+CD25^-$ T cells triggered florid pneumonitis alongside a decrease in pathogen burden. This lethal pneumonitis was prevented by the transfer of nT_{regs} (Hori *et al*., 2002).

1.3.8 Regulatory: effector cell balance in host-pathogen interactions

A recognised consequence of nT_{reg} control of excessive immune responses is enhanced pathogen survival, in some cases chronic persistence (Belkaid *et al*., 2000). The advantage to the host is the avoidance of the cellular damage that may accompany pathogen eradication (Belkaid and Rouse, 2005). An example of this 'compromise' reached between host and pathogen is demonstrated in a mouse model of *Leishmania major* infection in which nT_{regs} facilitate pathogen survival and persistence and simultaneously benefit the host by promoting long term-immunity (Belkaid *et al*., 2000b). C57BL/6 mice infected with a low dose of parasites develop discrete lesions which are self-healing, and immunity to re-infection requires persistent infection (Belkaid et al., 2000b). nT_{regs} accumulate at sites of infection and limit the efficacy of effector T cell responses, thus promoting pathogen persistence and transmission. Depletion of nTregs results in pathogen eradication but at the expense of enhanced pathology and with a loss of immunity to re-infection. Thus, the equilibrium established between effector and regulatory T cells in sites of chronic infection might reflect both parasite and host survival strategies. (Belkaid *et al*., 2000, 200b; Belkaid and Rouse, 2005).

However, some studies have concluded that T_{regs} may be detrimental to the host-pathogen immune balance. For example, T_{reg}-deficient mice infected with herpes simplex virus (HSV) were found to mount a more effective antiviral response than T_{reg} replete mice, and did not suffer any collateral tissue damage of note (Suvas *et al*., 2003). Hiraki et al reported that T_{reg}

depletion, achieved with anti-IL-10 and/or anti-TGF-β, improved survival of septic mice post caecal ligation and puncture (Hiraki *et al*., 2012). Sawant et al explored the role of T_{regs} following low-dose infection with the intestinal helminth parasite Trichuris muris, which yields a chronic infection because of inefficient induction of T_H2 responses (Else *et al*., 1991). Early T_{reg} depletion post infection was associated with an enhanced T_H2 response and expedited worm clearance (Sawant *et al*., 2014). Interestingly this was not the case if T_{regs} were depleted later, once the infection was established. The investigators concluded that the impact of T_{reg} depletion is context and time dependent, and can be beneficial to the host in situations where T_H1 responses should be limited in favour of T_H2 responses (Sawant *et al*., 2014). These studies collectively suggest that continual accumulation of T_{regs} at foci of infection can, in some circumstances, negatively impact on the homeostasis of the infected organ and cause local immunosuppression which hinders pathogen clearance.

An alternative view is that for some infective processes T_{regs} are beneficial to bacterial clearance and/or to controlling collateral inflammatory damage during infection. One of the first demonstrations of this was when Heuer et al reported that, in a CLP model of polymicrobial sepsis, adoptive transfer of *ex vivo*-stimulated T_{regs} improved survival. This survival advantage was associated with greater peritoneal TNF-α levels at 24 h post-CLP, greater mast cell recruitment into the peritoneum and enhanced bacterial clearance in animals that received T_{regs} (Heuer *et al*., 2005). This could indicate that, under septic conditions, T_{regs} may have unique, and as yet undefined functional roles in the innate immune response. In support of these findings, Okeke et al reported an increase in T_{regs} following LPS-induced inflammation, and increased mortality post LPS challenge if T_{regs} were depleted prior to the insult (Okeke *et al*., 2013). Consistent with the protective role of T_{regs} they showed that CD25 KO mice, which do not have functional T_{regs}, are hypersensitive to LPS challenge. Adoptive transfer of T_{regs} rescued these mice from LPS-induced death. Perhaps most importantly they demonstrated a possible protective role during infection with live *E. coli*; depletion of T_{regs} resulted in acute death from an otherwise sub lethal dose of this pathogen (Okeke *et al*., 2013).

1.3.9 T_{reg} stability and plasticity

The significant heterogeneity within the regulatory T cell lineage so far discussed could be defined as a type of 'T_{reg} plasticity'. The core T_{reg} identity, revolving around stable Foxp3 expression and maintenance of suppressive capacity, is endured, but the pliable nature of the cells themselves allows them to adapt, phenotypically and functionally, to carry out an extensive range of immunoregulatory activities appropriate to a variety of pathological situations (Sakaguchi *et al*., 2013). Such heterogeneity/plasticity extends to the demonstrated existence of different T_{reg} sub-populations taking up residency in diverse anatomical locations, in part facilitated by the differential expression of chemokine receptors that support T_{reg} migration into these non-lymphoid sites (Burzyn *et al*., 2013; Cipolleta *et al*., 2012, 2015).

Different to the concept of plasticity is the issue of stability. Accumulating evidence suggests that T_{regs}, exposed to certain environmental cues, can become unstable, lose Foxp3 expression and their suppressive capabilities and take on features analogous to effector T cells (Yang *et al*., 2008b). Yang and colleagues reported that T_{reg} exposure to an inflammatory microenvironment, specifically cytokines IL-6 and IL-1, precipitated downregulation of Foxp3 and stimulated production of IL-17 (Yang *et al*., 2008b). It appears that this destabilising event facilitates a change in behaviour of the T_{reg}, designated "ex-T_{reg}" to secrete immune effector cytokines (IFN-γ, IL-4, and IL-17) and promote, as opposed to suppress, the inflammatory response (Yang *et al*., 2008b; Komatsu *et al*., 2009).

1.3.10 T_{reg} migration and tissue tropism

The distribution and function of T_{regs} within non-lymphoid tissues continues to be explored as there are several gaps in our understanding of this aspect of T_{reg} biology. Using GFP-expressing Foxp3.DTR mice (Kim *et al*., 2007) it has been demonstrated that T_{regs} are distributed throughout the body and can be isolated from a wide array of non-lymphoid tissues, in the steady state, in the absence of inflammation (Feuerer *et al*., 2009; Cipolleta *et al*., 2012, 2015; Mackay *et al.,* 2016). During an inflammatory response, such as that invoked by bacterial infection, T_{regs} undergo extensive expansion, during which their ability to migrate to non-lymphoid tissues is enhanced (Kadioglu *et al*., 2004; Sather et al., 2007;

Thorburn *et al*., 2010). Sather et al postulated that this may occur because of the increased migration of mature APCs to draining lymph nodes and subsequent enhanced presentation of cognate antigen and T cell costimulatory molecules to T_{regs}. T_{regs} then migrate from LN to non-lymphoid tissue where they limit effector T-cell responses, allowing for effective pathogen control, whilst protecting the organ from collateral tissue damage and immunopathology (Sather *et al*., 2007).

T_{regs} have been shown to express diverse patterns of homing receptors that facilitate this migration to non-lymphoid sites (Huehn et al., 2004; Siegmund et al., 2005). For example, CD103 has been suggested as a marker of "effector memory" T_{reg} cells that preferentially home to non-lymphoid sites including skin and lungs (Huehn et al., 2004). CC-chemokine receptor 4 (CCR4) is also established to be important; a large proportion of $Foxp3^+$ T_{regs} in non-lymphoid tissues are $CCR4^+$, indeed T_{regs} isolated from skin have a unique $CD103^{hi}$ $CCR4^+$ immune phenotype (Sather et al., 2007). CCR4 appears to contribute to the accumulation of T_{regs} in the airways and lung tissue. In accordance with this, the CCR4 ligand CCL17 (thymus and activation-regulated chemokine) is constitutively expressed by bronchial epithelial cells in the lung and its expression increases significantly in the inflamed lung (Sekiya et al., 2000). Mice, deficient in CCR4 expression within their T_{reg} compartment, exhibited impaired accumulation of T_{reg} cells within the airways and also in skin (Baekkevold et al., 2005). These mice developed severe inflammatory disease associated with $CD4^+$ $Foxp3^-$ T cell infiltration to lungs and skin (Sather et al., 2007). In a mouse model of allergic lung disease, the allergic phenotype of mice with CCR4 deficiency (CCR4KO mice) was similar to that observed in anti-CD25-treated mice rendered deficient in T_{regs}. Furthermore, the exacerbated allergic inflammation of CCR4KO mice correlated with impaired migration of T_{regs} to the airways, suggesting that CCR4 is critically involved in the trafficking of T_{regs} to the inflamed lung (Faustino *et al*., 2013).

Individual tissues have been demonstrated to impose unique features on tissue-resident lymphocyte populations that are relevant to those tissues (Bapat *et al*., 2015; Cipolleta *et al*., 2015). In a seminal study, Feuerer et al identified unique fat-residing Tregs (visceral adipose tissue (VAT) Tregs) in mouse and human adipose tissue, which in contrast to the 10-15% fraction of the peripheral lymphoid compartment, accounted for nearly 50% of the fat $CD4^+$ T cells (Feuerer *et al*., 2009) and expressed the transcription factor PPAR-γ

(Vasanthakumar *et al*., 2015; Cipolleta *et al*., 2015). PPAR-γ, acknowledged to be the 'master regulator' of adipocyte differentiation, orchestrates their distinct transcriptional profile which enables their accumulation in adipose tissue where they are important regulators of local and systemic inflammation and metabolism (Bapat *et al*., 2015; Cipolleta *et al*., 2012, 2015). Murine VAT T_{regs} have been shown to be of thymic origin with little to no conversion of naive T cells into visceral VAT T_{regs} (Kolodin *et al*., 2015). The TCR repertoire of VAT T_{regs} is different from conventional VAT T cells and has been shown to represent only a restricted subset of lymph node T_{regs} TCR repertoire (Feurer *et al*., 2009), suggestive of a VAT specific distinct TCR repertoire of T_{regs} (Sharma and Rudra, 2018). Adoptive transfer of congenically marked T_{regs} confirmed that VAT T_{regs} are not significantly derived from circulating T_{regs} (Kolodin *et al*., 2015).

T_{regs} are also enriched in intestinal lamina propria in large and small bowel; colonic T_{regs} are largely developed against microbial antigens (Kim *et al*., 2016). Germ-free mice, devoid of any microbiota, have been shown to have significantly fewer colonic T_{regs} than specific pathogen-free (SPF) mice (Atarshi *et al.,* 2011; Geuking *et al.,* 2011). Sequencing of colonic T_{reg} TCRs of genetically engineered mice with limited polyclonal repertoires revealed that colonic T_{reg} TCR differed to that of other tissues, adding weight to the hypothesis that they represent a distinct T_{reg} subset (Lathrop *et al*., 2011). Another separate subset of T_{regs} have been demonstrated to infiltrate injured and regenerating skeletal muscle. Gene expression profiling using microarray revealed that these muscle-resident T_{regs} have a transcriptome more similar to VAT T_{regs} than to T_{regs} from lymphoid sites. They also have a skewed TCR repertoire, which is distinct from that of splenic T_{regs}, and shows clear signs of clonal expansion (Burzyn *et al*., 2013). A distinct subset of T_{regs} expressing high levels of pro-inflammatory cytokine IL18 receptor (IL18R) has recently been described in the lung (Arpaia *et al*., 2015). Arpaia and colleagues showed that IL18R+ T_{regs} can proliferate early in the course of lung infection or injury and enhance tissue repair by producing the tissue repair protein amphiregulin, in an "innate" manner, independent of TCR engagement (Arpaia *et al*., 2015). Mice rendered deficient in T_{reg} specific amphiregulin exhibited a rapid decline in lung function following intranasal influenza virus infection, though antiviral immune response was unaffected (Arpaia *et al*., 2015).

An important void in the existing knowledge of tissue resident T_{regs} is identification of their natural ligands or antigens. Establishment of the specific cognate ligands that support survival and proliferation of these T_{reg} subsets may facilitate induction of therapeutic tissue-specific modulations (Sharma and Rudra, 2018). A recent study has demonstrated that T_{regs} with defined antigen specificity (chimeric antigen receptor- (CAR-) Tregs), have potent localised immunosuppressive functions, but do not induce generalised immunosuppression (MacDonald et *al*., 2016).

1.3.11 Tissue-resident memory T (T_{RM}) cells

T_{RM} cells express conventional, diverse, MHC-restricted αβTCRs but differ from recirculating memory T cells in blood and secondary lymphoid organs in a number of ways (Clark 2015, Stary *et al*., 2015). Firstly, they reside in mucosal tissues and concentrate in their highest numbers at sites of infection or barrier breach (Jiang *et al*., 2012). Secondly, they are acutely sensitive to their tissue microenvironment, readily responding to locally produced stimuli to adapt their phenotype. For example, epidermal T_{RM} cells have been demonstrated to adopt a dendritic morphology akin to that of Langerhans cells and to patrol the epidermis, providing a surveillance function for the host parenchyma (Zaid *et al*., 2014). Thirdly, on meeting their cognate antigen, T_{RM} cells secrete soluble factors which serve to generate a sense of alert within their tissue of residence, resulting in the activation and recruitment of other immune cells to provide a robust response to infection or injury, one that extends far beyond that specific to the cognate antigen (Schenkel *et al*., 2013, 2014; Ariotti *et al*., 2014). Tissue-resident memory $CD8^+$ T-cells in skin expressed high levels of HOBIT and BLIMP 1 for up to 30 days post herpes simplex virus (HSV) infection. In mixed-bone-marrow chimeric mice infected with HSV both BLIMP1 and HOBIT had to be present for the development of T_{RM} cells but not of circulating memory T cells, suggesting that BLIMP1 and HOBIT cooperate to specifically promote T_{RM} cell development. (Mackay *et al*., 2015).

1.3.12 B Cells

B lymphocytes have traditionally been associated with humoral immunity, however it is now known that they also play an important role in cellular immunity (Hoffman *et al*., 2016). B cells can i) function as APCs which enhance T cell-mediated immune responses, ii) serve as cellular effectors that produce inflammatory cytokines (IFN-γ, TNF-α, IL-17) and iii) exhibit regulatory activities through the secretion of IL-10 (regulatory subset termed BREGS) (Bouaziz *et al*., 2008; Wojciechowski *et al*., 2009; Lund and Randall, 2010; Chalasani and Rothstein, 2014). Three subclasses of B cells are recognised in both mice and humans: B1 B lymphocytes are generated in fetal liver and develop as a self-renewing population with minimal input from the BM; B2 B lymphocytes are BM-derived, developing from transitional 2 (T2) B cells and differentiating into marginal zone (MZ) or follicular (FO) lineages in the spleen (Montecino-Rodriguez and Dorshkind, 2012; Cerutti *et al*, 2013).

B cell development involves the rearrangement of immunoglobulin heavy- and light-chain gene segments (variable V, diversity D, joining J) to achieve cell surface expression of an IgM B cell receptor (BCR) which can bind antigens (Thomas *et al*., 2006). Subsequent B cell maturation is influenced by the strength of signal encountered; strong BCR signals induce Bruton tyrosine kinase (BTK) and support maturation to FO B cells, while weaker BCR signals allow expression of neurogenic locus notch homolog protein 2 (NOTCH2) giving rise to MZ B cells (Pillai and Cariappa, 2009; Cerutti *et al*., 2013). MZ B cells are retained in the spleen whilst FO B cells migrate to secondary lymphoid tissues (Thomas *et al*., 2006; Pillai and Cariappa, 2009; Cerutti *et al*., 2013).

The BCRs of individual maturing B cells exhibit huge variation as a consequence of the process of random rearrangement of immunoglobulin genes during early development, which facilitates B cell recognition of a vast array of antigens (Thomas *et al*., 2006). This includes recognition of self, which must be regulated if autoimmunity is to be avoided. Immature B cells with strong reactivity to self-antigen undergo clonal deletion, or receptor editing, which involves further rearrangement of their immunoglobulin gene segments. This so called central tolerance curtails self-reactivity and allows entry to the transitional B-cell pool (Wardemann *et al*., 2003). In the spleen, additional selection mechanisms (clonal deletion and anergy) ensure that transitional B cells capable of recognising peripheral self-antigens are eliminated, so called peripheral tolerance (Cambier *et al*., 2007). Transitional B

cells depend on B cell–activating factor (BAFF) for survival and differentiate into mature B cells in the spleen. Elevated levels of BAFF and/or T cell help of anergic B cells can rescue autoreactive B cells and allow them to persist (Meffre and Wardemann, 2008). A further checkpoint exists however, involving $CD4^+$ T cell-mediated elimination, via Fas-receptor-Fas ligand and CD40-CD40L signaling and Treg/Breg interactions (Rathmell *et al*., 1996; Herve *et al*., 2007).

Mature B cells that are activated by foreign antigen and enter germinal center reactions give rise to isotype-switched memory B cells and plasma cells. This requires the integration of a number of influences: nature of antigen, TLR recognition and cytokine/co-stimulation signals (Nutt *et al.,* 2015). B1 and MZ B cells react to glycolipid or polysaccharide antigens, such as pneumococcal capsular polysaccharides, and generate predominantly low affinity IgM or isotype-switched IgG antibodies independent of T cell help. This facilitates clearance of blood-borne microorganisms early in the infection process (1-3 days), prior to the emergence of FO B cell-derived IgG antibodies (7 days) (Cerutti *et al*., 2013). FO B cells react to protein antigens and utilise helper signals from antigen-specific CD4 T cells (Cerutti *et al*., 2012, 2013). T cell help involves the production of cytokines (IFNγ, IL-4, IL-21) and provision of costimulatory ligands (CD40L) that stimulate B cell proliferation and differentiation (Cerutti *et al*., 2012). MZ B cells have been demonstrated to participate in responses to T-dependent protein antigens in addition to their T-independent activities (Arnon *et al*., 2013).

Antibodies (immunoglobulins) produced by B cells are glycosylated protein molecules which reside on the B cell surface and function as antigen receptors (BCR), or are actively secreted to bind with and neutralise their target antigen in the extracellular space. An antibody is made up of two ‘heavy’ and two ‘light’ chains joined by disulfide bonds with an N-terminus region providing the site for antigen binding; it is this region which varies between molecules and thus dictates antibody specificity (Murphy, 2012). There are 5 recognised isotypes, distinguished by the non-variable C-terminus regions of the heavy chains (Fc): IgM, IgD, IgG, IgA and IgE. IgG subclass 1-4 have been identified (Schur, 1988). Antibody effector function involves direct target neutralization, immune effector cell activation via Fc receptor (FcR) binding and activation of the classic pathway of the complement system via binding to C1q. Variability in binding affinity for activating or inhibitory FcR on immune cells and the

nature of the heavy chain isotype determines the dominant effector mechanism for a given antibody, for example IgM is an efficient activator of compliment, whereas IgE activates mast cells (Murphy, 2012).

1.4 *Streptococcus pneumoniae*

1.4.1 Clinical spectrum of disease caused by S. pneumoniae

S. pneumoniae is an important human pathogen capable of causing significant morbidity and mortality (Spellerberg and Brandt, 2011). The wide variety of pathologies to which it contributes includes sinusitis, otitis media, osteomyelitis, septic arthritis, endocarditis, meningitis, sepsis and pneumonia (Kadioglu *et al*., 2002; Weiser *et al.,* 2018). In developed countries, *S. pneumoniae* has been credited as the main cause of community acquired pneumonia (CAP) and meningitis in children and the elderly (Van de Beek *et al*., 2006) and has been reported as being responsible for 30% of all adult pneumonia cases, carrying a mortality rate of 10% to 40% (Bridy-Pappas *et al*., 2005). In undeveloped countries, pneumococcal infections are responsible for deaths approaching one million per annum in infant populations under the age of five years (O'Brien *et al*., 2009). *S. pneumoniae* is known to cause secondary bacterial infection in patients with influenza and has been isolated from biological fluids of in-patients being treated for this illness (McCullers *et al*., 2010), as well as patients hospitalised with infective exacerbations of chronic obstructive pulmonary disease (COPD) (Domenech *et al*., 2013).

The bacterium readily colonises the upper respiratory tract and is asymptomatically carried by healthy individuals; carriage rate varies between 5 and 70%, depending on age of acquisition, the microenvironment of the host respiratory tract and the existence of other respiratory pathogens (Obaro *et al*., 1996; Wu *et al*., 1997). Colonisation is the prequel to infection and also serves as a reservoir, facilitating transmission to other susceptible individuals (Kadioglu *et al*., 2002). The first step in colonisation of respiratory surfaces is bacterial adherence, followed by cytokine-induced cell activation and subsequent invasion into host cells (Hakansson *et al*., 1996). Pneumococcal factors identified as being important for the process of colonisation include cell-wall structural proteins, permeases and pneumolysin (Rubins *et al*., 1998; Kadioglu *et al*., 2008). The exact mechanisms underlying

the observed variability in the tendency of *S. pneumoniae* to transition from nasopharyngeal colonisation to disease-causing pathogen are not well defined.

1.4.2 *Biological characteristics of S. pneumoniae and associated virulence factors*

S. pneumoniae cells are Gram-positive, lancet-shaped cocci, usually seen in pairs (diplococci), but they may also occur singly and in short chains. The organism is a fastidious bacterium, grown optimally in 5% carbon dioxide (Ryan and Ray, 2004). It requires a source of catalase, such as blood, to neutralize the large amount of hydrogen peroxide produced during growth (Percione *et al*., 2000). When cultured on blood agar *S. pneumoniae* is alpha haemolytic (Ryan and Ray, 2004). This is one of the criteria which has been used for identification and distinction of pneumococci from other streptococci. Minimum criteria include Gram-positive staining, haemolytic activity and bile or optochin sensitivity, however optochin-resistant and bile-insoluble pneumococcal isolates and optochin-susceptible non-pneumococcal alpha-hemolytic streptococci have been reported (Kellogg *et al*., 2001). To overcome these problems, pneumococcus-specific PCRs targeting genes encoding virulence factors like pneumolysin (*plyA*) and autolysin (*lytA*) have been utilised (El Aila *et al*., 2010).

S. pneumoniae has a capsule composed of polysaccharide which completely envelopes the pneumococcal cells. 97 different capsule types have been identified and form the basis of antigenic serotyping of the organism (Daniels *et al*., 2016). Serotyping of pneumococci can be determined by the quellung (Neufeld's) reaction; specific antibodies react with capsular antigens on the surface of the organism being tested, causing capsular swelling (Obaro *et al*., 1996). The capsule is identified as a virulence factor by virtue of its antiphagocytic activity. It interferes with phagocytosis by preventing complement C3b opsonisation of the bacterial cells (Bridy-Pappas *et al*., 2005).

Beneath the capsule, the cell wall of *S. pneumoniae* is composed of peptidoglycan, teichoic acid and N-acetylmuramic acid. Teichoic acid contains phosphorylcholine, which has been recognised to play a central role in the biology of the pneumococcus by its specific adherence to choline-binding receptors, present on virtually all human cells (Severin *et al*., 1997). Other respiratory pathogens such as Haemophilus and Pseudomonas also have phosphorylcholine on lipopolysaccharide, proteins or pili, suggesting a shared mechanism

for the invasion of the respiratory tract (Tuomanen, 1999). Pneumococcus also expresses choline-binding proteins (CBPs) on its cell surface which are non-covalently bonded to the choline moiety of the cell wall. The CBP family contains important determinants of virulence such as PspA (protective antigen), CbpA (an adhesin) and Lyt A, B and C (autolysins) (Canvin *et al*., 1995). The process of autolysis is fundamental to pneumococcal biology and plays a critical role in the release of pneumolysin from the bacterial cytoplasm. Pneumolysin is a 53Kd protein produced by virtually all clinical isolates of the pneumococcus (Kalin *et al*., 1987). When present at high concentrations it functions as a pore-forming toxin by interactions with host cell membrane cholesterol (Boulnois *et al*., 1991). At lower, sublytic concentrations it has a range of effects: induction of apoptosis, complement activation and promotion of host immune cell pro-inflammatory responses (Mitchell *et al*., 1991; Cockeran *et al*., 2001; Kadioglu *et al*., 2004, 2008).

1.4.3 Host immune response to S. pneumoniae lung infection

In the initial stages of lung infection with *S. pneumoniae* the first cells likely to contest invasion are the resident alveolar macrophages (AM). AM release IL-8 (or mouse equivalent KC) and TNF-α, which attract neutrophils to the infected area. Neutrophils phagocytose pneumococci through complement C3 deposition and its associated receptors (opsonophagocytosis), and themselves provide an additional source of TNF-α which further enhances neutrophil recruitment (Garvy and Harmsen, 1996). In the latter stages of lung infection the pneumococci are lysed by activation of Lyt-A, or by neutrophils, causing a release of factors (including pneumolysin) into the tissue. These factors have a range of cytotoxic and inhibitory effects on host tissue and immune cells and increase the probability of pneumococcal survival and growth *in vivo* (Kadioglu *et al*., 2000).

Accumulating evidence in both preclinical and clinical settings indicates a role for innate-like T cells, particularly iNKT cells and MAIT cells, in host defense against lung pathogens (Nakamatsu *et al*., 2007; Le Bourhis et al., 2010; Meierovics et al., 2013; Barthelemy *et al*., 2017; Kurioka *et al*., 2018). iNKT KO (*Jα18*−/−) mice have been shown to be more susceptible to infection with *S. pneumoniae* serotype 1 or 3 strains than WT controls (Nakamatsu *et al*., 2007; Barthelemy *et al*., 2017). iNKT cell protective effects appear to be

driven by the production of IFN-γ and IL-17A which enhance neutrophil recruitment and control bacterial elimination (Nakamatsu *et al*., 2007; Ivanov *et al*., 2012). The initial step of iNKT cell activation depends on activating cytokines, pneumococcal-derived Ag, or both, according to the strain studied (Kinjo *et al*., 2011; Ivanov *et al*., 2012; Barthelemy *et al*., 2017). Common to several *S. pneumoniae* strains is cell wall glycolipid (α-glucosyldiacylglycerol) which can serve as a CD1d- restricted iNKT cell Ag and has been demonstrated to be important *in vivo* for host response to *S. pneumoniae* (Kinjo *et al*., 2011; Ivanov *et al*., 2012). Other studies have shown that $CD103^+$ DC-generated IL-12 is also important for iNKT cell activation (Ivanov *et al*., 2012; Barthelemy *et al*., 2017). iNKT cells have been demonstrated to support B cells in mounting antibody responses (Leadbetter *et al*., 2008) including in the production of specific anti-pneumococcal antibodies and class switching in response to pneumococcal vaccines in experimental models (Miyasaka *et al*., 2013).

Interest in MAIT cell participation in host defense against *S. pneumoniae* has grown since genomic and transcriptomic analyses have revealed that multiple strains of pneumococcus produce enzymes involved in the synthesis of riboflavin metabolites (Le Bourhis et al., 2010, 2013) as well as a highly conserved riboflavin operon (Kurioka *et al*; 2018), recognizable to MAIT cells via MR1-restricted presentation. Recently, Hartmann and colleagues demonstrated that variation in riboflavin metabolism between different clinical isolates of *S. pneumoniae* resulted in differential activation of MAIT cells. The investigators proposed that this depended on genetic variations in isolate expression of the riboflavin pathway, suggesting a role for MR1-dependent Ags in this model (Hartmann *et al*., 2018). Conversely, Kurioka and colleagues showed that MAIT cell activation, in response to pneumococcus-infected THP-1 cells, was unchanged upon MR1 blockade but was fully abrogated following IL-12 and IL-18 neutralization. They concluded that certain cytokines may be more crucial for MAIT cell activation than MR1-dependent Ags (Kurioka *et al*; 2018).

In line with the paucity in clinical data on the role of iNKT cells in patients with severe pneumococcal pneumonia, understanding of phenotype and dynamics of human MAIT cell behaviour in this illness is limited. It has been shown that the levels of circulating MAIT cells in patients admitted to intensive care with severe bacterial infections were markedly decreased compared to age-matched healthy controls (Grimaldi *et al*., 2014). However, the

reduction in MAIT cell frequency was less pronounced in cases of streptococcal infections, including pneumonia, than in non-streptococcal infections, which may indicate a more minor role for MAIT cells during *S. pneumoniae*-induced pneumonia in humans than first thought (Grimaldi *et al*., 2014).

Humoral immune response is also fundamental to host defense against *S. pneumoniae*. Patients with genetic or acquired defects in immunoglobulin production are more susceptible to infection with this pathogen and tend to develop more severe sepsis syndromes than immunocompetent individuals (Oksenhendler *et al*., 2008). Murine studies looking specifically at lung immune responses, by utilising a non-invasive *S. pneumoniae* serotype (19F strain) which causes lung infection only (without sepsis), showed that a combination of humoral immunity and Th17 $CD4^+$ cell-mediated immunity is required for protection (Wilson *et al*., 2015). Development of adaptive immunity, including antibody production, appears to follow on from nasopharyngeal colonisation in infancy (Simmel *et al*., 2002) and continue into adulthood (Goldblatt *et al*., 2005). Data from longitudinal studies of serum antibody responses in toddlers (aged less than 2 years) demonstrated the development of anti-pneumococcal antibodies to both capsular and protein antigens post colonisation events (Turner *et al*., 2013). This has also been shown to be the case in mice (Richards *et al*., 2010; Wilson *et al*., 2015). Observation that colonisation induces both anti-capsule and anti-protein antibodies is noteworthy as, historically, the capsular polysaccharide was considered the most important virulence factor and ideal vaccine target (Shinefield and Black, 2000).

Antibodies against serotype-specific polysaccharides are found as subtypes IgM, IgA, and IgG. IgG antibodies may be from subclasses 1-4, however IgG2 is reported to be the most efficient subclass for stimulating phagocytosis of pneumococcal antigens (Lortan *et al*., 1993). In circulation, IgM and IgG antibodies play a fundamental role in pathogen elimination, while on mucosal surfaces IgA antibodies are protective against *S. Pneumoniae* colonisation and invasion (Zhang *et al*., 2010). In addition to the response to polysaccharide antigens, a robust response to S*. pneumoniae* protein antigens results in transudation of serum antibodies to mucosal sites such as the nasopharynx and middle ear, which constitutes a major defense component (Xu et al., 2015). Further augmentation of the immune response involves active upper respiratory mucosal tissues such as tonsils and

adenoids, which contribute to local antibody production in the nasopharynx; specific *S. pneumoniae* protein antigens have been demonstrated to elicit robust mucosal responses in an infection or ex-vivo stimulation setting (Zhang *et al*., 2010). The incidence of *S. pneumoniae* with age exhibits a U-shaped curve, with highest frequency in the very young and the elderly (Melegaro *et al*., 2006). This pattern supports the concept of adaptive immune responses maturing with age, but ultimately waning due to immunosenescence, a phenomenon demonstrated in mice and humans (Boyd and Orihuela, 2011; Puchta *et al*., 2016). A reduction in the number and activity of IgM memory B cells has been demonstrated to be contributory to the impairment of immunity to *S. pneumoniae* seen with advancing age (Shi *et al*., 2005).

1.4.4 Strategies for treatment of S. pneumoniae infection

The mainstay of treatment of *S. pneumoniae* infection is prompt initiation of empirical antibiotic therapy, whilst the organism is cultured and antibiotic sensitivities determined. Over the last 30 years there has been a rapid emergence of antibiotic resistance to *S. pneumoniae* (Ramirez and Anzueto, 2011; Kim *et al*., 2016), which can be attributed, in part, to the organism's natural transformation system which serves as a mechanism for genetic exchange (Griffith, 1928). In the pneumococcus, natural transformation occurs during a genetically programmed differentiated state termed competence (Tomasz and Hotchkiss, 1964). A competent state can be reached in exponentially growing cells at a critical cell density (Tomasz and Hotchkiss, 1964). During this competent state, pneumococci secrete Competence-Stimulating-Peptide (CSP), a peptide pheromone which distributes competence throughout the pneumococcal population (Havarstein *et al*., 1995; Martin *et al*., 2006). Competence promotes genome plasticity and adaptability (Popa and Dagan, 2011). An example of this is the important issue of penicillin resistance due to altered penicillin binding proteins (PBPs) that exhibit a low affinity for β-lactam antibiotics. Comparison of the nucleotide sequences encoding PBPs in *S. pneumoniae* and *S. mitis* revealed that horizontal gene transfer had occurred between these two bacteria (Dowson *et al*., 1994). Of interest, specifically studied in *S. pneumoniae*, some antibiotics induce competence, which may serve to enhance bacterial resistance to eradication (Prudhomme *et al*., 2006).

S. pneumoniae can also develop antibiotic resistance by mutation and selection. The bacterium's relatively fast rate of growth and the cell density achieved in an infectious setting facilitates both the occurrence of natural transformation and the emergence of spontaneous mutants resistant to the antibiotic being used (Brown, 2002; Johnsborg *et al*., 2007). However, the rate at which *S. pneumoniae* acquires genetic variation through recombination is much higher than the rate at which the organism transforms through spontaneous mutations (Chaguza *et al*., 2015).

1.5 *Pseudomonas aeruginosa*

1.5.1 Clinical spectrum of disease caused by P. aeruginosa

Pseudomonas aeruginosa is a Gram negative bacteria which is an environmental opportunist, readily isolated from fresh water and soil. It is rarely problematic in immunocompetent healthy individuals, but it is an important pathogen in patients with underlying illness or impaired immunity, and colonization is frequently selected by broad-spectrum antimicrobial usage in the hospital setting (Richards et al., 1999; Hoeffken and Niederman, 2002). Newly acquired *P. aeruginosa* infection or colonization is particularly prevalent in patients who have experienced a breach in cutaneous or mucosal barriers, such as occurs with mechanical ventilation and tracheostomy placement (Gupta *et al*., 2017), operative procedures (Ranjan *et al*., 2010) and severe burns (Erol *et al*., 2004; Park *et al*., 2017). As a cause of ventilator-associated pneumonia (VAP), *P. aeruginosa* has a high mortality compared with other pathogens (Chastre and Fagon, 2002).

Impairment of local immune defenses carries a high risk for colonisation by this organism and predisposes individuals to more acute forms of infection such as tracheobronchitis and pneumonia; mortality rate is reportedly increased by 40% among immunocompromised patients with *P. aeruginosa* pneumonia (Afessa and Green, 2000). *P. aeruginosa* is also a recognised cause of CAP and is especially prevalent in residents of nursing homes, patients with underlying lung disease and patients recently discharged from hospital (Ruiz *et al*., 1999). Chronic infection with *P. aeruginosa* can be particularly problematic in patients with cystic fibrosis (CF), a multi-system disorder characterized by mutations in the cystic fibrosis transmembrane (CFTR) gene, which almost invariably affects the lungs. Acquisition of *P.*

aeruginosa by CF patients is associated with a deterioration in lung function and consequent increased morbidity and mortality (Hauser *et al.,* 2011).

1.5.2 Biological characteristics of P. aeruginosa and associated virulence factors

P. aeruginosa is a ubiquitous and highly versatile organism, able to survive in diverse environmental settings owing to its minimal nutritional requirements and ability to tolerate a variety of physical conditions (Frimmersdorf *et al*., 2010). It is generally believed to be an extracellular pathogen, however its gene products are readily internalised by host cells and stimulate a wide range of innate immune receptors (Wei and Ma, 2013). The bacterium possesses a variety of factors which facilitate the process of infection: Type IV pili and flagella, required for motility and initiation of biofilm formation (Murray *et al*., 2010), TLR agonists: lipopolysaccharide and lipoproteins and three different secretion systems responsible for the release of proteases, lipases and toxins that act as virulence factors (Bleaves *et al*., 2010). Bacterial production and translocation of Type 3 secretion system (T3SS) effector molecules (Yahr *et al*., 1996) has been linked to increased severity of illness and poor survival outcomes in human patients infected acutely with *P. aeruginosa* and mouse models of *P. aeruginosa* pneumonia (Hauser *et al*., 2002).

An example of a molecule, secreted by *P. aeruginosa*, which is directly toxic to immune cells is pyocyanin. This redox-active phenazine has been demonstrated to trigger neutrophil apoptosis *in vitro* (Prince *et al*., 2008) and *in vivo* (Allen *et al*., 2005). It follows that bacteria unable to produce pyocyanin are more easily cleared from the infected lungs of mice than wild-type organisms (Allen *et al*., 2005). It is worthy of mention that clinical and laboratory strains of *P. aeruginosa* exhibit significant variability in their expression of surface structures and appendages such as flagella, exoproteins and exotoxins, which should be taken into consideration when interpreting experimental outcomes (Murray *et al*., 2010).

1.5.3 Host immune response to P. aeruginosa lung infection

P. aeruginosa is recognised by LPS-mediated stimulation of TLR4, flagellin-mediated triggering of TLR5 and MyD88-mediated induction of an IL-1 dependent pathway (Mijares *et*

al., 2011). Secretion of the pro-inflammatory cytokine interleukin -1β (IL-1β) is a critical early host response to *P. aeruginosa* lung infection, in part because subsequent IL-1R-dependent signaling promotes neutrophil recruitment to the site of infection (Wangdi *et al*., 2010; Mijares *et al*., 2011). Clearance of *P. aeruginosa* is significantly decreased in the absence of IL-1R signaling (Wangdi *et al*., 2010). Of note, in humans, mutations in the adaptor protein MyD88 and the downstream kinase IRAK-4 have been linked to increased susceptibility to a small selection of lung pathogens which includes *S. pneumoniae* and *P. aeruginosa* (Picard *et al*., 2010).

Neutrophils play a pivotal role in *P. aeruginosa* clearance, as clearly evidenced in murine studies by the susceptibility of neutropenic animals to this pathogen (Koh *et al*., 2009) and clinical studies involving patients rendered neutropenic by chemotherapy (Kim *et al*., 2014). Recruited neutrophils must be capable of killing the organism if they are to be of benefit to the host, this can be influenced by both host and bacterial factors that directly affect neutrophil phagocytosis and survival (Diaz *et al*., 2008; Morris *et al*., 2009). Neutrophil serine proteases are important for *P. aeruginosa* elimination (Hirche *et al*., 2008). In a murine model of acute *P. aeruginosa* pneumonia, mice deficient in neutrophil elastase exhibited increased susceptibility to the organism, associated with impaired bacterial clearance, compared to wild-type controls (Hirche *et al*., 2008). However, neutrophil elastase and other serine proteases (cathepsin G and proteinase 3) attack host cells as well as targeting the bacteria themselves, which can exacerbate the inflammatory response and directly contribute to collateral tissue damage (Lavoi *et al*., 2011). Given the adaptability of the organism, it is not surprising that *P. aeruginosa* has evolved mechanisms for evading neutrophil-mediated clearance. An example of this is biofilm formation which protects the bacterial population from neutrophil surveillance and uptake by enveloping it in extracellular proteins, alginate, and DNA (Mai *et al*., 1993).

Another key contributor to neutrophil recruitment and bacterial clearance is the alveolar macrophage (AM), likely to be the first immune effector cell to encounter *P. aeruginosa* in the lung. Not only do AMs have the capability of engulfing and killing the pathogen (Descamps *et al*., 2012), they also produce neutrophil recruiting chemokines (IL-8 or mouse homologue KC) and IL-1β, sensed by airway epithelial cells, which in turn secrete further neutrophil chemokines (Mijares *et al*., 2011). Impairment of neutrophil recruitment and

bacterial clearance was previously reported in a rodent model of *P. aeruginosa* acute lung infection in which alveolar macrophages had been depleted, suggesting the importance of their contribution to bacterial clearance (Hashimoto *et al*., 1996). However, in a more recent study, depletion of AMs improved bacterial clearance and survival in a murine model of *P. aeruginosa* pneumonia (Cohen and Prince, 2013). The investigators concluded that inflammasome signaling, induced by *P. aeruginosa* flagella, stimulated AMs to secrete IL-1β and IL-18 which did not contribute to bacterial clearance, but rather caused excessive lung pathology and increased mortality. Furthermore, IL-18 was observed to suppress IL-17-dependent antimicrobial peptide production from innate lymphoid cells such as γδ T cells, which may serve to perpetuate bacterial survival (Cohen and Prince, 2013).

It is well established that IL-17 production and associated neutrophil recruitment is crucial to the clearance of extracellular bacterial pathogens from the lungs (Jones and Chan, 2002; Liu *et al*., 2011, 2013; Xu *et al*., 2014). Indeed, the design of anti-*P. aeruginosa* vaccines has included gene products that specifically upregulate T_H17 cell signaling (Wu *et al*., 2012). There is accumulating evidence that the timing of IL-17 production is important. Upregulation of IL-17 early in the course of *P. aeruginosa* lung infection has been shown to enhance bacterial clearance (Xu *et al*., 2014). In contrast, pre-treatment with IL-17 neutralising antibody was associated with low levels of neutrophil recruitment and impaired bacterial clearance (Liu *et al*., 2011).

1.5.4 Strategies for treatment of P. aeruginosa lung infection

Treatment of community-acquired and nosocomial *P. aeruginosa* infections can present a significant challenge; selection of the appropriate antibiotic at the outset is key to minimising morbidity and mortality (Micek *et al*., 2005). However, antibiotic selection is often complicated by the ability of *P. aeruginosa* to develop resistance to multiple classes of antimicrobials, sometimes during treatment, a problem which has been shown to impact significantly on hospital stay and the cost of patient care (Dimatatac *et al*., 2003). The capacity of *P. aeruginosa* for multi-drug resistance is based on its intrinsically advanced antibiotic resistance mechanisms: multidrug efflux pumps and the low permeability of the bacterial cellular envelopes (Poole, 2004), in combination with its acquisition of resistance

genes via plasmids or through mutational processes that alter the expression and/or function of chromosomally encoded mechanisms (Lister *et al*., 2009). This not only makes it difficult to treat individuals infected with this organism, it impairs the process of pathogen eradication from medical equipment and surfaces in hospitals, which allows for cross contamination between medical equipment, health care professionals and patients (Russotto *et al*., 2015).

1.5.5 Potential role for cellular therapy in the treatment of bacterial lung infection

The lung is a complex organ with a limited capacity for regeneration and repair. It is readily damaged by the process of bacterial infection and associated inflammation yet its repair mechanisms are not well understood. In other organs such as skin, local stem cells with the capability of limitless self-renewal have been observed to contribute to tissue repair and regeneration (Blanpain and Fuchs, 2006). There is evidence to suggest this may also be the case in the lung and respiratory tract (Hong *et al*., 2004; MacPherson *et al*., 2005).

However, whatever the mechanism of endogenous repair in the lung, it is not sufficient to ameliorate chronic, progressive lung diseases such as bronchiectasis and pulmonary fibrosis or the acute lung injury which can occur as a consequence of severe pneumonia. There exists a growing body of research looking at the employment of extrinsic stem or 'stromal' cells, particularly those derived from adult bone marrow, to assist in lung repair and improve the response of lung tissue to injury. The next part of this introductory chapter will explore what is currently understood about this exciting cellular therapy.

1.6 Mesenchymal stem (stromal) cells (MSC)

1.6.1 From bench to bedside

The initial concept of what we now refer to as mesenchymal stem (or stromal) cells (MSCs) dates back to the 1800s when it was first demonstrated that transplantation of bone marrow (BM) to a range of anatomical niches could result in de novo generation of ectopic bone and marrow (Goujon, 1869). Tavassoli and Crosby went on to establish clear evidence

of the osteogenic potential of BM, but the question remained as to which BM cells were specifically involved in osteogenesis (Tavassoli and Crosby, 1968). Through a series of seminal studies in the 1960s and 1970s, Friedenstein (generally credited with the discovery of MSC) and his co-workers demonstrated that the osteogenic potential of bone marrow was associated with a minor subpopulation of BM cells (Friedenstein *et al*., 1968). These cells exhibited a fibro-blast like appearance in culture and were rapidly adherent to tissue culture flasks, which enabled them to be readily distinguished from the majority of hematopoietic cells (HSCs).

Friedenstein expanded his work based on the critical observation that BM in postnatal life is a reservoir of stem cells for mesenchymal tissues and further developed the field with the demonstration that seeding BM cell suspensions at clonal density resulted in the founding of discrete colonies initiated by single cells (the colony-forming unit fibroblastic, CFU-Fs) (Friedenstein *et al*., 1970). *In vivo* transplantation of these cells revealed that multiple skeletal tissues (cartilage, adipose tissue, and fibrous tissue) in addition to bone could be produced by the progeny of a single BM stromal cell, which Friedenstein and Owen called an osteogenic stem cell (Friedenstein *et al*., 1987) or a BM stromal stem cell (Owen and Friedenstein, 1988). The term “mesenchymal stem cell” was first coined by Caplan in the early 1990s (Caplan, 1991). This terminology remains the subject of debate within the field as it is argued by some that the nomenclature “mesenchymal stromal cells” better describes the characteristics of these cells that may be independent of their stem cell properties (Horwitz *et al*., 2005; Dominici *et al* 2006). In the last few years it has been shown that MSCs can be derived from both pericytes and adventitial progenitor cells from nearly all tissues (James *et al*., 2012; Crisan *et al*., 2012). Thus, it may be appropriate to refer to MSCs as “multipotent perivascular-derived cells” (Ankrum *et al*., 2014).

The realisation that MSCs with multi-lineage capabilities could be grown and expanded easily following isolation from human BM led to a flurry of interest in this subfield of cell therapy. Initially, research concentrated on their role as multipotent stem cells, which could be manipulated to differentiate into the different components of the haematopoetic niche when exposed to appropriate culture conditions (Pittenger *et al*., 1999). The scope of MSC potential was expanded further with the discovery that long-term cultures of cells with

mesenchymal stem cell characteristics could be generated from a range of organs and tissues from adult mice (brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus, pancreas), and also from blood vessels, large (aorta and vena cava) and small (venules within kidney glomeruli). In these studies, MSCs were not detected in peripheral blood (da Silva Meirelles *et al*., 2006; Beltrami *et al*., 2007). These populations had similar morphology, immunophenotype and growth properties to bone marrow-derived MSCs. Interestingly, their differentiation potential was related to the tissue of origin. It was concluded that the distribution of MSCs throughout the post-natal organism was related to their existence in a perivascular niche (da Silva Meirelles *et al*., 2006). MSC have been sourced from a variety of anatomic niches: adipose tissue (Fraser *et al*., 2006), heart (Beltrami *et al*., 2003), lung (Griffiths *et al*., 2005), skeletal muscle, synovial membrane and dental pulp (Chen et al., 2008; Sancricca *et al*., 2010) fetal tissues such as amniotic fluid, placenta and umbilical cord blood, (Wolbank *et al*., 2010; Legaki *et al*., 2106) the cervix (Eiro *et al*., 2014) and menstrual blood (Rossignoli *et al*., 2013).

In the last two decades studies have focused much more on the interactions of MSC with cells of the immune system and their release of paracrine mediators which impact on immune response and tissue repair. There is now a wealth of evidence showing that BM-MSCs have a variety of immunomodulatory properties (discussed in more detail in section 1.7). The current consensus of opinion is that it is their ability to exert broad immunomodulatory effects as opposed to their direct engraftment into damaged tissue that contributes to tissue repair (Uccelli *et al*., 2006; Dazzi *et al*., 2012). It is acknowledged that BM-MSC-engraftment in damaged tissue can occur, but at a very low rate (<0.1%), such that this process is unlikely to impact significantly on clinical outcome (Krause, 2005). However, it has been reported that MSCs can differentiate into epithelial and endothelial cells and engraft into damaged tissue, indicating a possible role in repair of the epithelial/endothelial layer in sepsis damaged organs (Ricciardi *et a*l., 2012; Wang *et al*., 2013).

1.6.2 MSC definition

MSCs are a heterogeneous subset of stromal stem cells that reside in bone marrow and other anatomic niches. They are self-replicating and multipotent with at least tri-lineage

differentiation potential (into osteoblasts, adipocytes and chondroblasts) *in vitro*. A position paper from the International Society for Cellular Therapy states human MSCs must be "plastic adherent when maintained in standard culture conditions and must express specific cell surface markers (CD105, CD73 and CD90) and lack expression of haematopoietic progenitor, endothelial and HLA-DR surface molecules" (Dominici *et al*., 2006).

The establishment of these minimal criteria for designating a cell an 'MSC' underpinned attempts to standardise practice in human MSC (hMSC) usage in preclinical studies and facilitate data comparison between studies. However, culture-expanded MSCs consist of a heterogeneous population of cells exhibiting a spectrum of phenotypes, function and immunogenicity. The functional capabilities of MSCs are influenced by a combination of factors including tissue, donor and species of origin, isolation technique, culturing protocols and maturity or passage number (Dazzi *et al*., 2012; Ankrum *et al*., 2014). Furthermore, current interest in the clinical application of MSCs revolves around their immunomodulatory properties as opposed to their regenerative 'stem cell' properties. MSCs produce extracellular vesicles (EVs) and a whole host of cytokines and growth factors that modulate immune responses by inhibiting (or supporting) B and T cell proliferation and monocyte maturation and by promoting generation of regulatory T cells and M2 macrophages (Francoise *et al*., 2012; Bernardo *et al*., 2013; Prockop, 2013). So, whilst it may be considered appropriate to define MSCs based on their differentiation potential and ability to support hematopoiesis (Bianco *et al*., 2013; Shen, 2013), others prefer a broader definition that places less weight on their 'stem' properties and more on their immunomodulatory potential (Dazzi *et al*., 2012; Keating, 2012; Fibbe *et al*., 2013).

1.6.3 MSC immunogenicity

The immune system's ability to distinguish self from non-self is fundamental to immune tolerance (Gorer *et al*., 1948; Billingham *et al*., 1953). A critical component of this is the human leukocyte antigen (HLA) or major histocompatibility complex (MHC), which encodes a collection of immune and non-immune related molecules (Benacerraf, 1981; Snell, 1981). The importance of the MHC became clear following early transplant studies in mice and humans that exposed the role of glycoproteins encoded by MHC in the identification of self

(Gorer *et al*., 1948). Recognition of foreign MHC from allogeneic cells was acknowledged as a determinant factor in the rejection of donor tissue by the host (Billingham *et al*., 1953; Burnet, 1959). Some years later, the role of MHC molecules was more specifically linked to antigen presentation (Doherty and Zinkernagel, 1975). MHC genes are clustered into three groups: Class I, II and III. In humans, MHC Class I molecules are ubiquitously expressed and function to present foreign cytosolic peptides to $CD8^+$ cytotoxic T cells (Townsend *et al*., 1985). MHC Class II molecule expression is restricted to the surface of APCs: macrophages, DCs and B cells (Cresswell *et al*., 2005). Class II molecules present exogenously derived antigens to $CD4^+$ T helper cells, generating an immune response, such as the stimulation of antibody production by B cells, to target the invading cell (Cresswell *et al*., 2005). MHC Class III contains a variety of genes that code for immune functions other than antigen presentation, such as components of the complement system and cytokines (Xie *et al*., 2003). In the 1990s, Matzinger and Anderson proposed that, in addition to the contribution from MHC molecules, APCs require the presence of "danger signals" as co-stimulant with the antigen to induce immune activation of T cells. Antigens presented without concomitant danger signal result in tolerance (Matzinger, 1994; Anderson and Matzinger, 2000).

In the resting state, human MSCs express low levels of MHC class I, negligible (or undetectable) amounts of MHC class II and Fas ligand and do not express costimulatory molecules (Pittenger *et al*., 1999; Deans and Moseley, 2000; Eliopoulos *et al*., 2005). They fail to stimulate allogeneic T cells *in vitro* and exhibit immunosuppressive properties, specifically inhibition of the proliferation of active and inactive T-cells (Klyushnenkova *et al*., 1998; Tse *et al.,* 2000). Interestingly, hMSCs analysed immediately post isolation from human bone marrow were demonstrated to potently express MHC class II, which was then lost following culture expansion *in vitro* (Jones *et al.,* 2002). Murine MSCs have been shown to constitutively express MHC Class I (utilised as a positive marker for characterization of murine MSC) but not MHC Class II or costimulatory molecules, though MHC II expression is inducible by IFN-γ (Romieu-Mourez *et al*., 2007).

There is a body of evidence that MSCs have the capacity to evade host immune recognition *in vivo* and are capable of inhibiting allogeneic T cell responses independent of MHC, in line with the immunosuppressive effects observed *in vitro* (Di Nicola *et al.,* 2002; Krampera *et*

al., 2003; Le Blanc *et al*., 2003). This 'immunoprivileged' state allows MSCs to escape immune surveillance and consequently expands their potential usage to include immunocompetent MHC-mismatched recipients (Le Blanc *et al*., 2003b). A number of clinical studies have been undertaken to investigate co-transplantation of MSCs in allogeneic hematopoietic stem cell transplantation (HSCT), with the aim of enhancing HSC engraftment, by preventing host-versus-graft reactivity and suppressing graft-versus-host reactions (Angelopoulou *et al*., 2003; Le Blanc *et al*., 2007). Attenuation of graft versus host disease (GVHD) has been demonstrated by allogeneic MSCs in mice and humans (Le Blanc *et al*., 2004; Ball *et al*., 2007; Dazzi *et al*., 2008; Macmillan *et al.,* 2009).

Alongside the studies of MSC impact on donor HSC engraftment, hMSCs have themselves been observed to engraft in utero in sheep; MSCs engrafted into the fetus of sheep after transplantation intraperitoneally, and persisted in the host organs for as long as 13 months without immunosuppression. Of interest, engraftment was reported to be successful even when the transplant was performed after the fetuses became immunocompetent (Liechty *et al*., 2000). hMSCs have also been reported as successfully engrafting in the brains of albino rats (Azizi *et al*., 1998). Allogeneic MSCs have been shown to prolong skin graft survival in immunocompetent baboons (Bartholomew *et al*., 2002). *In vitro* study of these BM-MSCs, isolated from baboon, yielded results consistent with human MSC studies: MSCs failed to elicit a proliferative response from allogeneic lymphocytes and when added into a mixed lymphocyte reaction, or to mitogen-stimulated lymphocytes, MSCs led to a greater than 50% reduction in proliferative activity (Bartholomew *et al*., 2002). Others subsequently reported that donor-derived MSCs promote tolerance to other transplanted tissues, including pancreatic islets (Ito *et al*., 2010) and heart allografts (Casiraghi *et al*., 2008).

For hMSCs, exposure to an inflammatory environment, or 'licensing' by IFN-γ, has been shown to upregulate expression of MHC Class I and induce the expression of MHC Class II molecules, but not the costimulatory molecules CD40, CD80 and CD86, which remain undetectable (Tse *et al.,* 2000; Le Blanc *et al*., 2003b; Eliopoulos *et al*., 2005). Importantly, IFN-γ-induced expression of MHC class II does not result in alloreactivity (Le Blanc *et al*., 2003b). Furthermore, fully differentiated MSC: adipocytes, osteoblasts, and chondrocytes, like undifferentiated MSC, were not immunogenic as they did not stimulate allogeneic

lymphocytes in co-culture experiments but retained the capacity to reduce lymphocyte proliferation (Le Blanc *et al*., 2003b). Murine MSCs have also been shown to upregulate MHC Class II expression and MHC-II mediated antigen presentation on exposure to IFN-γ (Romieu-Mourez *et al*., 2007), though this observation is not consistent amongst studies and may be dependent on cell density: favoured by culturing at high density (Romieu-Mourez *et al*., 2007). Of interest in this study, the investigators observed the opposite effect in human MSCs: IFN-γ-induced MHC II expression occurred at the highest levels in low-density cultures. They concluded that MHC II-related immunostimulatory functions in MSC are critically regulated by culture conditions such as cell density, (Romieu-Mourez *et al*., 2007) which highlights the importance of standardisation of MSC expansion and culture techniques if data from different studies is to be compared. In contrast to hMSCs, mMSCs have also been found to express co-stimulatory molecule CD80 when exposed to IFN-γ (Krampera *et al*., 2003).

There has been much controversy about MSC immunogenicity; the concept of MSCs being so called 'immunoprivileged' has been heavily debated. Work from Jacques Galipeau's group strongly supports the notion that MHC-mismatched murine MSCs are immunogenic in immunocompetent allogeneic mice (Eliopoulos *et al*., 2005). His group demonstrated that MSCs derived from Bl/6 mice, genetically engineered to produce erythropoietin (EPO) and injected into Balb/c mice, failed to lead to the sustained rise in haematocrit (Hct) seen in the Bl/6 mice treated with syngeneic EPO-producing MSCs. The allogeneic MSCs administered to the Balb/c mice provoked a transient increase in Hct, which on return to baseline, failed to respond to further MSC injections, illustrating a refractory effect. In addition, the Balb/c mice produced antibodies to EPO, Bl/6 mice did not. Analysis of Balb/c splenocytes showed increased numbers of activated $CD8^+$ T cells, NK cells and NKT cells compared with Bl/6 mice. It was concluded that the immunosuppressive ability of the murine MSCs in question was not sufficient to prevent their own rejection, and thus, in the murine system, MSCs cannot serve as a "universal donor" in cell therapy (Eliopoulos *et al*., 2005).

In a later study, injection of allogeneic EPO-MSCs into immune-competent hosts resulted in sensitisation to the EPO antigen and an anti-EPO immune response (Campeau *et al*., 2009). Nauta and colleagues showed that irradiated mice receiving allogeneic bone marrow

transplant (BMT) treated with their own (syngeneic) MSCs exhibited enhanced HSC engraftment compared to control mice (not receiving MSC treatment). However, mice receiving allogeneic BMT treated with allogeneic MSCs from the donor mouse exhibited transplant rejection. Nauta attributed this observation to a memory T cell response which he went on to demonstrate in immunocompetent naïve mice treated with donor MSCs (Nauta *et al*., 2006).

A question which comes out of these data is why the conflicting results in MSC immunogenicity between studies? There are a several possible explanations for this. Lack of immunogenicity may be explained by implantation of MHC-mismatched MSCs into immunoprivileged sites such as the brain (Azizi *et al*., 1998; Djouad *et al*., 2003). An alternative explanation may be the pre-conditioning of the host, for example irradiated monkeys receiving allogeneic simian MSCs exhibiting cell retention (Devine *et al*., 2003; Mahmud *et al*., 2004). Saito and colleagues, described immunologic tolerance to mouse MSC which facilitated engraftment in the myocardium of immunocompetent rats who had suffered myocardial infarction (MI), suggesting that the cells impart tolerogenic properties in that xenogenic environment (Saito *et al*., 2002). A study with contradictory results, siting retention of human MSCs in non-immunocompetent rats but not in immunocompetent rats, noted a significant inflammatory immune response dominated by macrophage infiltrate in the myocardium of the immunocompetent cohort (Grinnemo *et al*., 2004). The investigators suggested that species-specific differences in MSC source could account for the discrepant results, highlighting some of the differences between human and murine MSC mechanistics (Grinnemo *et al*., 2004).

hMSCs have been demonstrated to constitutively secrete the immune-inhibitory molecule HLA-G, recognized to be important in materno-fetal tolerance (Nasef *et al*., 2007). The secretion of HLA-G, in combination with other immunosuppressive factors, may be an additional mechanism by which MSCs induce their own tolerance. Of course, immune recognition does not necessarily result in immune rejection. It may be that xenogeneic and allogeneic MSC can exert immunosuppressive effects by surviving for a short time, time enough to secrete soluble factors which continue to impact on immune effector cells, beyond the lifetime of the MSC, the so-called hit-and-run mechanism (von Bahr *et al*., 2012;

Prockop, 2013). Indeed, it has yet to be shown clinically that increased MSC persistence leads to enhanced efficacy of MSC therapy (Lalu *et al*., 2012).

Application of bioluminescence imaging and donor DNA/RNA analysis demonstrated that the persistence of human MSCs in mice with severe combined immunodeficiency (SCID) was limited, with the most cells dying within 48 hours of being infused. This was also the case with mMSC in syngeneic mice and rat MSCs in allogeneic rats. (Toma *et al*., 2009; Lee *et al*., 2009). A separate study utilising luciferase-expressing mMSCs injected into syngeneic and allogeneic hosts showed that the majority of syngeneic mMSCs were detectable for the 40-day duration of the experiment, in contrast to a 20-day detection limit in the allogeneic setting (Zangi *et al*., 2009). Furthermore, mice previously treated with allo-MSCs showed accelerated rejection of fibroblasts from the same donor (at day 2 compared to day 14 in naive mice), and these allo-MSC–treated mice harbored more $CD4^+$, $CD122^+$, $CD44^+$ $CD62L^{low}$ T cells, suggesting T-cell memory development (Zangi *et al*., 2009). Corrobatory evidence of a lack of persistence of allo-MSCs has been observed in a variety of species: rat (Schu *et al*., 2012), rhesus macaque (Isakova *et al*., 2010) and pig (Poncelet *et al*., 2007).

Whilst MSC do not appear to be truly immune privileged, rejection of allo-MSCs occurs more slowly than rejection of other allogeneic cell types and the extent of MSC rejection can be influenced by the balance in expression of MSC immunogenic and immunosuppressive factors (Kidd *et al*., 2009; Waterman *et al*., 2010; Bianco *et a*l., 2013; Dembinski *et al*., 2013). For example, allo-MSCs can persist *in vivo* within the tumour micro environment which is relatively immune suppressed, but fail to persist in other tissues within the same animal (Kidd *et al*., 2009; Dembinski *et al*., 2013) This may suggest that local immune suppression is required to mask MSC immunogenicity (Dembinski *et al*., 2013). It may therefore be appropriate to consider MSCs ‘immune evasive’ as opposed to ‘immune-privileged’, meaning that whilst treatment with allo-MSC remains attractive, the concept of ‘off the shelf therapy’ from a ‘universal donor’ has limitations, necessitating further exploration.

1.6.4 MSCs and toll-like receptors

Both human and murine MSCs have been reported to express TLRs, believed to contribute to MSC sensing of their microenvironment and play a part in modulating MSC behaviour (Cho *et al*., 2006; Pevsner-Fischer *et al*., 2007; Wang et al., 2009). TLRs 1, 2, 3, 4, 5 and 6 have been demonstrated as being expressed by human MSCs isolated from a variety of anatomic niches; the presence of TLR-7, 8, 9 and 10 is controversial (Liotta *et al*., 2008; Tomchuck *et al*., 2008; van den Berk *et al*., 2009; Opitz *et al*., 2009). TLR- 1-8, but not TLR-9 has been demonstrated in mouse BM-derived MSC (Pevsner-Fischer *et al*., 2007). This area of MSC biology has been challenging to study since TLR detection at the mRNA level is often not matched at a protein level, making detection by flow cytometry difficult (DelaRosa *et al*., 2012).

Interestingly, when stimulated with a variety of TLR agonists, MSCs from different tissues responded in a diverse manner. For example, TLR activation in human MSCs derived from adipose tissue either had no impact, or abrogated proliferation (Cho *et al*., 2006) whereas TLR triggering in human MSCs derived from bone marrow resulted in enhanced proliferation (Pevsner-Fischer *et al*., 2007). These data stress the importance of cell source as well as species when TLR profile is considered. The study by Pevsner-Fischer and colleagues also demonstrated that prototypic TLR-2 ligand Pam3Cys inhibited MSC differentiation into osteogenic, adipogenic, and chondrogenic cell types whilst sparing their immunosuppressive effect. The investigators concluded that specific TLR ligands could be used to suppress MSC differentiation triggered by exogenous mediators and instead maintain the cells in an undifferentiated proliferating state (Pevsner-Fischer *et al*., 2007).

Several studies have demonstrated that activating MSC TLRs by different TLR ligands can generate significant changes in basic cell functions: cell proliferation, differentiation, migration, apoptosis and secretion of immunomodulatory factors (Cho *et al*., 2006; Pevsner-Fischer *et al*., 2007; Wang *et al*., 2009; Opitz *et a*l., 2009). Tomchuck and colleagues demonstrated that migration of hMSCs *in vitro* is promoted by TLR ligand exposure (specifically TLR-3 activation) and disruption of TLRs by neutralizing TLR antibodies compromised hMSC migration (Tomchuck *et al*., 2008). The group also showed that

stimulation of hMSCs with TLR agonists promotes activation of downstream signaling pathways, including NF-κB and MAPK, which in turn trigger the induction and secretion of cytokines and chemokines (Tomchuck *et al*., 2008).

Opitz and colleagues showed that TLR3 and TLR4 ligation enhanced the immunosuppressive properties of hMSC by significantly increasing tryptophan degradation and kynurenine production via induction of indoleamine-2,3-dioxygenase-1 (IDO1). Interestingly this involved an autocrine IFN-β signaling loop, which was independent of IFN-γ (Opitz et al., 2009). In contrast, Liotta and colleagues demonstrated that TLR3 and TLR4 ligation on hMSC inhibit their ability to suppress T-cell proliferation, but this had no impact on MSC immunophenotype or differentiation potential (Liotta *et al*., 2008). These contradicting results have been addressed by Raicevic and colleagues who highlighted the use of PBMCs versus $CD4^+$ purified T cells as responder cells in the allogeneic mixed lymphocyte reactions (MLRs) set up by the two groups of investigators. In the Opitz study, non–T cells could influence MSC–T cell interaction, whereas in the Liotta study, this influence is avoided (Raicevic *et al*., 2010). The issue of differences in experimental systems provoking MSCs to behave in different ways supports the concept that MSCs function in a dynamic manner, tailoring their response to the specific environmental conditions in which they find themselves (DelaRosa *et al*., 2012).

It is pertinent to remember that the behaviour and function of MSCs derived from human tissue versus mouse can differ significantly; this appears to be the case with regard to TLR expression, TLR-ligand interactions and the subsequent modulation of MSC activity. For example, whilst TLR activation enhanced hMSC migration in the Tomchuck study (Tomchuck *et al*., 2008), it impaired or had no effect on mMSC migration in other reports (Pevsner-Fischer *et al*., 2007; Lei *et al*., 2011). Indeed, the role of IDO (discussed above as being pivotal to hMSC-mediated immunosuppression) is species dependent (Meisel *et al*., 2011). Utilising IDO-deficient mice, Lanz and colleagues demonstrated that IDO activity is not required for mMSC-mediated immunosuppressive effects, in contrast to hMSCs (Lanz *et al*., 2010).

Focusing on murine MSCs, Wang and colleagues investigated the effect of lipopolysaccharides (LPS), a ligand of TLR4, on the survival of mMSCs *in vitro* and discovered that appropriate doses of LPS protected MSCs from hydrogen peroxide/serum deprivation-induced apoptosis via phosphatidylinositol 3-kinase (PI3K)/Akt- and NF-kB-dependent mechanisms. They concluded that pro-inflammatory LPS-mediated stimulation of TLR-4 on MSC protects against apoptosis induced by oxidative stress, whilst simultaneously enhancing MSC proliferation (Wang *et al*., 2009). In line with work by Raffaghello's group, Cassatella and colleagues reported that activation of TLR-3 and TLR-4 enhanced MSC capacity to delay neutrophil apoptosis through the induction of IL-6, IFN-γ and GM-CSF (Raffaghello *et al*., 2008; Cassatella *et al*., 2011).

Exposure to an inflammatory environment may also modulate the pattern and function of TLRs expressed by MSCs; expression of TLRs-2, 3 and 4 was demonstrated to increase in hMSCs following exposure to a combination of pro-inflammatory cytokines (IFN-α, IFN-γ, TNF-α and IL-1β), whilst TLR-6 was downregulated (Raicevic *et al*., 2010). Ligation of specific TLRs has been reported to generate MSCs with either pro-inflammatory (MSC1) or anti-inflammatory (MSC2) phenotypes (Romieu-Mourez *et al*., 2009; Waterman *et al.,* 2010). MSC1 'polarisation', stimulated by activation of TLR-4 (by LPS), resulted in upregulation of pro-inflammatory cytokines (IL-6 and IL-8) whilst MSC2 polarisation, via TLR-3 stimulation, led to the production of anti-inflammatory cytokines (IL-4 and PGE2). TLR-3-activated MSC2 maintained T cell immunosuppressive effects *in vitro*, while TLR-4-primed MSC1 did not, in fact it was demonstrated that these MSC were capable of T cell activation (Waterman *et al*., 2010).

This concept of TLR-mediated MSC polarisation is likely to be an oversimplification of a complex process where MSCs continuously adapt to their environment. The *in vivo* anti-microbial and anti-inflammatory therapeutic effects reported in mouse models of infection and LPS-induced lung injury strongly contradict the polarising phenomenon demonstrated *in vitro* (Gonzalez-Rey *et al*., 2009; Mei *et al.,* 2010; Nemeth *et al*; 2009, 2010). However, Waterman's group experimented with pre-polarised MSC1 and MSC2 in a murine model of acute lung injury and found that, when compared to untreated (non-polarised) BM-MSCs, MSC1 aggravated the inflammation and MSC2 abrogated it (Waterman *et al*., 2010). In

conclusion, the inflammatory milieu MSCs encounter *in vivo* influences how they behave, mediated, at least in part, by TLR ligation, but questions remain unanswered about the nature of the inflammatory environment (cytokines, TLR ligands) best suited to stimulating optimal MSC immunomodulatory effects (Dela Rosa *et al*., 2012).

A recent study has demonstrated that MSC can express retinoic acid inducible gene-I (RIG-I)-like receptors (RLR), in addition to TLRs, and that this is the case for MSCs isolated from a variety of sources: bone marrow (BM), adipose tissue (AT), Wharton's jelly (WJ) and foreskin (FSK). MSC RLR mRNA expression was increased in the presence of an inflammatory environment across all sources, and RLR activation stimulated the production of Type I IFN (IFN-β) and Type III IFN (IFN-λ1) via TBK1/IKK-ε signaling and IRF7 phosphorylation (Raicevic *et al*., 2017). Type I IFNs are known to play an essential role in defense against viral infection by inducing the transcription of genes involved in apoptosis, as well as stimulating innate and adaptive immune cell activation (Theofilopoulos et al., 2005; Stetson and Medzhitov, 2006). MSC source was shown to influence response to RLR triggering, with AT-MSCs identified as the most responsive, secreting the highest levels of pro-inflammatory cytokines and interferons compared with the other MSC types. In line with this pro-inflammatory phenotype, RLR activation enhanced the immunomodulatory potential of AT-MSCs by increasing production of IDO1 and leukemia inhibitory factor (LIF), implicating AT-MSCs as being the most potent for virus recognition and stimulation of an antiviral immune response. (Raicevic *et al*., 2017).

1.6.5 MSC trafficking

A remarkable feature of MSCs is their ability to migrate or 'home' towards sites of tissue damage or inflammation. *In vitro* studies have demonstrated a role for growth factors, cytokines and chemokines in directing MSC trafficking: stromal-derived factor-1 (SDF-1), epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF) and angiopoiten-1 (Ang-1) (Fiedler *et al*., 2002 and 2006; Forte *et al*., 2006; Ponte *et al*., 2007; Johnson *et al*., 2017). These chemotactic factors are relevant for MSC homing since their accumulation within tissues has been demonstrated following tissue injury (Askari *et al*., 2003; Ji *et al*., 2004; Li *et al*., 2013;

Hocking *et al*., 2015). Ponte and colleagues highlighted the difference in migration capacity between stimulated and resting MSCs; for unstimulated hMSCs, growth factors (PDGF-AB and IGF-1) were more potent chemo attractants than the chemokines investigated (SDF-1), whereas migration toward chemokines was enhanced in TNFα-stimulated MSC (Ponte *et al*., 2007). A recent study demonstrated that the interaction of human tonsillar ILC3 cells with hMSC *in vitro* induced expression of VCAM-1 by MSCs, whist its ligand (a4b7 integrin) was expressed by activated ILC3s. Consequently, ILC3s could be maintained in the tissues by MSCs and MSCs retained by adhesion to the epithelium, thus enhancing ILC3-MSC interaction and MSC protective effect (Van Hoeven *et al*., 2018).

The capability of MSC to sense changes in their environment via TLR-ligand interactions allows them to react to invading microorganisms by recognizing foreign proteins belonging to, or secreted by, the pathogen (Pevsner-Fischer *et al*., 2007; Wang *et al*., 2009). This process of recognition is followed by migration towards the bacteria, or bacteria-infected cells (Li *et al*., 2013; Fakhari *et al*., 2014). An example of this is *S. aureus*-infected intestinal epithelial cells reported to induce directed migration of hMSCs via the secretion of TNF-α (Li *et al*., 2013b). *In vitro* work has shown that the migration of hMSCs is promoted by TLR ligand exposure (specifically TLR-3 activation); disruption of this interaction by the addition of neutralising TLR antibodies impaired hMSC migration (Tomchuck *et al*., 2008).

1.6.6 MSC licensing

The concept, now widely accepted, that MSCs are not constitutively inhibitory, but acquire their immunosuppressive functions following exposure to the inflammatory environment stems from the seminal observation that anti-IFN-γ receptor antibodies can revert the suppressive effect of hMSCs (Krampera *et al*., 2006). Krampera and colleagues reported that the ability of IFN-γ to induce the suppressive effect of MSCs on T cell and NK cell proliferation involved the stimulation of MSC-mediated IDO activity, and that this took 3 days, suggesting that there may need to be a crosstalk established between MSC and target cells in order for MSC immunomodulation to occur (Meisel *et al*., 2004; Krampera *et al*., 2006). They concluded that IFN-γ, produced by activated T cells or NK cells (but not B cells), was a key stimulant in the promotion of immunomodulatory activity of MSCs, which

ultimately resulted in the observed anti-proliferative effects on T cells and NK cells; B cells were not affected. In support of this is the important observation that the addition of exogenous IFN-γ to MSC-B cell co-cultures made the previously protected B cell proliferative response susceptible to the anti-proliferative effects of MSCs (Krampera *et al*., 2006).

Exposure to IFN-γ and other inflammatory cytokines such as TNF-α effectively 'licenses' the MSC to unleash its immunosuppressive armory (Ren *et al*., 2008). This licensing step can be exploited to increase the efficiency of MSC-mediated immunosuppression. This has been demonstrated in a murine model of graft-versus-host-disease (GvHD) in which MSCs, pre-treated with IFN-γ, had a 5-fold greater immunosuppressive effect than control MSCs (Polchert *et al*., 2008).

1.6.7 The role of MSCs in tissue regeneration and repair

Exogenously administered MSCs promote tissue regeneration and repair (Lange *et al*., 2005, Lee *et al*., 2006; Ramasamy *et al*., 2007; Ruffell *et al*., 2009). Ramasamy and colleagues demonstrated how MSC antiproliferative and antiapoptotic activities conferred a cytoprotective effect on tumour cells (Ramasamy *et al*., 2007). This cytoprotection, facilitates the recovery of residual stem cells within the parenchyma of damaged tissue and promotes tissue repair (Ruffell *et al*., 2009). This has been demonstrated in several organs, including kidney (Lange *et al*., 2005, Lee *et al*., 2006); pancreas (Lee *et al*., 2006) and lung (Ortiz *et al*., 2007). Of note, the predominant mechanism by which MSCs enhance tissue repair is thought not to be the result of trans-differentiation into the appropriate cell type, or direct engraftment, but to involve MSC trophic effects on residual host parenchymal stem cells, which serves to nurture their regenerative potential - a type of graft initiated endogenous repair process (Ramasamy *et al*., 2007; Urban *et al*., 2008). Indeed, Darwin Prockop's group observed an improvement in cardiac function in mice with acute myocardial infarction following intravenous treatment with hMSC, despite a lack of evidence of engrafted donor cells 3 weeks post injection (Iso *et al*., 2007).

MSCs express proangiogenic factors and growth factors, such as vascular endothelial growth factor (VEGF) and angiopoietin-1 (AP-1), which work in concert to stimulate capillary

proliferation, vessel remodeling and recruitment of HSCs from BM to aid tissue repair (Hattori *et al*., 2001; Tang *et al*., 2006). MSCs also express a variety of neuroregulatory proteins and neuropeptides which maintain the integrity of nervous tissue and guide the innervation of nerve fibers during the reparative process (Phinney and Prockop, 2007). In summary, it appears that MSCs effect tissue repair by a variety of mechanisms: promoting the survival and proliferation of endogenous cells (Lange *et al*., 2005; Lee *et al*., 2006), inhibiting pro-inflammatory immune responses (Zappia *et al*., 2005; Ortiz *et al*., 2007), inducing angiogenesis (Tang *et al*., 2006), reducing apoptosis (Hung *et al*., 2007) and engaging in mitochondrial transfer to rescue host cell bioenergetics (Islam *et al*., 2012; Ahmad *et al*., 2014; Jiang *et al*., 2016).

1.7 MSC immunomodulatory effects

The properties of MSC have been carefully dissected over the last 20 years, from their differentiation potential and regenerative capabilities to their anti-inflammatory and antimicrobial effects in a variety of experimental systems. One of the key features of MSCs, responsible for generating considerable interest, is their ability to exert a range of immunomodulatory activities on almost all types of immune cell studied. Of interest, MSC exposure to an inflammatory environment is critical to their activation status, effectively licensing them to exert their immunoregulatory effects (Krampera *et al*., 2006). There is accumulating evidence that the complex interactions between MSC and the immunoregulatory networks engaged during inflammation underpins the delivery of MSC-mediated anti-inflammatory and immunosuppressive effects (Aggarwal and Pittenger, 2005; Selmani *et al*. 2008; Trento and Dazzi, 2010; van Hoeven *et al*., 2018).

1.7.1 MSC and T cells

The interactions of MSCs with T cells are the best studied of all the immune effector cells from both innate and adaptive immune systems. There is strong evidence that MSCs from various species (rodents, primates and humans) and anatomical sources (bone marrow, adipose tissue, umbilical cord) can inhibit the proliferation of T cells *in vitro* (Klyushnenkova *et al.,* 1998; Di Nicola *et al*., 2002; Krampera *et al*., 2003; Tse *et al*., 2003). MSC-mediated

suppression of T cell response has been reported in the context of mitogenic and poly-clonal stimuli (Di Nicola *et al*., 2002; Bartholomew *et al*., 2002) and stimulation by cognate peptide (Krampera *et al*., 2003). A study using MSC from third-party donors i.e, cells obtained from neither stimulator nor responder cell source (demonstrated to be fully-mismatched for the MHC haplotype of the responder T cells) reported MSC-mediated suppression in T cell response, suggesting that this MSC effect is not cognate dependent (Le Blanc *et al*., 2003). Le Blanc and colleagues also demonstrated that the suppressive activity of both undifferentiated and differentiated MSC increased when the cells were exposed to IFN-γ, and that this finding was independent of MHC expression (Le Blanc et al., 2003b).

There appears to be a lack of selectivity in MSC suppression of T cell response, T helper cells and cytotoxic T cells (Glennie *et al*., 2005), naïve and memory T cells (Krampera *et al*., 2003) are equally vulnerable. Glennie and colleagues characterized the phenotype of these T cells by dissecting the effect of MSCs on T cell proliferation, activation and effector function (Glennie *et al*., 2005). They found that the inhibitory effect of MSCs was directed mainly at cell proliferation. The expression of early activation markers CD25 and CD69 was unaffected, but T cell IFN-γ production was diminished. T cell effector function recovered following removal of MSCs and restimulation with cognate peptide, but T cell proliferative capacity did not. Analysis of the cell cycle showed that T cells, stimulated in the presence of MSCs, were arrested at the G1 phase, as a result of downregulation of cyclin D2. Interestingly, this induction of T cell unresponsiveness was associated with prevention of T cell apoptosis (Jones *et al*., 2007) and could not be reversed by exogenous IL-2, as observed in the classical form of 'anergy' (Essery *et al*., 1988). The investigators concluded that co-culture with MSC induces an unresponsive T cell profile, consistent with that seen in division arrest anergy, first described in $CD4^+$ T cells (Wells *et al*., 2001). In this system, a proportion of $CD4^+$ cells fail to divide post stimulation with anti-CD3 and APCs, despite expressing markers of activation (CD69). The non-dividing cells expressed CD25 but did not respond to exogenous IL-2 (Wells *et al*., 2001).

In addition to their T cell immunosuppressive effects, MSCs have been shown to stimulate a shift in T cell response from T_H1 to T_H2 cell predominance (Aggarwal and Pittenger, 2005, Bai *et al*., 2009). Naive T cells, activated to differentiate into T_H1 effector cells in the

presence of hMSCs, secreted significantly lower amounts of IFN-γ than their counterparts cultured without hMSC. In contrast, naive T cells, activated to differentiate into T_H2 effector cells in the presence of hMSCs, secreted significantly higher levels of IL-4 than newly differentiated T_H2 cells cultured in the absence of hMSC. Mechanistically, the hMSCs were found to secrete prostaglandin E2 (PGE2), inhibition of which mitigated hMSC-mediated effects on T cells and other immune cells investigated (which included DCs and NK cells) (Aggarwal and Pittenger, 2005).

1.7.2 MSC and T_{regs}

MSC-mediated immunosuppression is not only related to a direct action on immune effector cells, but can also result from the recruitment of other immune-regulatory populations, such as T_{regs}. *In vitro* studies demonstrate that MSC recruit T_{regs} from both naive and memory T cells (Di Ianni *et al*., 2008; Prevosto *et al*., 2007; Lee *et al*., 2017). MSC-mediated T_{reg} recruitment is also evident *in vivo* in a variety of experimental models: experimental autoimmune encephalomyelitis (Zappia *et al*., 2005), experimental arthritis (Gonzales *et al*., 2009), diabetes (Madec *et al*., 2009) and asthma (Nemeth *et al*., 2010).

There are many ways in which MSC induce T_{reg} expansion. MSC secrete or express molecules such as transforming growth factor (TGF)-β or non-classic human leukocyte antigen class I molecule HLA-G that directly activate and expand T_{regs} (Nemeth *et al*., 2010). Selmani and colleagues demonstrated *in vitro* expansion of a population of $CD4^+CD25^{high}Foxp3^+$ T cells among allostimulated T cells when MSCs were added to MLR, which was at least partly dependent on MSC secretion of HLA-G soluble isoform HLA-G5. IL-10 was identified as a key cytokine upregulating both expression and secretion of HLA-G5 by MSCs; in addition to HLA-G5 secretion, expansion of $CD4^+CD25^{high}$ $Foxp3^+$ T cells required a preliminary direct cell-to-cell contact between MSC and T cells (Selmani *et al*. 2008). This study also highlights that in addition to their action on the adaptive immune system, MSCs, through HLA-G5, affect innate immunity by inhibiting both NK cell-mediated cytolysis and IFN-γ secretion (Selmani *et al*. 2008). MSCs also recruit further immune-regulatory networks (monocytes and DCs) that actively engage T_{regs} by producing tolerogenic cytokines (Aggarwal and Pittenger, 2005).

MSCs are also reported to interact with fully polarized tissue-infiltrating human T_H17 cells and induce a regulatory phenotype (Ghannam *et al*., 2010).

The stress responsive enzyme haem oxygenase-1 (HO-1), demonstrated to be part of the MSC secretome of rats (Blancou *et al*., 2010) and humans (Mougiakakos *et al*., 2011), has been demonstrated to stimulate the differentiation of induced regulatory T cells such as IL-10^+ type 1 regulatory T (Tr1) cells and TGF-β^+ T helper 3 (Th3) cells (Mougiakakos *et al*., 2011). A recent study demonstrates that in addition to cell contact-dependent mechanisms and secreted soluble factors, hMSC express a cell surface molecule, ICOS ligand (ICOSL), belonging to the B7 family of co-stimulatory molecules, which is important for T_{reg} induction *in vitro (*Lee *et al.,* 2017*)*. ICOS signaling has previously been demonstrated as being required for active suppression by T_{regs} (Strauss *et al*., 2008). ICOSL expressed by APCs, epithelial cells, and tumor cells is reported to directly drive T_{reg} expansion and activation (Martin-Orozco *et al*., 2010). In Lee's study, ICOSL-ICOS signaling promoted T_{reg} differentiation from $CD4^+$ T cells through activation of the phosphoinositide 3-kinase-Akt pathway (Lee *et al*., 2017). This signaling pathway was significantly upregulated following MSC priming with IL-1β, in line with previous observations that upregulation of ICOSL in MSCs occurs under inflammatory conditions (Lee *et al*., 2017).

The real relevance of MSC-mediated T_{reg} recruitment or expansion remains undetermined because these studies have not addressed the question of whether and how T_{regs} are required for MSC activity. An important part of my thesis is the exploration of this area of MSC biology and the significance of MSC-T_{reg} interaction in the context of bacterial lung infection.

1.7.3 MSC and B cells

The impact of MSC on B cell proliferation and function remains controversial. Corcione and colleagues reported that human MSCs inhibit B cell proliferation and differentiation into plasma cells *in vitro* (Corcione *et al*., 2006). Glennie and colleagues also reported MSC inhibition of B cell proliferation in mouse (Glennie *et al*., 2005). The mechanism proposed was one of cell cycle arrest, whereby MSCs induce a block in the G0/G1 phase of the cell

cycle, reminiscent of their effect on T cells. This was thought to be achieved via hMSC production of soluble factors, as demonstrated by transwell experiments (Corcione *et al*., 2006). MSCs have also been demonstrated to inhibit B cell differentiation to mature plasma cells capable of antibody production and impair B cell chemotaxis by downregulating chemokine receptors CXCR4, CXCR5 and CCR7 (Deng *et al*., 2005; Corcione *et al*., 2006). Interestingly, hMSC had no effect on B cell costimulatory molecule expression or cytokine production (Corcione *et al*., 2006).

However, MSC effect on B cells is likely to be more multifaceted than first thought given the more recent evidence that hMSCs can in fact induce polyclonal expansion and differentiation of B cells from healthy donors and from patients with systemic lupus erythematosus (SLE) (Traggiai *et al*., 2008). This data is in line with a report by Rasmusson and colleagues, who observed an increase in the proliferation and differentiation of enriched B cells isolated from peripheral blood and spleen and co-cultured with MSCs *in vitro* (Rasmusson *et al*., 2007). These data incite important questions about the use of BM-MSC as a therapeutic tool in autoimmune conditions in which B cell activation is implicated in disease pathogenesis, such as SLE. Possible explanations for these contradictory observations are the different cell purification procedures, experimental conditions, and timing of analysis employed in the different studies. Previous reports have demonstrated that the interaction between MSCs and cells of the adaptive immune system can vary quite significantly, depending on the microenvironment in which it occurs (Krampera *et al*., 2006; Chan *et al*., 2006). As the result of alternative types of B cell stimulation, B cell cytokine secretion could actually impact on MSC function. Thus, instead of MSCs suppressing B cell proliferation and differentiation, they may become polarised towards a pro-inflammatory 'M1' phenotype and exert stimulatory effects on neighboring B cells (Rasmusson *et al*., 2007; Traggiai *et al*., 2008; Trento and Dazzi, 2010).

1.7.4 MSC and Macrophages

MSCs also interact with cells of the innate immune system such as macrophages, neutrophils, monocytes, DCs and NK cells. Macrophages are among the first responders to pathogen invasion or tissue injury and initiate an inflammatory response to protect against

infection or damage (Aderem and Underhill, 1999). It follows that careful regulation of macrophage activation is important if the host is to be protected from an unbridled inflammatory response and consequent collateral tissue damage (Wang *et al*., 2006; Sindrilaru *et al*., 2011). MSCs are excellent candidates for this regulatory job since they are well known to respond to inflammatory microenvironments by reigning in excessive inflammatory processes downstream from the innate monocyte/macrophage response (Singer and Caplan, 2011; Prockop and Oh, 2012; Dazzi *et al*., 2012).

Studies have shown that treatment with MSCs impacts on macrophage polarisation to produce a predominantly anti-inflammatory, regulatory M2 phenotype (Kim and Hematti, 2009; Nemeth *et al*., 2009; Maggini *et al*., 2010). Kim and Hematti investigated the *in vitro* effect of human BM-derived MSCs on human peripheral blood monocyte-derived macrophages and defined MSC-educated macrophages (IL-10 high, IL-12 low, IL-6 high, TNF-α low) as a novel type of alternatively activated macrophage (Kim and Hemati, 2009). Nemeth investigated the *in vivo* effect of mouse BM-derived MSCs in a murine model of polymicrobial sepsis and demonstrated that LPS-stimulated macrophages produced more IL-10 when co-cultured with BM-derived MSCs. This MSC effect is at least partially attributed to soluble mechanisms and Prostaglandin E2 (PGE-2) has been strongly implicated as one of the paracrine mediators involved (Nemeth *et al*., 2009; Maggini *et al.*, 2010). The production of PGE-2 by MSC increases under inflammatory conditions (Hoogduijn *et al*., 2010) suggesting an increased efficacy of MSC to induce regulatory macrophages in the context of tissue inflammation. TNF-α-induced protein 6 (TSG-6) is another soluble mediator implicated in MSC-macrophage interactions (Lee *et al*., 2009; Qi *et al*., 2014).

There is increasing evidence to suggest that besides the secretion of soluble molecules such as PGE-2, MSCs secrete extracellular vesicles (EVs). In a recent study, Sicco and colleagues demonstrated that EVs isolated from human adipose-derived MSCs were efficiently internalised by responding BM-derived macrophages, eliciting their switch from a M1 to a M2 phenotype (Sicco *et al*., 2017). In addition to inducing changes in macrophage phenotype and cytokine profile, Maginni *et al* demonstrated that MSCs can inhibit the up-regulation of CD86 and MHC class II in LPS-stimulated macrophages, impairing their ability to activate antigen-specific $CD4^+$ T cells. Interestingly, this immunosuppressive effect was

accompanied by increased uptake of apoptotic thymocytes by MSC-treated macrophages (Maggini *et al*, 2010), which suggests that MSCs can enhance macrophage phagocytosis.

1.7.5 MSCs and Neutrophils

The interaction of MSCs with neutrophils is less thoroughly explored than that of MSCs with other immune effector cells. However, MSC/neutrophil co-culture studies have demonstrated an MSC-protective effect against neutrophil apoptosis and inhibition of excessive oxidative metabolism which may well contribute to observed MSC anti-inflammatory effects (Raffaghello *et al*., 2008). In this study, the anti-apoptotic activity of MSC did not require cell-to-cell contact. The key MSC-derived soluble factor identified as responsible for neutrophil protection was IL-6, which signaled by activating transcription factor STAT-3. IL-6 has been shown previously to protect neutrophils from apoptosis (Asensi *et al*., 2004).

MSC impact on neutrophil Reactive Oxygen Species (ROS) production is intriguing, because in the context of infection, ROS production is an essential step required for the elimination of invading pathogens by neutrophils (Dinauer and Orkin, 1992). However, when activation of the respiratory burst is dysregulated or excessive, ROS can cause significant host tissue damage, such as that seen in acute respiratory distress syndrome, atherosclerosis and ischemia-reperfusion injury (Babior, 2000). It follows that ROS production must be carefully modulated if it is to contribute to maintenance of the inflammatory response when appropriate, but not at the expense of tissue integrity (Fialkow *et al*., 2007). Of note, in Raffaghello's study, neutrophils exposed to opsonized zymosan (OPZ) were not susceptible to MSC inhibitory effects on the respiratory burst, unlike resting and N-formyl-methionyl-leucyl-phenylalanine (f-MLP)-stimulated neutrophils, allowing the efficient phagocytosis of the opsonized particles. Raffaghello concluded that MSCs could preserve necessary neutrophil effector functions whilst protecting against overzealous and potentially damaging inflammatory reactions (Rafaghello *et al*., 2008).

Rafaghello and colleagues reported no MSC effect on neutrophil phagocytosis, expression of adhesion molecules or chemotaxis in response to established chemoattractant molecules

such as IL-8 or complement component C5a (Raffaghello *et al*., 2008). In contrast to these data, Hall and colleagues found that, in a CLP model of polymicrobial sepsis, MSCs enhanced the phagocytic activity of neutrophils, which led to improved bacterial clearance in the peritoneum and blood. Furthermore, depletion of neutrophils prior to CLP and MSC treatment abrogated MSC positive effects on bacterial clearance and survival (Hall *et al*., 2013). Cassatella and colleagues also reported MSC-mediated enhanced neutrophil phagocytosis which involved the combined action of paracrine mediators IL-6, interferon-β (IFN-β), and granulocyte macrophage colony-stimulating factor (GM-CSF) (Cassatella *et al*., 2011). In studies of acute lung injury MSC treatment has been shown to reduce lung inflammation by inhibiting the transmigration of neutrophils into alveoli (Ortiz *et al*., 2007; Lee *et al*., 2009). This is also the case in mouse models of sepsis where MSCs limit neutrophil infiltration into liver and kidneys (Gonzalez-Rey *et al*., 2009; Nemeth *et al*., 2009). Taken altogether it is reasonable to suggest that MSCs may have the ability to limit potentially damaging neutrophil infiltration, whilst harnessing the inflammatory potential of activated neutrophils. This may be achieved by inhibition of neutrophil apoptosis and reactive oxygen species production alongside stimulation of neutrophil phagocytic activity.

1.7.6 MSC and Monocytes / Dendritic Cells (DCs)

MSC can indirectly modulate immune responses by acting on APCs. Several studies have demonstrated that MSCs interfere with DC maturation and function (Zhang *et al*., 2004; Aggarwal and Pittenger, 2005; Jiang *et al*., 2005; Nauta *et al*., 2006b; Ramasamy *et al*., 2007). There is *in vitro* evidence of $CD34^+$ haematopoietic precursor cells and monocytes being blocked from differentiating into mature DCs by MSCs (Jiang *et al*., 2005; Nauta *et al*., 2006b). Similar effects to those employed by MSC on cell cycle progression in T cells have been demonstrated to occur in MSC-monocyte co-culture; monocyte inability to differentiate into mature DC depended on a block from entering the G1 phase of the cell cycle with a progressive accumulation of cells in the G0 phase (Ramasamy *et al*., 2007). The resultant immature DCs exhibited decreased expression of MHC class II and co stimulatory molecules CD80 and CD86 and produced reduced amounts of IL-12, thus impairing their capacity to present antigen. Instead they acquired regulatory features, secreting significant amounts of IL-10 (Aggarwal and Pittenger, 2005). As a consequence of this, MSC have been

shown to have further indirect regulatory effects by IL10-mediated engagement of T_{regs} (Li *et al*., 2008).

A variety of molecules have been implicated in the mechanistics of MSC impact on monocyte differentiation and DC maturation and function. STAT 3 activity in APCs is enhanced by co-culture with MSC, which induces regulatory DCs that secrete IL-10, resulting in impaired T-cell responses (Gur-Wahnon *et al*., 2007). The same tolerogenic phenotype is inducible via activation of suppressor of cytokine signaling 1 (SOCS1), which is primarily mediated by IL-6 (Deng *et al*., 2014). Interestingly, SOCS1 has been shown to be important in M2 macrophage polarisation (Whyte *et al*., 2011). Previous *in vitro* work defined a role for Notch as a molecular switch between pro-inflammatory and anti-inflammatory T_H1 cell functions (Rutz *et al*., 2008). DCs co-cultured with MSC have been reported to induce the generation of T_{regs} through Notch activation (Li *et al*., 2008).

MSCs have also been reported as utilising monocytes in host defense against pathogen invasion (Krasnodembskaya *et al*., 2012). It has been demonstrated that one of the mechanisms by which MSCs promote bacterial clearance is through enhancing monocyte phagocytosis (Krasnodembskaya *et al*., 2012). This concept is supported by microarray data from Duncan Stewart's group showing down-regulation of inflammation related genes and up-regulation of genes involved in promoting phagocytosis and bacterial killing in a CLP model of polymicrobial sepsis treated with intravenous MSCs (Mei *et al*., 2010).

1.7.7 MSC and NK cells

MSCs have been shown to have inhibitory effects on NK cell activation, cytokine production and cytotoxicity (Aggarwal and Pittenger, 2005; Poggi *et al*., 2005; Spaggiari *et al*., 2006; Sotiropoulou *et al*., 2006; Krampera *et al*., 2006). Aggarwal and Pittenger demonstrated that hMSCs, co-cultured with IL-2–stimulated NK cells, induced a sizable reduction in the levels of IFN-γ produced by the NK cells, compared with NK cells cultured in the absence of hMSC (Aggarwal and Pittenger, 2005). Sotiropoulou and colleagues produced some corroborative data, demonstrating that at low NK-to-MSC ratios, MSCs suppress NK cell proliferation and cytokine secretion. Some of these effects were noted as requiring cell-to-cell contact,

whereas others were found to involve MSC secretion of soluble factors (TGF-β1 and PGE2), suggesting that a range of mechanisms is likely to be responsible for MSC-mediated NK cell suppression (Sotiropoulou *et al*., 2006). The inhibitory effect of hMSCs on NK cell proliferation was reported to require the presence of IFN-γ, demonstrated to act by enhancing MSC IDO activity (Krampera *et al*., 2006).

Sotiropoulou's study also highlighted that MSCs are susceptible to lysis by activated NK cells (Sotiropoulou *et al*., 2006, Poggi *et al*., 2005). Le Blanc's group investigated NK-MSC cell interactions a few years earlier, testing the ability of freshly isolated NK cells to lyse KIR-ligand mismatched MSCs. They found no such effect of NK cells on MSCs (Rasmusson *et al*., 2003). Sotiropoulou's data agreed with this finding, indeed, MSCs could not be lysed by freshly isolated NK cells. However, NK cells cultured in the presence of IL-2, IL-15 or a combination of IL-12/IL-18, so called activated NK cells, could effectively lyse MSCs, including MSCs autologous to the activated NK cells (Sotiropoulou *et al*., 2006). Spaggiari and colleagues discovered that the activating NK receptors: NKp30, NKG2D, and DNAM-1 represented the major receptors involved in the induction of NK cell lysis of MSCs. Furthermore, MSCs were found to express Unique Long 16 Binding Proteins (ULBPs), Poli Virus Receptor (PVR), and Nectin-2, the ligands for these NK receptors. Interestingly, the process of NK-mediated MSC lysis was inhibited when licensed MSCs, primed by IFN-γ and consequently expressing higher levels of MHC class I on their cell surface, were used as target cells (Spaggiari *et al*., 2006).

NK-cell function is regulated by a combination of activating and inhibitory signals, recognised and processed by cell surface receptors. MSC-NK cell co-culture has been reported to alter the expression levels of some of these receptors (Sotiropoulou *et al*., 2006), for example, 2B4 (involved in cytotoxicity and IFN-γ production) (Tangye *et al*., 2000) and NKG2D (also important in mediating cytotoxicity and recognition of cancer cells) (Smyth *et al*., 2002). Both receptors were downregulated when NK cells were exposed to MSCs in a co-culture system which allowed direct cell-to-cell contact to occur. The investigators concluded that this reduction in receptor availability may account for the observed MSC-mediated inhibition of NK cell function. (Sotiropoulou *et al*., 2006).

Initial data supported the concept of MSC immunosuppressive effects extending to NK cell cytotoxicity (Krampera *et al*., 2006; Sotiropoulou *et al*., 2006). NK cells pre-cultured for 5 days with MSCs were partially inhibited in their ability to lyse MHC class I negative K562 target cells (Krampera *et al*., 2006). IL-15–stimulated NK cells from 4-day co-culture with MSCs showed no alteration in cytotoxic potential against K562 cells. However, when MHC-class I positive tumour targets were used, NK cytotoxicity was significantly reduced (Sotiropoulou *et al*., 2006). A recent study comparing BM-MSC with AT-MSC isolated from human volunteers demonstrated that MSC from both sources induced a similar inhibition of NK cell proliferation, but only BM-MSC suppressed NK cell cytotoxic activity and showed a trend for a greater reduction in NK-cell IFN-γ secretion (Blanco *et al*., 2016). Controversy persists because it has also been reported that NK-cell lysis of MHC I positive allogeneic targets is not inhibited by MSC (Rasmusson *et al*., 2003).

MSC have also been demonstrated to influence NK cell cytokine production by inducing the release of IFN-γ and TNF-α (Poggi *et al*., 2005). Relevant to host defense against pathogen invasion, it is important to consider that the cytokines secreted by NK cells play a key role in the eradication of microbes and infected cells, and influence the adaptive immune response, thereby facilitating a dynamic interaction between innate and adaptive immunity (Koc, 1999). Whilst MSC-mediated suppression of NK cell activity may initially have its impact by blunting the innate immune response, it may secondarily suppress the function of cells of the adaptive immune system (Sotiropoulou *et al*., 2006).

1.7.8 MSC and Tissue Resident Lymphocytes

A recent study investigated whether a specific class of innate lymphoid cells, ILC3s, may contribute to the therapeutic effect of MSCs. Co-culture of ILC3s isolated from human tonsils with human BM-MSCs enhanced the proliferation and IL-22 production of the ILC3s. Reciprocally, ILC3s promoted ICAM-1 and VCAM-1 expression on MSCs, which may perpetuate MSC retention in the target tissue (Van Hoeven *et al*., 2018). MSC-iNKT cell interactions have also been investigated (Gazdic *et al*., 2017; Milosavljevic *et al*., 2017). In a murine model of Con A and α-GalCer-induced acute liver injury, treatment with mMSCs significantly reduced hepatocyte damage and attenuated the influx of inflammatory (T-bet^{+},

TNFα, IFN-γ-producing and GATA3^{+}, IL-4-producing) iNKT cells; serum levels of TNFα, IFN-γ and IL-4 were also reduced in MSC-treated mice. mMSC treatment attenuated expression of apoptosis-inducing ligands on liver iNKT cells and suppressed the expression of pro-apoptotic genes in the livers of α-GalCer-treated mice. Inhibition of soluble mediators IDO and iNOS abrogated MSC hepato-protective effects, both *in vivo* and *in vitro,* leading the investigators to conclude that MSCs can protect from acute liver injury by attenuating the cytotoxicity of liver iNKT cells and reducing their pro-inflammatory effects in an iNOS- and IDO-dependent manner (Gazdic *et al*., 2017). The role of IDO in MSC hepato-protective effect was highlighted again in a study which investigated MSC interaction with IL-17-producing iNKT (NKT17) cells in mouse models of neutrophil and iNKT cell–mediated acute liver failure. Milosavljevic and colleagues demonstrated that MSC treatment reduced serum levels of IL17 and increased IL10, whilst reducing the number of NKT17cells and increasing the presence of forkhead box P3^{+} IL10-producing natural killer T regulatory cells (NKT_{regs}). Interestingly, the capacity of neutrophils and T lymphocytes for IL17 secretion was not altered by MSC therapy. Treatment with MSC-CM resulted in a similar augmentation of NKT_{reg}/NKT17 ratio in the liver, attenuated hepatitis *in vivo* and reduced hepatotoxicity of NKT cells *in vitro*, blocked by inhibition of IDO (Milosavljevic *et al*., 2017).

Musso and colleagues investigated the interaction between hMSC and γδ T cells and found that MSCs inhibited γδ T cell-mediated lymphoid cell killing and that this was NKG2D-dependent. Interestingly, rituximab-induced antibody-dependent γδ T cell-mediated cytotoxicity was spared. NKG2D-dependent killing activity was recovered with pre-treatment of MSC with aminobisphosphonates (N-BPs), pamidronate or zoledronate, due to inhibition of MSC secretion of TGF-β and enhancement of IL-15 production. N-BP-treated MSC stimulated γδ T cells to differentiate into effector memory cells producing Th1-type cytokines *in vitro* (Musso *et al*., 2014).

1.8 Mechanisms of MSC-mediated immunomodulation

A variety of mechanisms have been proposed as contributing to the wide range of MSC immunoregulatory activities so far discussed: cell-to-cell contact (Krampera *et al* 2003; Beyth *et al*., 2005; Lee *et al*., 2017) secretion of soluble factors (Di Nicola *et al*., 2002; Djoud

et al., 2003; Milosavljevic *et al*., 2017), release of extracellular vesicles (Legaki *et al*., 2016; Sicco *et al*., 2017) and mitochondrial transfer (Islam *et al*., 2012; Ahmad et al., 2014; Jiang *et al*., 2016). It seems likely that the different mechanisms employed are determined, at least in part, by the local microenvironment to which the MSCs are first exposed; it has been suggested that these mechanisms may be used in concert as opposed to exploited individually at any given time point (Augello *et al*., 2005, Ryan *et al*., 2007, Trento and Dazzi, 2010; Lee *et al*., 2017).

1.8.1 Immunomodulation by cell-cell contact

Over the last 20 years there have been conflicting observations about the importance of cell-cell contact between MSC and immune effector cells, versus MSC secretion of soluble factors. Data from Di Nicola and colleagues suggested that cell-cell contact is not a mandatory requirement for MSC-mediated suppression of T cell proliferation, instead soluble factors such as TGF-β1 and HGF were demonstrated to be important for MSC effect (Di Nicola *et al*., 2002). Djoud's study provided corroborative data, demonstrating a role for soluble mediators in the observed MSC immunomodulatory effects, but TGF-β1 and HGF were not found to be significant contributors (Djoud *et al*., 2003). In contrast, Krampera and colleagues concluded that MSC inhibitory effect required cell contact, based on the observations that MSC activity was dose-dependent and transient, being entirely dependent on the presence of MSCs. They hypothesized that, through cell-cell contact, MSCs may physically hinder T cells from interacting with APCs (Krampera *et al*., 2003). Beyth and colleagues went on to demonstrate that hMSCs could inhibit T cells indirectly by induction of regulatory APCs with T cell-suppressive properties and that this was contact-dependent (Beyth *et al*., 2005). Augello and colleagues concluded that the mechanisms by which BMSC exert their immunomodulatory effects are pleiotropic and redundant, encompassing the activation of the inhibitory programmed death 1 (PD-1) pathway in a cell-to-cell contact fashion, and the secretion of TGF-β1 as a key soluble mediator (Augello *et al*., 2005).

Lee et al demonstrated that hMSC expression of ICOS ligand (ICOSL) contributed to T_{reg} induction *in vitro* when hMSCs were co-cultured with $CD4^+$ T cells. Furthermore, when cultured in a transwell plate, MSCs failed to induce T_{regs}, suggesting that cell contact

between MSCs and T cells was crucial to MSC-mediated T_{reg} induction (Lee *et al*., 2017). Recent data suggests that MSC immunomodulatory activity is reliant on MSC apoptosis, which is shown to be dependent upon MSC having physical contact with activated cytotoxic cells (Galleau *et. al*, 2017). Another recent study demonstrated the importance of cell-cell contact in MSC interaction with ILC3 cells in a murine model of Chagas cardiomyopathy (van Hoeven *et al*., 2018).

The strength of requirement for an initial cell contact phase between MSC and target cell remains controversial, but the importance of secreted paracrine mediators for ultimate signal generation is well established (Sato *et al*., 2007; Ryan *et al.,* 2007, Trento and Dazzi, 2010). Over the last decade studies have looked at the ways in which these bioactive factors are secreted by MSCs, particularly the role of extracellular vesicles (EVs). These include: exosomes which are small (30-100nm), homogenous and of endosomal origin; microvesicles (MVs) which are larger (100-1000nm), plasma membrane-generated non-homogenous entities and apoptotic bodies (Thery *et al*., 2009; Park *et al.*, 2019). Using stem cell-derived therapy such as EVs or MSC-conditioned media instead of actual MSCs is attractive because it avoids the potential complications of using live cells. These include the risk of tumour formation (Ridge *et al.,* 2017) and haemodynamic instability, specifically pulmonary embolism (Furlani *et al*., 2009), due to their physical properties.

Relevant to their application in treating infection, there is evidence that MSC EVs can have direct anti-microbial effects: porcine BM-MSC EVs have been demonstrated to exhibit anti-viral activity in the lungs of pigs infected with influenza (Khatri *et al*., 2018). Human BM-MSC EVs had antibacterial activity and anti-inflammatory effects in an *in vivo* murine model of *E Coli* pneumonia (Monsel *et al.,* 2015) and in an *ex vivo* perfused human lung injured with severe *E. coli* pneumonia (Park *et al*., 2019). Plasma membrane-generated MVs contain a variety of proteins, lipids, mRNAs, microRNAs and organelles, representative of those present in the cells from which they originate (Zhu *et al*., 2014).

Bruno *et al* demonstrated that human MSC-derived MVs were renal protective following kidney insult (Bruno *et al*., 2011). Subsequent work suggested that the therapeutic effect of these MVs could be attributed to the transfer of mRNA and miRNA to the injured renal epithelium, as opposed to representing a response to an individual paracrine mediator (Bruno *et al*., 2012). Indeed, Zhu et al demonstrated in an *ex-vivo* perfused human lung

model of ALI that MSC-MVs expressed the mRNA for keratinocyte growth factor (KGF), an MSC-secreted paracrine factor previously shown to restore alveolar fluid clearance in *E. coli* endotoxin and bacteria-induced acute lung injury. KGF siRNA-pretreatment of MSC partially reduced the therapeutic effect of MVs released by MSCs. The investigators concluded that the benefit of MVs in this model appears to be in part through KGF, via transfer of the mRNA to the injured alveolar epithelium and subsequent expression of the protein (Zhu *et al*., 2014). In line with this data, Lee et al observed that murine MSC EVs had protective effects on vascular remodeling in a mouse model of hypoxic pulmonary hypertension; influx of inflammatory cells and induction of pro-inflammatory mediators was significantly reduced. The biologically active component of the MSC CM was demonstrated to be exosomes, not paracrine soluble mediators; removal of the exosomes abrogated therapeutic effect (Lee *et al*., 2012). This fits with the data from Zhu et al where the therapeutic effect of MSC MVs mirrored that of the cells themselves (Zhu *et al*., 2014).

1.8.2 Immunomodulation by soluble factors

Two of the earliest soluble factors recognized as contributory to MSC immunosuppressive effects are TGF-β1 and HGF (Di Nicola *et al*., 2002). TGF-β1 is well known to have potent immunosuppressive properties, including the ability to inhibit pro-inflammatory cytokine secretion by APCs and to induce T_{regs} (Gollnick *et al*., 1995). HGF has been reported to support hematopoiesis and protect against apoptosis (Kuroiwa *et al*., 2001). hMSC have been demonstrated to constitutively express TGF-β1, HGF and IL-10 at immunosuppressive concentrations (Ryan *et al.* 2007). Consistent with the concept of MSC licensing being a fundamental first step in MSC immunomodulatory activities, stimulation with IFN-γ led to a significant increase in MSC secretion of TGF-β1 and HGF; IL-10 levels were unchanged (Ryan *et al*., 2007). In contrast, MSC pre-treatment with aminobisphosphonate pamidronate has been shown to decrease TGF-β1 secretion and stimulate production of IL-15 (Musso *et al*., 2014). Di Nicola and colleagues observed that simultaneous neutralisation of TGF-β1 and HGF restored T cell proliferation (Di Nicola *et al*., 2002). This was not the case in subsequent studies which demonstrated that neutralisation of these factors failed to prevent MSC-mediated suppression (Tse *et al*., 2003, Ryan *et al*., 2007). Ryan and colleagues concluded

that there may be redundancy in the mechanisms of immunosuppression employed by MSC, or alternative more dominant mechanisms at work (Ryan et al., 2007).

PGE_2 is also acknowledged to be an important mediator of MSC immunosuppressive activities (Aggarwal and Pittenger, 2005; Nemeth *et al*., 2009). It has been demonstrated to coordinate quite complex and far reaching effects, which result in transformation of an inflammatory environment into an anti-inflammatory environment by modulating DC and T cell cytokine secretion (Harris *et al*., 2002; Aggarwal and Pittenger, 2005). Prostaglandins are derived from arachidonic acid by the enzyme cyclo-oxygenase (COX) (Harris *et al*., 2002). hMSC have been observed to express both isoforms of this enzyme, COX-1 and COX-2 constitutively, with significant levels of PGE2 detected in the supernatants of these cultures (Ryan *et al*., 2007). A number of studies have reported that the immunosuppressive effects of hMSCs can be significantly blunted by inhibiting PGE_2 synthesis (Aggarwal and Pittenger, 2005; Spaggiari *et al*., 2006; Ryan *et al*., 2007).

The roles of the various factors identified differ between murine and human MSCs (Meisel *et al*., 2004; Sato *et al*., 2007). An example of this is indoleamine 2,3-dioxygenase (IDO), a potent immunosuppressive enzyme which exerts prominent effects in humans but is not utilised to the same extent by murine MSCs (Meisel *et al*., 2004); instead mMSC secrete nitric oxide (NO) as a key mediator of T cell suppression (Sato *et al*., 2007; Ren *et al.,* 2009). IDO has long been established as important in controlling human T cell responses to autoantigens and alloantigens (Munn *et al*., 1998; Hwu *et al*., 2000). IDO promotes tryptophan degradation and kynurenine synthesis, which deprives immune cells of nutrients and inhibits their growth and function (Munn *et al*., 1998). hMSC, when exposed to an inflammatory environment, have been reported to secrete IDO, which has been implicated in the observed anti-proliferative effects on T cells (Meisel *et al*., 2004; Ryan *et al*., 2007), NK cells (Spaggiari *et al*., 2006) and B cells (Krampera *et al*., 2006). Addition of tryptophan was shown to mitigate hMSC immunosuppressive effects, restoring allogeneic T cell proliferation (Meisel *et al*., 2004). Tryptophan-derived catabolites may also have direct toxic effects on T cells and NK cells (Frumento *et al*., 2002). IDO has been reported as working in synergy with PGE2 to inhibit T cell proliferation (Di Nicola *et al*., 2002).

NO, utilised by mMSCs, is synthesized by the inducible isoform of the enzyme NO synthase (iNOS); the induction process in MSC appears to require contact with activated T cells (Sato

et al., 2007). NO reduces phosphorylation of transcription factor Stat 5, which is pivotal to T-cell development and function (Bingisser *et al*., 1998). Thus, mMSC-secreted NO is an important contributor to mMSC antiproliferative effects. Indeed, Sato and colleagues demonstrated that MSC deficient in iNOS (iNOS $^{-}/^{-}$), and therefore unable to generate NO, were less effective at suppressing T cell proliferation than WT MSC (Sato *et al*., 2007).

There is evidence to suggest that human and murine utilisation of IDO versus iNOS is dependent on the local cytokine milieu. It has been shown that under T_H1 and T_H2 conditions IDO is important for hMSC-mediated immunomodulation whereas mMSCs use iNOS (Meisel *et al*., 2004; Ren *et al*., 2009; Ling *et al*., 2009). However, under T_H17 conditions, murine MSCs do not produce NO, instead their immunosuppressive effects are mediated through the production of other mediators, including IDO (Tatara *et al*., 2011). Tatara and colleagues demonstrated that MSCs can suppress the generation of effector T_H17 cells in an IDO-dependent manner (Tatara *et al*., 2011). This concept is supported by data showing that IDO can act as a critical molecular switch that stimulates the immunosuppressive properties of T_{regs} and blocks the reprogramming of T_{regs} into IL17-producing effector T cells (Matteoli *et al*., 2010).

Another factor secreted by hMSCs demonstrated to contribute to their immunosuppressive properties is histocompatibility locus antigen HLA-G (Nasef *et al*., 2007; Selmani *et al*., 2008). HLA-G is well established as an important contributor to immune tolerance, assisting fetal allografts to evade maternal rejection, (Rouas-Freiss *et al*., 1997). HLA-G is a nonclassical MHC class I molecule, expressed in both membrane-bound and soluble isoforms, which can interact with DCs, NK cells and T cells to induce a tolerogenic state (Rouas-Freiss et al., 1997; Carosella *et al*., 2000). Soluble HLA-G (HLA-G5), secreted by hMSC stimulated with IL-10, has been shown to inhibit NK cell and $CD8^+$ T cell-mediated cytolysis, induce a T_H2 cytokine profile in $CD4^+$ T cells and induce the expansion of T_{regs} (Selmani et al., 2008). Membrane-bound HLA-G, derived from hMSCs, has been shown to suppress T cell proliferation in MLRs (Nasef *et al*., 2007). Significant restoration of T cell proliferation was observed following the addition of HLA-G neutralising antibody, suggesting that HLA-G may be partly responsible for hMSC-mediated anti-proliferative effects *in vitro* (Nasef *et al*., 2007).

Anti-inflammatory protein TNF-α-induced protein 6 (TSG-6) is another soluble mediator associated with MSC protective effects (Lee *et al*., 2009b; Qi *et al*., 2014). Prockop's group demonstrated the intriguing mechanism by which hMSCs, administered intravenously, lodged in the lung and became activated to secrete TSG-6; TSG-6 exerted potent anti-inflammatory effects in a mouse model of myocardial infarction (MI) (Lee *et al*., 2009b). MSC protective effects were partially negated by knockdown of the TSG-6 gene prior to infusion of the cells. Furthermore, administration of human recombinant TSG-6 was observed to partially emulate MSC effect (Lee *et al*., 2009b). hMSC-released TSG-6 has also been shown to play a role in accelerating wound healing and reducing tissue fibrosis in a mouse model of full-thickness skin wounds (Qi *et al.*, 2014). In this study, MSC-derived TSG-6 suppressed macrophage TNF-α synthesis, disrupting the previously reported autocrine amplification of macrophage activation (Wang *et al*., 2006; Sindrilaru *et al.*, 2011). Interleukin-1 receptor antagonist (IL-1ra) is also recognised as an MSC paracrine mediator with immunosuppressive activity towards macrophages (Ortiz *et al*., 2007). IL-1ra competitively competes with IL-1β for IL-1 receptor binding; IL-1β is a potent inflammatory cytokine regularly present in the pulmonary oedema fluid in patients with acute lung injury (ALI) (Geiser *et al*., 2001). Ortiz and colleagues demonstrated that MSC-conditioned media could suppress proliferation of an IL-1α-dependent T cell line and inhibit production of TNF-α by activated macrophages *in vitro* (Ortiz *et al*., 2007).

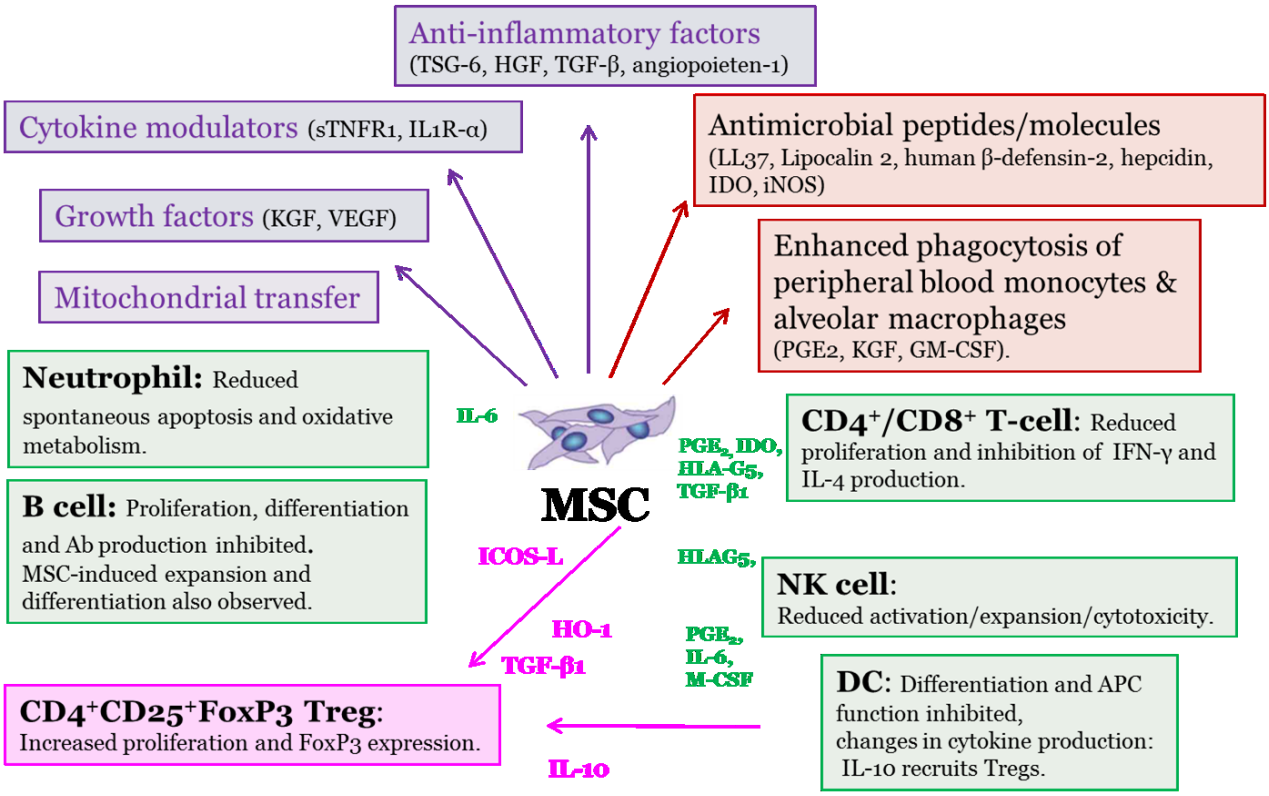


Figure 1.1 Schematic diagram representing the wide range of MSC immunomodulatory activities and some of the paracrine mediators involved. Adapted from Najib El Haddad (2011).

Di Nicola *et al*., 2002; Bartholomew *et al*., 2002; Rafaghello *et al*., 2008; Spaggiari *et al*., 2006; Aggarwal and Pittinger, 2005; Glennie *et al*., 2005; Marigo *et al*., 2011; Ramasamy *et al*., 2007; Corcione *et al*., 2006; Krasnodembskaya *et al* ., 2010 & 2012; Meisel *et al*., 2011, Mougiakakos *et al*., 2011; Islam *et al*., 2012; Alcayaga-Miranda *et al*., 2015; Sung *et al*., 2016; Lee *et al*., 2017.

1.8.3 Restoration of host cell bioenergetics by mitochondrial transfer

Another intriguing mechanism of MSC protective effect is the transfer of mitochondria from exogenously administered MSC to injured host cells (Islam *et al*., 2012; Ahmad *et al*., 2014; Jiang *et al*., 2016; Jackson *et al*., 2016; Morrison *et al*., 2017). This was first demonstrated in a study by Islam and colleagues in which mice, exposed to airway instilled LPS and treated with locally administered mBMSCs, underwent live optical imaging of MSC interaction with alveolar cells. Fluorescently labeled mitochondria within the MSCs were shown to traffic within microvesicles to injured alveoli via connexin 43 (Cx43)-containing gap junctional channels formed between MSC and alveolar epithelium. Mitochondrial transfer increased alveolar ATP, restoring host cell bioenergetics. Futhermore, the attenuation of LPS-induced lung injury observed following treatment with wild type MSCs was abrogated by treatment with mutant MSC unable to form gap junctional channels with neighboring epithelial cells, or by mBMSCs with dysfunctional mitochondria. The investigators concluded that restoration of alveolar bioenergetics through Cx43-dependent alveolar attachment and mitochondrial transfer is one of the mechanisms by which MSCs exert their protective effect in acute lung injury (ALI) (Islam *et al*., 2012).

The relative contributions of mitochondrial transfer versus paracrine secretions have yet to be clarified. It seems plausible though that both could be in play, if not by the same MSC at a specific time point. Mitochondrial loss in alveolus-attached BMSCs may decrease ATP in these cells, impairing their secretory capacity. However, non-attached mitochondria-replete BMSCs may continue to provide protective paracrine secretions (Islam *et al*., 2012). Subsequent to this study, Ahmad and colleagues published their evidence of intercellular mitochondrial movement from MSC to epithelial cells in mouse models of airway injury and asthma (Ahmad *et al*., 2014). They attributed regulation of this transfer process to Miro 1, a mitochondrial Rho-GTPase. Over expression of Miro 1 in MSC enhanced mitochondrial transfer and rescue of epithelial injury, while Miro 1 knockdown resulted in loss of MSC protective effect. Importantly, anti-inflammatory MSC products such as PGE2, TGF-β1 and IL-10 were unchanged by Miro 1 overexpression, excluding non-specific paracrine effects (Ahmad *et al*., 2014).

Another recent study demonstrated the process of mitochondrial transfer between MSCs and epithelial cells in the cornea (CECs). Transfer occurred via formation of tunneling nanotubes between MSCs and CECs, tubular connections that allowed direct intercellular communication. Separation of MSCs and CECs by a transwell culture system abrogated mitochondrial transfer from MSCs to CECs (Jiang *et al*., 2016). Interestingly, mitochondrial transfer efficiency was improved following exposure of CECs to Rotenone (Rot)-induced oxidative stress. Rot is a specific inhibitor of mitochondrial respiratory chain complex I, it causes mitochondrial dysfunction by enhancing production of mitochondrial reactive oxygen species (Li *et al*., 2003; Ahmad *et al*., 2014). This observation suggests that MSCs can respond to signals from 'stressed' epithelial cells and proactively engage with them to assist in restoration of cellular bioenergetics in an effort to prevent cellular damage (Jiang *et al*., 2016). Consistent with these data discussed, an unsupervised network analysis (Benjamini *et al*., 2001) of genome-wide expression microarray data on the transcriptional response to MSC treatment of sepsis revealed a host of findings, including the normalisation of pathways responsible for maintaining mitochondrial function (dos Santos *et al*., 2012).

1.8.4 MSC apoptosis

A recent study has addressed the conundrum that MSCs can exert potent immunosuppressive and anti-inflammatory effects, despite their lack of engraftment (Eliopoulos *et al*., 2005; Nauta *et al*., 2006; von Bahr *et al*., 2012) and their apparent disappearance within hours of administration (Lee *et al*., 2009; Ankrum *et al*., 2014). Using a murine model of GvHD Francesco Dazzi's group demonstrated that recipient cytotoxic T cells actively induced infused hMSC to undergo caspase activation and perforin-dependent apoptosis, in the lungs of MSC-treated mice, and that this is critical for MSC-induced immunosuppression. The absence of *in vivo* MSC apoptosis in naïve and BM mice confirmed that MSC apoptosis is not simply the result of xenogeneic recognition of human MSC, because it is detected only in GvHD mice (Galleau *et al*., 2017). Recipient phagocytes engulfed apoptotic MSC (apoMSC) and produced IDO (Galleau *et al*., 2017), which is well known to be a potent immunosuppressor (Munn *et al*., 1998; Hwu *et al*., 2000). McGaha et al have previously shown that the injection of irradiated thymocytes into mice resulted in efferocytosis by recipient macrophages and induction of IDO (McGaha *et al*., 2011). In

Galleau's study, depletion of recipient macrophages, or the inhibition of IDO activity, impaired the therapeutic activity of infused MSC, highlighting the importance of MSC efferocytosis for treatment effect (Galleau *et al*., 2017). Other studies have demonstrated MSC lysis *in vitro* (Spaggiari *et al*., 2006) and MSC clearance *in vivo (Eliopoulos et al., 2005; Nauta et al., 2006;* Moll *et al.,* 2012; Li *et al*., 2012*)* but these studies have not clearly demonstrated the link between *in vivo* MSC apoptosis and induction of MSC immunosuppressive activities.

Another interesting observation from Galleau's study is that the effectiveness of apoMSC was dependent on the route of administration, i.p injection achieved the best treatment response. Despite being phagocytosed, apoMSC injected i.v. did not induce IDO production, perhaps because these MSC failed to engage with a specific subpopulation of phagocytes (Galleau *et al*., 2017). Of clinical interest, GvHD patients with high levels of cytotoxic activity were observed to have a better response to MSC therapy than patients with low levels, adding weight to the concept that MSC interaction with cytotoxic T cells is key to treatment efficacy. The investigators suggest that cytotoxic activity could be used as a potential biomarker to predict patient response to MSC treatment. They conclude with the proposal that patients should be stratified for MSC treatment according to their ability to kill MSC, or that all patients could be treated with MSC rendered apoptotic *ex vivo* prior to infusion (Galleau *et al*., 2017).

1.9 MSC Clinical Applications

The first human study of allogeneic bone marrow-derived MSC transplant was in children with osteogenesis imperfecta, a genetic disorder in which osteoblasts produce defective type I collagen, leading to osteopenia, bony deformities, fractures and stunted growth (Horwitz *et al*., 1999). Prior to this, bone marrow transplantation had not been considered as a means of treating disorders involving cells of mesenchymal origin, merely haematopoietic cell disorders. Horwitz and colleagues demonstrated that mesenchymal progenitors in transplanted marrow could migrate to bone and give rise to functional osteoblasts, and that this correlated with an enhancement in bone mineral content,

integrity and fracture resistance. Importantly they showed that low levels of MSC engraftment (1.5-2% at 3 months) was sufficient to achieve satisfactory synthesis of normal collagen to convert the severe form of the condition to a much milder phenotype (Horwitz *et al*., 1999). Subsequent studies in patients with inborn errors of metabolism, such as Hurler syndrome, a genetic disorder that results in the accumulation of glycosaminoglycans (GAGs) due to a deficiency of a specific enzyme, were encouraging (Koc *et al*., 2002). The investigators demonstrated a lack of adverse effects and some improvement in nerve-conduction velocity, however no survival advantage or notable improvement in clinical status was observed (Koc *et al*., 2002).

Consistent with *in vitro* studies of MSC immunomodulatory effects, murine allogeneic MSC were found to be effective in treating a variety of murine models of human disease, for example experimental autoimmune encephalomyelitis (EAE) (Constantin *et al.,* 2009) and Type 1 diabetes mellitus (Fiorina *et al*., 2009). Human MSCs also proved to be an effective cellular therapy when administered systemically to animal models of multiple sclerosis (Bai *et al*., 2009), inflammatory bowel disease (Gonzalez-Rey *et al*., 2009), diabetes (Lee *et al*., 2006) and rheumatoid arthritis (Gonzalez *et al*., 2009). The last 10-20 years has seen a surge in attempts at clinical application of MSC therapy. To date, over 700 MSC-related clinical trials are currently registered (May 2018; ClinicalTrials.gov), of which a significant proportion are focused on treatment for autoimmune diseases, hematopoietic disorders and transplantation-related conditions such as graft-versus-host disease (GvHD). A comparatively small number are dedicated to MSC treatment for sepsis and sepsis-related complications such as Adult Respiratory Distress Syndrome (ARDS).

1.9.1 Graft versus host disease (GvHD)

The clinical application of MSC is best studied in GvHD, a condition where donor HSC attack the immune cells of the recipient (Korngold and Sprent, 1978; Socie and Blazar, 2009). The first successful usage of MSC therapy for this disorder was in a nine-year-old boy suffering from a severe form of GvHD, resistant to treatment with conventional steroids, who received haploidentical third-party MSC, harvested from his mother. The boy was treated 73 days post bone marrow transplantation and again 100 days later. On both occasions he

responded well and survived beyond 1 year. This is in contrast to the 24 other patients with acute grade IV GvHD treated at the same center, but not with MSC therapy, who died an average of 2 months after bone marrow transplantation. (Le Blanc *et al*., 2004). This landmark case study provided an early glimpse of MSCs' therapeutic potential. Following this, MSC therapy was trialed in a larger group of patients with steroid resistant GvHD. Again, results were encouraging, demonstrating a survival advantage with MSC treatment (Ringden *et al*., 2006; Le Blanc *et al*., 2008). MSCs have also been used to treat chronic GVHD with positive effects (Weng *et al*., 2010). They have been utilised for their wound healing and anti-inflammatory activities in addition to their immune inhibitory effects. For example, MSCs have been demonstrated to reverse tissue toxicity such as hemorrhagic cystitis, pneumo-mediastinum and colon perforation with peritonitis (Ringden *et al*., 2007).

By 2010 allogeneic MSCs were being successfully employed for treatment of severe steroid-refractory GVHD in children with response rates of more than 60% at 28 days post infusion, correlating with 100-day survival, suggesting that MSC therapy had an excellent risk–benefit ratio (Guo *et al*., 2009; Macmillan *et al*., 2009). Co-transplantation of MSCs and HSCs as a means of GVHD prophylaxis has subsequently been explored. The protocols of these trials involve co-transplantation of HSCs and third-party MSCs, or transplantation of both cell types from the same donor (Baron *et al*., 2010). MSC protective effect has been attributed to the secretion of immunosuppressive factors such as IDO, IL-10, TGF-β1 and PGE2, which can inhibit the proliferation and function of innate and adaptive immune cells: macrophages, neutrophils, NK cells, DCs, T cells, and B cells and promote the expansion of T_{regs} (Nemeth *et al*., 2009; Maggini *et al*., 2010; Luz-Crawford *et al*., 2013; Bernardo and Fibbe, 2013). A recent study has demonstrated that in addition to these previously noted mechanisms of action, MSCs also have the capacity to stimulate the proliferation and IL-22 secretion of innate lymphoid cells, specifically ILC3s, which may contribute the protective effects shown in GvHD (van Hoeven *et al.,* 2018). ILCs have previously been shown to have a protective role in the development of GvHD, thought to be attributable, in part, to their secretion of IL-22 (Munneke *et al*., 2014; Lindemans *et al*., 2015).

1.9.2 Lung disease

Many of the principles discussed above are relevant to the application of MSCs to lung disease, acute and chronic. Following acute lung injury (ALI) caused by severe infection or exposure to toxin, some of the paracrine soluble factors produced by MSCs have been shown to have a therapeutic role by restoring alveolar fluid clearance and lung permeability; candidates include keratinocyte growth factor (KGF) (Lee *et al*., 2009) and angiopoietin-1 (Mei *et al*., 2007). In addition, MSC-derived immunomodulation of innate and adaptive immune cells may reduce alveolar inflammation (Ortiz *et al*., 2007; Nemeth *et al*., 2009; Zheng *et al*., 2014; Zhu *et al*., 2014; Park *et al*., 2019).

Cystic fibrosis (CF) is a chronic suppurative lung disease with multisystem involvement inherited in an autosomal recessive manner. The major defect in the conductance regulator (CFTR) protein has recently been addressed by corrector and potentiator drugs such as ivacaftor, lumacaftor and tezacaftor (Davies *et al*., 2013, 2018). Whilst this has been a huge step forward for CF treatment, essentially 'normalising' the airway surface fluids and mucous viscosity by facilitating sodium and chloride transportation, these drugs do not specifically target the inflammatory process which underpins the lung tissue damage associated with this condition. Inflammation, even in the absence of infection, is thought to be an independent risk factor for CF disease progression and has been demonstrated in the uninfected lungs of infants in the first few months of life (Armstrong *et al*., 1997; Frayman *et al*., 2017). These children do better if their lower airway microbiota remains relatively diverse; decreased microbial diversity in older patients has been shown to correlate with severe lung disease (Delhaes *et al*., 2012; Dickson *et al*., 2014). Specific pathogens, including *Pseudomonas aeruginosa*, are associated with increased airway inflammation (Gangell *et al*., 2011).

Components of the MSC secretome with anti-inflammatory properties have the potential to redress the balance of the CF lung, which has been demonstrated to exhibit increased production of bioactive lipids such as ceramide and down regulation of antioxidant genes such as HO1 (Caretti *et al*., 2014). In addition to the anti-inflammatory impact of reducing ceramide it has been demonstrated that its reduction enhances expression of key genes

involved in pathogen clearance, specifically *A. fumigatus* (Caretti *et al*., 2016). It follows that these effects could be additionally enhanced by MSC / MCV (EV) direct antimicrobial activity against the pathogens most associated with lung function decline: *S. aureus, P. aeruginosa* and *Burkholderia cepacia* complex**.** Zulueta et al showed that EVs isolated from human lung MSCs downregulated transcription and protein expression of a range of pro-inflammatory cytokines (IL-1β, IL-8 and IL-6) in a cellular model of cystic fibrosis stimulated with TNF-α (Lannan *et al*., 2015; Zulueta *et al*., 2018). The specific vesicular mediators were not identified in this study, but the investigators concluded that the pathway pivotal to the MSC anti-inflammatory effect was the PPARγ axis, which was upregulated. This inhibits downstream effector NF-κB, an established driver of inflammation (Zulueta *et al*., 2018). An interesting concept highlighted by this work is that the MSC EVs do not act as a "specifc drug" because of the wide range of mediators contained within the vesicles, which may target multiple molecular mechanisms.

1.9.3 Sepsis

There remains a relative paucity in clinical trials dedicated to MSC treatment in sepsis compared to their use in immune and inflammatory diseases. The first clinical study of MSCs in sepsis commenced in 2013 in Russia. The investigators evaluated the 28-day efficacy of intravenous MSC treatment in 30 patients with sepsis complicated by severe neutropenia. They concluded that MSC treatment improved short-term survival but not organ failure and eventual mortality (Galstian *et al*., 2015). A Canadian trial (NCT02421484) is currently recruiting patients to evaluate the safety of allogeneic BM MSC for the treatment of septic shock by screening for adverse effects at different MSC doses. Safety data from a Belgian Phase 1 trial (NCT02328612) in which allogeneic adipose-derived MSC treatment was given to healthy volunteers pre-administration of bacterial endotoxin (LPS) was encouraging, with no significant adverse effects reported. Adipose-derived MSC have also been demonstrated as safe and well tolerated in a Phase 1 clinical trial of sepsis-induced ALI (NCT01902082). However, the clinical impact at the dose used was not significant (Zheng *et al*., 2014).

1.9.4 Future considerations

In the last decade interest has grown in the bioactive factors that MSCs produce, the MSC secretome, and the potential clinical application of this cell-free therapy. The MSC secretome, and conditioned media from MSCs, has key advantages over stem-cell based applications: immune compatibility, reduced tumourigenicity, lack of emboli formation and reduced risk of infection (Eiro *et al*., 2014; Bermudez *et al*., 2015, 2016). In addition, components of the MSC secretome may be more accurately assessed for safety, potency and dosage than aliquots of live MSCs. Storage of secretome components does not require the toxic cryopreservative agents that live cells need for preservation long-term, nor is potency as affected by storage (Bermudez *et al*., 2016; Vizoso *et al*., 2017). Mass-production may even be possible by using tailor-made cell lines under calculated laboratory conditions, providing a reliable supply of bioactive factors, which could be modified as desired by manipulation of the cell-line. This could facilitate treatment of acute conditions such as ischaemic stroke, myocardial infarction and sepsis with bespoke "off-the-shelf secretome therapies" (Vizoso *et al*., 2017). The isolation of MSCs from more accessible anatomic niches such as the cervix (via pap smear) yielding human uterine cervical stem cells (hUCESCs) (Eiro *et al*., 2014; Bermudez *et al*., 2015) and menstrual blood (Rossignoli *et al*., 2013) makes the process of acquisition less invasive than for the more traditional bone-marrow or adipose tissue-derived MSC. This may increase both MSC availability and donor diversity which could serve to enhance the MSC secretome.

An alternative view point is that optimisation of MSC treatment may not be so much about identifying the most clinically effective MSC subpopulation, or MSC secretome components, but may revolve around patient selection. Galleau et al recently showed that patient-derived cytotoxic T cells were required to be in an activated state in order to induce MSC apoptosis and subsequent uptake by macrophages, which induced IDO-dependent immunosuppression (Galleau *et al*., 2017). The investigators raised the intriguing possibility that if apoptosis is indeed crucial for MSC immunomodulatory activity, then rendering them apoptotic *ex vivo*, prior to administration, could circumvent the need for patient-derived cells to play their part. Thus, *ex vivo* generated apoMSC may be effective in patients who are refractory to standard MSC treatment (Galleau *et al*., 2017).

1.10 MSC and infection

There is an increasing body of evidence that MSC therapy can protect against widespread apoptosis and endothelial/epithelial dysfunction and enhance bacterial clearance to improve clinical outcome following localised infection and sepsis. MSCs from a variety of anatomical sources have been observed to reduce bacterial burden in a range of preclinical models, independently of dose, timing and route of administration (Gonzalez-Rey *et al.,* 2009; Nemeth *et al.,* 2009; Krasnodembskaya *et al*., 2010 and 2012; Lee *et al*., 2013; Alcayaga-Miranda *et al*., 2015; Sutton *et al*., 2016).

1.10.1 MSC enhance bacterial clearance

It has been known for some time that BM-derived MSCs *in vivo* reside in a perivascular position, perfectly placed to detect microbial invasion and participate in initial inflammatory events (Paquet-Fitfield et al., 2009). It has been suggested that through their expression of selectin, integrin and chemokine receptors, BMSCs exposed to pathogen may directly engage invading microbes, effectively holding them hostage whilst host immune effector cells prepare for attack (Auletta *et al*., 2015). In addition, MSCs may impede pathogen growth by directly competing for nutrients or by making the local environment in some way hostile to pathogen persistence (Auletta *et al*., 2015; Zhang *et al*., 2016). One way in which MSCs have been reported to do this is by secretion of antimicrobial peptides/proteins (AMPs).

AMPs or host-defense peptides/proteins (HDPs) are a diverse group of evolutionary conserved endogenous molecules produced as an initial defense by organisms from prokaryotes to humans (Brogden, 2005; Zhang *et al*., 2016). As many as 2700 AMPs/HDPs have been recorded (http:// aps.unmc.edu/AP/main.php). Collectively, they exhibit a broad spectrum of antimicrobial and immunomodulatory activities against a wide range of pathogens: bacteria, viruses, fungi and yeasts (Zhang *et al*., 2016). They display various mechanisms of action to achieve their goal of pathogen elimination, some of which are influenced by the local environment, making factors such as pH and salt concentration important for their activity (Brogden, 2005; Dutta & Das, 2016). AMP-mediated pathogen

killing can occur at the cell surface, by destroying membrane integrity, or within the cell, by targeting certain intracellular organelles or inhibiting DNA/RNA/protein synthesis (Brogden *et al*., 2005). Of clinical relevance is that some AMPs have been identified as active against bacteria which are resistant to conventional antibiotics, making them attractive for treating multi-drug resistant infections (Seo *et al*., 2012). In addition to their antimicrobial effects, certain AMPs have immunomodulatory functions, such as modulation of pro-inflammatory cytokines, anti-endotoxin activities and angiogenic properties, which help to resolve inflammation which may be tissue damaging (Frew *et al*., 2011; Zhang *et al*., 2016).

MSCs have been demonstrated to constitutively express four AMPs: cathelicidin LL-37 (Krasnodembskaya *et al*., 2010; Sutton *et al.,* 2016), lipocalin-2 (Gupta *et al*., 2012), human β-defensin-2 (Sung *et al*., 2016) and hepcidin (Alcayaga-Miranda *et al*., 2015). This constitutive expression can be upregulated by exposure to bacteria or inflammatory stimuli during the infection process (Krasnodembskaya *et al*., 2010; Alcayaga-Miranda *et al*., 2015; Sutton *et al.,* 2016). In one of the early studies exploring the mechanistics of MSC antimicrobial effect, Krasnodemskaya and colleagues demonstrated that human BMSCs could inhibit *in vitro* bacterial growth of a range of pathogens, including *P. aeruginosa*, through secretion of human cathelicidin antimicrobial peptide, hCAP (also known as LL37). Blocking LL37 with monoclonal antibody abrogated MSC effect. Furthermore, MSC-induced LL37 impeded bacterial growth *in vivo* in a murine model of *E. coli* pneumonia (Krasnodembskaya *et al*., 2010). LL-37 has subsequently been observed to exhibit immunomodulatory, chemotactic, angiogenic and anticancer effects in addition to its antimicrobial activities (Wang. 2014). Derangement in its levels has been observed to contribute to various disease processes, including in the lungs, where reduced levels are found in patients with asthma (Xiao *et al*., 2005; Hiemstra *et al*., 2016). In an *in vivo* murine model of CF lung infection and inflammation, Sutton and colleagues demonstrated that LL-37 secreted by hMSCs slowed the rate of growth of Gram negative and Gram positive pathogens: *P. aeruginosa, S. aureus,* and *S. pneumoniae*. In addition, the hMSCs appeared to enhance antibiotic sensitivity, thus increasing bacterial killing further (Sutton *et al*., 2016).

Michael Matthay's group demonstrated enhanced bacterial clearance from the lung in a murine model of *E. coli* pneumonia as early as 4 hours after MSC installation as a result of

upregulation of antibacterial peptide lipocalin 2. Whilst MSCs themselves were noted to be a source of this peptide, far higher concentrations were achievable by culturing MSCs and alveolar macrophages together, in the presence of LPS. This suggests that a synergistic effect exists between MSCs and macrophages in the production of this antimicrobial peptide (Gupta *et al*., 2012). Lipocalin 2 is not simply a bacterial killer but has an important role in innate defense against microbes (Dittrich *et al*., 2013). It has been shown to sequester siderophores, bacterial iron chelators, which prevents bacteria obtaining iron for growth, resulting in bacteriostasis (Zhang *et al*., 2016). Mice deficient in the gene encoding Lipocalin-2 have been shown to exhibit increased mortality to *E. coli*-induced sepsis, due in part to the overwhelming bacterial burden (Berger *et al*., 2006).

Akin to LL-37, Defensins have two major functions in host defense: direct inhibition/elimination of invading microorganisms and modulation of innate and adaptive immune response to these pathogens (Doss et al., 2010). β-Defensins: hBD-1, 2 and 3 have been identified as important functional defensin peptides in humans (Wang *et al*., 2014), secreted by a range of cell types including hMSC (Sung *et al*., 2016). hBD-1 and 2 target Gram negative bacteria predominantly; hBD-3 is more broad spectrum (Mattar *et al*., 2016). The expression of hBD 2 and 3 has been shown to be inducible following exposure to pathogen or pro-inflammatory stimuli (Duits *et al.,* 2003). This feature is attractive when considering clinical application, particularly in conditions where AMP generation is impaired, such as asthma, COPD and CF (Doss *et al*., 2010). Hepcidin is another HDP, produced mainly by hepatocytes, but also by MSCs, which has a range of antimicrobial activity against bacteria and fungi (Alcayaga-Miranda *et al*., 2015). Hepcidin synthesis is induced by systemic iron levels and by inflammatory stimuli. During infection, like Lipocalin-2, its induction causes depletion of extracellular iron, which results in the withholding of iron from invading microorganisms (Michels *et al*., 2015). In a CLP model of polymicrobial sepsis, hepcidin-secreting MSCs derived from menstrual blood (MenSCs) enhanced bacterial clearance, reduced organ injury and improved survival when administered in combination with antibiotics. Hypoxic pre-conditioning of MenSC caused hepcidin to be downregulated which resulted in the loss of MSC antibacterial activity, implying a hepcidin-dependent mechanism. This study reported a lower bacterial burden in septic mice treated with MSCs compared with those given targeted antibiotic therapy. Of note, the group which received

antibiotic in combination with MSCs had the highest survival rate, indicating a synergistic beneficial effect of MSCs and antibiotics (Alcayaga-Miranda *et al*., 2015).

Another important molecule, secreted by human but not murine MSCs, and not classified as an AMP or HDP, is IDO. IDO is well known to have potent immunosuppressive effects on T-cells via its catabolism of tryptophan (Munn *et al*., 1998; Hwu *et al*., 2000), a process which is also recognised to impede bacterial growth (Meisel *et al*., 2011). Of note, the concentration of tryptophan required for bacteria to grow is approximately 25 times higher than that required for T cells (Meisel *et al*., 2011). It may assist MSCs in balancing direct attempts at pathogen elimination with immunoregulatory activities geared to controlling the inflammatory response if IDO can have its antimicrobial impact before its T cell suppressive effects (Mezey and Nemeth, 2015). Mouse BM-MSCs have also been demonstrated to secrete IL-17 which had potent antifungal activity against *Candida albicans*, partially abrogated by anti-IL-17 antibodies (Yang *et al*., 2012).

A series of studies conducted by Michael Matthay's group explored the impact of hMSCs on murine and human models of *E coli*-induced ALI, focusing on the specific paracrine mediators involved and the mechanism of interaction between MSC and the injured lung cells. MSC treatment resulted in an improvement in alveolar fluid clearance, decreased inflammation and reduced bacterial growth in the lung (Lee *et al*., 2013; Zhu *et al*., 2014; Park *et al*., 2019). The mechanisms by which MSCs had their effects included promotion of bacterial phagocytosis by alveolar macrophages, in part through secretion of KGF. *In vitro* studies suggested that MSCs protect blood monocytes expressing KGF receptor from apoptosis and enhance GM-CSF secretion, further augmenting MSC antimicrobial effect (Lee *et al*., 2013).

In line with this data, there is an increasing body of evidence that a key mechanism by which MSCs promote bacterial clearance is through enhancing monocyte/macrophage phagocytosis (Hematti *et al*., 2009; Mei *et al*., 2010; Krasnodembskaya *et al*., 2012; Jackson *et al*., 2016, Morrison *et al*., 2017). Krasnodembskaya and colleagues demonstrated that in a mouse model of peritoneal sepsis induced by gram-negative bacteria there was a marked reduction in the number of bacterial colony-forming units of *P. aeruginosa* in the blood of

MSC-treated mice compared with fibroblast 3T3-treated and PBS-treated control groups. Enhanced bacterial clearance in MSC-treated mice was associated with improved survival at 48h post septic insult. *In vitro* phagocytic activity was increased in blood monocytes isolated from mice treated with MSC compared with the 3T3 and PBS groups. Furthermore, monocytes isolated from MSC-treated mice exhibited upregulation of the phagocytosis receptor CD11b (Krasnodembskaya *et al*., 2012). Hematti et al demonstrated that human MSCs co-cultured with macrophages induced an M2 macrophage phenotype, characterised by enhanced phagocytic activity and anti-inflammatory properties (Kim and Hematti, 2009).

A recently identified mechanism of MSC antimicrobial activity linked to enhanced host cell phagocytosis is mitochondrial transfer (Jackson *et al*., 2016; Morrison *et al*., 2017). Jackson et al. showed that MSC transfer of their mitochondria to macrophages, via the formation of cytoplasmic bridges, induced an increase in macrophage mitochondrial metabolism and phagocytic index. Inhibition of MSC mitochondrial transfer reduced MSC antimicrobial effect (Jackson *et al*., 2016). In a model of ALI the same group showed that mitochondrial transfer can occur via MSC EVs, resulting in enhanced macrophage phagocytosis and a more anti-inflammatory M2 phenotype (Morrison *et al*., 2017).

The concept of MSCs enhancing monocyte/macrophage phagocytosis is supported by microarray data from Duncan Stewart's group showing down-regulation of inflammation related genes and up-regulation of genes involved in promoting phagocytosis and bacterial killing in a CLP model of polymicrobial sepsis treated with intravenous MSCs (Mei *et al*., 2010). Mei and colleagues demonstrated that whilst treatment with MSCs significantly reduced CFU counts in the spleen at 28 hours post CLP compared with the CLP/saline group, the ability of MSCs to directly phagocytose fluorescently-labeled bacteria *in vitro* was negligible. This implied that MSCs may not directly participate in bacterial clearance, but may indirectly modulate the ability of the host's phagocytes to clear bacteria. Macrophages co-cultured with MSCs *in vitro* did not exhibit enhanced phagocytic activity. However, $CD11b^{+}$ (monocyte and macrophage) subpopulations isolated from MSC-treated animals (exposed to MSCs *in vivo*) showed increased phagocytic activity compared with cells from septic animals not treated with MSCs. This was the case for both gram-positive and gram-negative bacteria. Significant improvement in survival was only seen when imipenem was

administered alongside MSC injection (Mei *et al*., 2010). MSC treatment in combination with targeted antibiotic therapy has been shown to be superior to MSC treatment alone in prolonging survival in subsequent studies (Alcayaga-Miranda *et al*., 2015; Wu et al., 2016). Of particular interest in Mei's study was the observation that MSCs significantly reduced all systemic cytokine and chemokine (IL-6, IL-1β, IL-10, KC, JE, and CCL5) levels measured in this model, in some instances approaching levels observed in sham-operated mice. Perhaps contrary to what might be expected, MSC-mediated enhancement of bacterial clearance was in fact accompanied by a reduction in inflammation (Mei *et al*., 2010). This study had a significant impact on the field as it was one of the earliest to demonstrate antibacterial activity of MSCs in addition to immunomodulation.

Two recent studies by Michael Matthay's group reported that MVs released by human MSCs were responsible for the observed antimicrobial effects in *E. coli* LPS and live bacteria-induced ALI in mice and in *ex vivo* perfused human lung (Zhu *et al*., 2014; Park *et al*, 2019). MSC antimicrobial activity was enhanced by pretreatment with Poly (I:C) and this was associated with a lesser influx of neutrophils to the injured alveolus. This data fits with the MSC licensing study conducted by Waterman et al which demonstrated that TLR-3 activation with Poly (I:C) increased the immunomodulatory potential of MSC, while TLR-4 activation reduced it (Waterman et al., 2010). Zhao et al also demonstrated a more potent immunosuppressive effect of umbilical cord MSCs pre-treated with Poly (I:C) (Zhao *et al*., 2014). Subsequent data from Matthay's group showed that Poly (I:C) pretreatment of MSC MV impacted on bacterial clearance by enhancing macrophage/monocyte phagocytosis and reducing inflammatory infiltrate, a so-called phenotypic M2 change (Monsel *et al*., 2015). This M2 change was further investigated by Song et al who showed recently that pretreatment of MSC with IL1β increased levels of anti-inflammatory miR146 contained in MSC exosomes. miR146, once delivered to host macrophages, induced an M2 phenotype by blocking the pro-inflammatory signaling pathways IL-1R-associatedkinase (IRAK1), TNF receptor-associated factor 6 (TRAF6), and Interferon regulatory factor 5 (IREF 5) (Song *et al*., 2017). This data collectively highlights the potential for using MVs as a viable alternative to live cells for the treatment of pneumonia-induced lung injury, and also the option to modify MVs via pretreatment of the parent MSCs to enhance antimicrobial activity.

The application of MSC therapy in chronic bacterial infections has recently been explored (Johnson *et al*., 2017). Johnson et al treated mice with chronic *S. aureus* implant infections with intravenous MSC, with or without pre-activation and antibiotic supplementation. The most effective treatment strategy was identified as activated MSC co-administered with antibiotic therapy, suggestive of synergism between the treatment approaches. MSC bactericidal activity was found to be partly attributed to the secretion of cathelicidin and was significantly increased by co-administration of antibiotics. Activated MSC could be detected in the wound margins several days after systemic delivery. Host macrophages of MSC treated mice assumed an M2 phenotype, in contrast to the untreated group whose infected tissues contained predominately M1 macrophages (Johnson *et al*., 2017). These findings are supported by another study demonstrating MSC synergistic effects with antibiotic therapy which resulted in enhanced survival in a mouse model of sepsis (Alcayaga-Miranda et al., 2015). Furthermore, experiments utilising domestic dogs suffering with spontaneous chronic multi drug-resistant wound infections demonstrated clearance of bacteria and accelerated wound healing following multiple infusions of activated allogeneic canine MSC (Johnson *et al*., 2017). These data suggest that systemic treatment with activated MSC in combination with conventional antibiotic therapy may represent a novel approach to treating chronic multi-drug resistant bacterial infections.

1.10.2 MSCs reduce inflammation whilst enhancing bacterial clearance

In severe infection, the sometimes overzealous reaction of immune effectors can be deleterious to the haematopoietic progenitor and mature immune cells of the host, as well as to host parenchymal cells and tissues (Hotchkiss *et al*., 2013; Skirecki *et al*., 2015). It has been suggested that by modulating the post-infection cytokine profile to become more anti-inflammatory than pro-inflammatory, MSCs may enhance bacterial clearance in part by preventing immune system self-degradation. In line with this concept, sensitivity of MSCs to their environment may allow for reversal of their phenotype from immunosuppressive to immunostimulatory when encountering dysfunctional immune cells in the latter stages of severe infection/sepsis (Li *et al*., 2012; Le Burel *et al*., 2017).

This apparent adaptability of MSCs when facing lymphocytes in different states of 'health' is an interesting recent development in our understanding of MSC biology. It has its roots in a study conducted a decade ago in which T cell proliferative capacity was inhibited or stimulated by co-culture with MSCs, depending on the activation status of the donor T cells. Actively proliferating T-cells, stimulated by cytokines IL-2, IL-7 or Il-15, were inhibited by MSCs, whereas T cells in a low proliferative state were stimulated by MSCs (Bocelli-Tyndall *et al*., 2009). In corroboration with this data Li et al demonstrated that MSC behaviour could be influenced by the level of NO generated by surrounding pro-inflammatory immune cells; high levels stimulated MSC immunosuppressive effects, low levels (and iNOS blockade) resulted in MSC promotion of T cell proliferation (Li *et al.,* 2012). Le Burel and colleagues recently demonstrated that hMSCs co-cultured with human PBMCs exhibited inhibitory effects towards healthy PBMCs (reduced proliferation of PHA-activated lymphocytes) and supportive effects toward LPS-intoxicated PBMCs (enhanced T cell proliferation and survival). This latter observation was associated with an increase in the expression of IFN-γ, interpreted as maintenance of theT_H1 phenotype of the LPS-injured PBMCs treated with MSC. Furthermore, MSC priming with a combination of pamidronate and low dose LPS prior to treatment enhanced this immune supportive phenotype (Le Burel *et al*., 2017). The observed MSC adaptability or "plasticity" may support the notion that MSC are more geared to preservation of homeostasis than to their traditionally understood suppressive role (Bocelli-Tyndall *et al*., 2009; Li *et al*., 2012; Le Burel *et al.,* 2017).

In a seminal study conducted a decade ago Nemeth and colleagues demonstrated that MSCs respond to TLR signaling and produce soluble mediators such as PGE2 which alters macrophage function by decreasing TNF-α and IL-6 production and increasing IL-10 levels. This, in turn, decreased neutrophil migration into the pro-inflammatory microenvironment and reduced myeloperoxidase expression in kidney and liver. This had the overall effect of prolonging survival, improving kidney and liver function and reducing bacterial burden in the blood (Nemeth *et al*., 2009). Another study, using a similar murine model of CLP-induced sepsis, looked specifically at MSC effect on acute kidney injury (AKI). The MSC treatment group exhibited decreased serum creatinine, urea and nitrogen levels, enhanced recovery of tubular function and improved survival. mRNA levels of TNF-α, IL-6 and IL-17 in kidney tissue were dramatically decreased following MSC treatment (Luo *et al*., 2014).

Pedrazza and colleagues demonstrated an MSC protective effect conferred by adipose-derived mMSC towards splenocytes in a mouse CLP model of sepsis, 12 hours post infection induction, which was associated with a survival advantage at 26 hours and a reduction in liver, kidney and pancreatic damage compared with untreated septic mice. These findings were in conjunction with MSC anti-inflammatory effects, which included a reduction in inflammatory cytokines (TNF-α, IL-6 and MCP-1) and an increase in anti-inflammatory IL-10 (Pedrazza *et al*., 2014). Yuan and colleagues demonstrated a BMSC-mediated reduction in bacterial load in conjunction with reduced production of inflammatory cytokines (IL-6, IL-1β and IL-10) in rats infected with methicillin-resistant *Staphylococcus aureus* (MRSA). Rats receiving MSC treatment displayed faster and more robust wound healing compared with fibroblast-treated controls (Yuan *et al*., 2014).

MSC-derived EVs have been demonstrated to limit apoptosis and promote tissue healing in acute kidney injury (Bruno *et al*., 2009) and chronic wounds (Shabbir *et al*., 2015). Shabbir and colleagues found that internalisation of MSC exosomes by recipient skin cells resulted in a dose-dependent enhancement of proliferation and migration of fibroblasts derived from healthy donors and patients with chronic skin wounds, which accelerated the healing process. The exosomes were demonstrated to induce the expression of growth factors: hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF1), nerve growth factor (NGF), and stromal-derived growth factor-1 (SDF1) and to activate several signaling pathways relevant to wound healing (Akt, ERK, and STAT3) (Shabbir *et al*., 2015).

MSC anti-apoptotic properties have been demonstrated in studies using different models of infection and tissue injury; specific proteins thought to mediate this MSC protective effect have been identified: B-cell lymphoma 2 (Bcl-2), survivin, and Akt (Okazaki *et al*., 2008; Wang *et al*., 2012). MSC secretion of Bcl-2 has been demonstrated to alter the ratio of Bcl-2 to Bcl-2-associated X protein (Bax) (Okazaki *et al*., 2008), which is known to influence the sensitivity of cells to a pathological stimulus (Oltvai *et al.,* 1993). Increasing the ratio of Bcl-2 to Bax impairs the release of caspase activators, which makes cells less likely to respond to apoptotic signaling (Green and Reed, 1998). In a rat model of myocardial infarction, Tang and colleagues detected downregulated Bax expression in the ischemic myocardium following autologous MSC transplantation (Tang *et al*., 2005). Pan and colleagues also

showed lower expression of Bax, TNF receptor superfamily, member 6 (FAS), and caspase 3 (CASP3) in hepatocytes injured with hydrogen peroxide and treated with MSC–CM; MSC treated hepatocytes exhibited less apoptosis than untreated control cells (Pan *et al*., 2012).

Collectively, these data show that MSCs, from a variety of sources, not only enhance bacterial clearance by the secretion of AMPs and augmentation of host phagocytic response, they also attenuate the inflammation induced by microbial invasion and limit host cell apoptosis and subsequent tissue damage. MSCs achieve this by decreasing inflammatory cytokine/chemokine production and migration of additional immune effector cells responding to infection, which, if left unchecked, may induce tissue injury by their release of pro-inflammatory mediators and free radicals. The result is enhanced pathogen clearance with preservation of tissue integrity and organ function. MSCs have been described as having "sensors" facilitating the detection of pathogen invasion which triggers their migration to the site on infection, "bioactive pumps" which control the continuous secretion of AMPs and "watch towers" which govern the release of bespoke combinations of AMPs as dictated by the stimulus (Alcayaga *et al*., 2017). MSC pre-conditioning, with selected cytokines (IFN-γ, TNFα, IL-22) or growth factors, prior to administration of the cells themselves or their EVs, may increase the expression of advantageous AMPs, thus enhancing MSC antimicrobial and anti-inflammatory potential (Gupta *et al*., 2012; Sutton *et al*., 2016; Alcayaga-Miranda *et al*., 2017).

Genetic modification of MSCs has also been demonstrated to enhance their therapeutic potential by increasing their paracrine actions (Nolta, 2016; Silva *et al.,* 2018). In a murine model of Chagas disease cardiomyopathy (characterized by myocardial inflammation/fibrosis due to chronic parasitic infection), mMSCs manipulated to over-express G-CSF (MSC_G-CSF) exhibited an increased migration capacity to the hearts of mice infected with *Trypanosoma cruzi* compared with regular mMSCs. MSC_G-CSF were more potent in reducing the number of infiltrating leukocytes and in suppressing levels of inflammatory mediators (IFN-γ, TNF-α). IL-10 production was enhanced to a greater degree by MSC_G-CSF than by regular MSC and this was associated with a significant increase in the percentage of T_{regs} in the hearts of MSC_G-CSF-treated mice (Silva *et al.,* 2018). Interestingly, T_{regs} have been implicated in controlling pathological T cell responses in

chronic Chagas disease cardiomyopathy in clinical studies of patients with a mild (asymptomatic) form of the disease. These patients were found to have increased numbers of T_{regs} in the peripheral blood compared to patients with severe cardiomyopathy (Yang *et al*., 2016).

1.11 Aims of this thesis

Over the last 20 years much effort has been devoted to trying to decipher the molecular mechanisms that underpin the vast array of observed MSC immunomodulatory effects. Significant progress has been made in our understanding of MSC biology but there remain many unanswered questions, particularly relating to MSC crosstalk with immune effector cells from both innate and adaptive immune systems. A putative mechanism by which MSCs have anti-inflammatory effects is via the recruitment of Tregs. A specific question addressed in this thesis is whether or not T_{regs} are pivotal for MSC effect?

This thesis explores the impact of MSC on host immunity in bacterial lung infection. Specific aims are:
(1) To isolate, characterise and validate murine MSCs for use in mouse models of bacterial lung infection,
(2) To study the impact of murine MSCs on bacterial clearance and the inflammatory response in mouse models of lung infection with *Streptococcal pneumoniae* and *Pseudomonas aeruginosa*,
(3) To study the impact of depletion of regulatory T cells (T_{regs}) on bacterial clearance and the inflammatory response to *Streptococcal pneumoniae* and *Pseudomonas aeruginosa*,
(4) To study the impact of depletion of regulatory T cells (T_{regs}) on MSC function in mouse models of lung infection with *Streptococcal pneumoniae* and *Pseudomonas aeruginosa*.

1.12 Thesis Hypotheses

(1) Systemic treatment with murine MSCs will enhance bacterial clearance and reduce the inflammatory response in mouse models of *S. pneumoniae* and *P. aeruginosa* lung infection.

(2) Depletion of T_{regs} prior to infection induction will impair bacterial clearance of *S. pneumoniae* but not *P. aeruginosa*.

(3) MSC protective effect will be impaired by T_{reg} depletion prior to infection with *S. pneumoniae* but not *P. aeruginosa*.

Chapter 2

2 *Materials and methods*

2.1 Primers

2.1.1 Primers for genotyping murine strains

Table 2.1: List of primers for genotyping different strains of mice.

All transgenic and knockout murine strains used in this study (Table 2.3) were maintained by

PCR genotyping of ear clip DNA prepared as described in Section 2.3.2 with primers listed below.

Target	**Strain of mice**	**Sense Primer Sequence (5'-3')**	**Anti-sense Primer Sequence (5'-3')**	**PCR product size (bp)**
Foxp3 WT	Foxp3.DTR or HLA-DQ8.Aβ0.DTR or IFN-γ reporter.DTR	TGGACCGTAGATG AATTTGAGTT	CCAGATGTTGTGGG TGAGTG	550
DTR	Foxp3.DTR or HLA-DQ8.Aβ0.DTR IFN-γ reporter.DTR	GGGACCATGAAGC TGCTGCCG	TCAGTGGGAATTAG TCATGCC	650
HLA-DQα	HLA-DQ8.Aβ0 or HLA-DQ8.Aβ0.DTR	TACCAGTTTTACGG TCCCTC	GGTAGCAGCGGTA GAGTTG	200
HLA-DQβ	HLA-DQ8.Aβ0 or HLA-DQ8.Aβ0.DTR	GCATGTGCTACTTC ACC	CACCGTGTCCACCG ACGCCCG	200
Luc2	IFN-γ reporter or IFN-γ reporter.DTR	ACAAGTACGACCTG AGCAAC	CTGGTAGCCCTTGT ACTTGAT	300

2.1.2 Primers for RT-PCR

Table 2.2: List of primers and probes used for RT-PCR.

In order to study the relative fold change in the expression of specific target genes: i) in *S. pneumoniae* and *P. aeruginosa* lung infection, ii) in tissue resident and non-resident regulatory T cells and iii) in the MSC secretome in resting versus stimulated MSCS, RT-PCR was performed as described in Section 2.4.11 using primers and probes as listed below. (TaqMan gene expression assays, ThermoFischer Scientific, UK).

Gene	Species	Assay ID
TNF-α	**Mouse**	**Mm00443258_m1**
IL-6	**Mouse**	**Mm00446190_m1**
IFN-γ	**Mouse**	**Mm01168134_m1**
IL-17F	**Mouse**	**Mm00521423_m1**
IL-10	**Mouse**	**Mm01288386_m1**
KC (Cxcl1)	**Mouse**	**Mm04207460_m1**
TGF-β	**Mouse**	**Mm01178820_m1**
T-bet (Tbx21)	**Mouse**	**Mm00450960_m1**
GATA 3	**Mouse**	**Mm00484683_m1**
RORγT (Rorc)	**Mouse**	**Mm01261022_m1**
Foxp3	**Mouse**	**Mm00475162_m1**
CCR4	**Mouse**	**Mm01963217_u1**
BLIMP 1 (Prdm1)	**Mouse**	**Mm00476128_m1**
S1pr1	**Mouse**	**Mm02619656_s1**

2.2 Bacterial Strains

2.2.1 *S. pneumoniae*

Bioluminescent *S. pneumoniae* Xen 10 (Xenogen, California, USA), derived from parental strain *S. pneumoniae* A66.1 was used for *in vivo* infection as described in Section 2.4.1. Bioluminescent transformation of the wild type (WT) parental strain was achieved by plasmid insertion of a gram-positive *lux* transposon cassette, Tn*4001 luxABCDE* Kmr (Francis *et al*., 2001).

2.2.2 *P. aeruginosa*

Bioluminescent *P. aeruginosa* Xen 41, derived from the parental strain *P. aeruginosa* PAO1 used for *in vivo* infection as described in Section 2.4.2. *P. aeruginosa* Xen 41 possesses a single stable copy of the *P. luminescens luxCDABE* operon on the bacterial chromosome (Francis *et al*., 2001).

2.3 Strains of mice

2.3.1 Animal husbandry

Table 2.3: List of murine strains used in thesis

Strain name	Phenotype	Background strain	Origin	Reference
HLA-DQ8.Aβ0	HLA-DQ8 transgenic, lacking endogenous mouse H2.	C57BL/6	Altmann lab	(Boyton *et al.*, 1998)
Foxp3.DTR	Inducible knockout of Foxp3+GFP+ cells.	C57BL/6	Rudensky lab	(Kim *et al.*, 2007)
HLA-DQ8.Aβ0.DTR	HLA-DQ8 transgenic, lacking endogenous mouse H2, inducible knockout of Foxp3+GFP+ cells.	C57BL/6	Boyton lab.	
IFN-γ reporter	Reporter of IFN-γ expression.	C57BL/6	Boyton lab.	(Reynolds *et al.*, 2019)
IFN-γ reporter-Foxp3.DTR	Reporter of IFN-γ expression and inducible knockout of Foxp3+GFP+ cells.	C57BL/6	Boyton lab.	(Reynolds *et al.*, 2019)

All strains of mice used in this thesis were either made in the Altmann or Boyton labs or supplied as kind gifts from the original producing lab (Table 2.3). Mice were bred on site and maintained in accordance with UK government Home Office guidelines in closed IVC housing. Conditions of project license (70/7708) were carefully adhered to for all experiments described in this thesis.

2.3.2 Genomic DNA extraction for genotyping

For each strain of mice the genotype was determined by PCR using extracted genomic DNA from ear clip biopsies. Genomic DNA was extracted using a high salt DNA extraction method. Mice were assigned a number for identification based on cage number and ear marking at the time of ear clipping so they could be easily identified for allocation to experiments. Tissue was digested overnight at 56°C in 400µl mammalian tissue lysis buffer (Section 2.5.1) and 10µl Proteinase K (100µg/ml; Sigma-Aldrich, UK – P6556). The next day, 200µl saturated NaCl solution was added and samples vigorously shaken for 30 seconds before centrifugation for 15 min, 15,000g. The supernatant was transferred into a clean tube and 600µl isopropanol added. The precipitated genomic DNA was pelleted by centrifugation at 9000g for 10 min. The pellet was washed in 750µl 70% ethanol and spun again at 9000g for 10 min before air-drying. Genomic DNA was resuspended in 100µl molecular biology water. The concentration of genomic DNA was measured on a NanoDrop spectrophotometer (NanoDrop, USA) using ND1000 V3.1.2 software. Samples were diluted to 40ng/µl for amplification by PCR. Samples were incubated briefly at 56°C for 15 minutes to ensure the DNA pellet was fully in solution and were then stored at 4°C for up to one week or -20°C for long term storage.

2.3.3 Genotyping PCR

The genotypes of the murine strains were determined from each litter by amplification of specific genes with appropriate primers (Table 2.1) using a BIOTAQ DNA polymerase (Table 2.4) with specific PCR thermal profiles (Table 2.5). PCR products were run on a 2% agarose gel made with 1X TAE buffer (Section 2.5.3) containing SYBR-Safe (ThermoFisher Scientific, UK – S33102) at 120V for approximately 45 min and visualized under UV light (Figure 2.1). Expected pattern of PCR products for each strain of mice are indicated in Table 2.6.

Table 2.4: Master-mix for BIOTAQ based genotyping PCR (for 1 reaction)

Component	Volume (µl)
10X buffer (BIOLINE Ltd, UK)	2.5
Magnesium (15mM) (BIOLINE Ltd, UK)	5
dNTPs (1mM) (BIOLINE Ltd, UK)	5
Molecular biology H2O	7.4
BIOTAQ polymerase (5U/µl) (BIOLINE Ltd, UK)	0.1
Sense primer (100µg/ml)	1.25
Anti-sense primer (100µg/ml)	1.25
Sample genomic DNA (40ng/µl)	2.5

Table 2.5: Thermal profiles for genotyping PCRs

Target	PCR Thermal Profile
Foxp3 WT	94°C 3 min, 35 cycles of (94°C 40 sec, 53°C 40 sec, 72°C 30 sec), 72°C 5 min
DTR	94°C 5 min, 35 cycles of (94°C 15 sec, 59°C 15 sec, 72°C 45 sec), 72°C 2 min
DQα	95°C 5 min, 32 cycles of (94°C 1 min, 55°C 1 min, 72°C 1 min), 72°C 8 min
DQβ	95°C 5 min, 32 cycles of (94°C 1 min, 55°C 1 min, 72°C 1 min), 72°C 8 min
H2 WT	95°C 5 min, 30 cycles of (94°C 45 sec, 60°C 45 sec, 72°C 1 min), 72°C 8 min
H2 KO	95°C 5 min, 30 cycles of (94°C 45 sec, 60°C 45 sec, 72°C 1 min), 72°C 8 min
Luciferase	95°C 5 min, 30 cycles of (94°C 45 sec, 60°C 45 sec, 72°C 1 min), 72°C 8 min

Table 2.6: Expected PCR products for genotyping transgenic mice.

Strain	Expected PCR product result
Foxp3.DTR	Foxp3 WT-, DTR+
DQ8.Aβ0	DQα+, DQβ+, H2 WT-, H2 KO+
DQ8.Aβ0x Foxp3.DTR	DQα+, DQβ+, H2 WT-, H2 KO+, Foxp3 WT-, DTR+
FN-γ reporter	Luc2+
IFN-γ reporter-Foxp3.DTR	Luc2+, Foxp3 WT-, DTR+

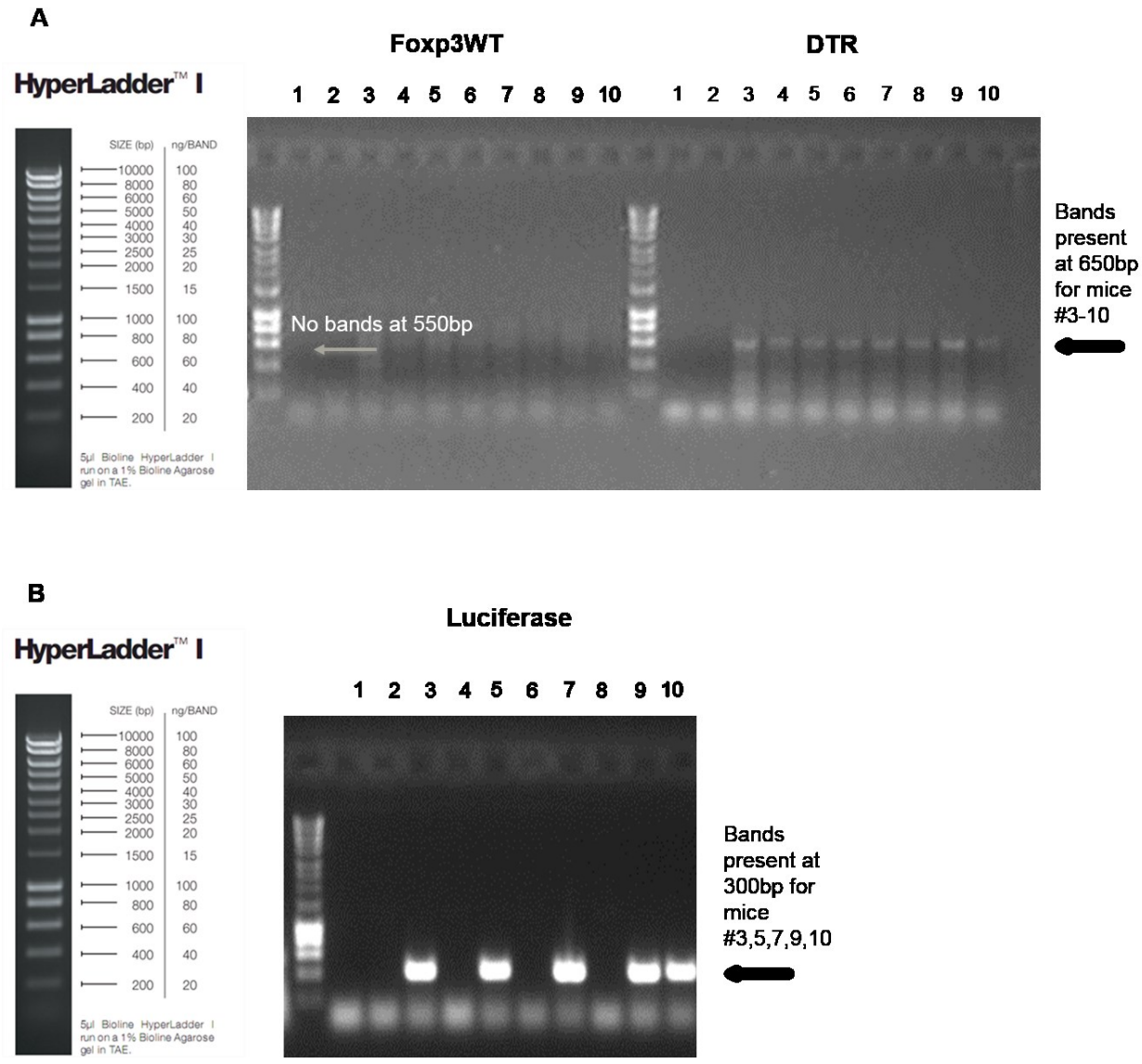


Figure 2.1. Representative PCR products for Foxp3.DTR and IFN-γ reporter transgenic mice. (A) Foxp3.DTR transgenic (Foxp3 WT-, DTR+), (B) IFN-γ reporter transgenic (Luciferase+).

2.4 Methods

2.4.1 Mouse model of lung bacterial infection (Strep pneumoniae, Xen 10)

Bioluminescent *S. pneumoniae* Xen10 was streaked from glycerol stocks stored at -80°C onto Columbia Blood Agar (CBA) plates (VWR, UK – EOLAPP0120-P090) and incubated overnight at 37°C, 5% CO_2. The next day, a single colony of Xen 10 was inoculated into each of 10, 50ml tubes of Brain Heart Infusion (Section 2.5.2) and incubated overnight (37°C, 5% CO2). Overnight cultures were washed in sterile Phosphate Buffered Saline (PBS) (Gibco, UK - 10010023) by centrifugation at 3000g, 10 min at 4°C. The bacterial pellets were pooled together and re-suspended in 2ml sterile PBS to make an inoculum of approximately 10^8 CFU/30ul, diluted to make an inoculum of $5x10^7$ CFU/30ul appropriate for infecting the mice. The bacterial inoculum was quantified initially by measurement of OD 600 (prior to infecting the mice) and ultimately by serial plating on to blood agar plates with evaluation of CFU count the following day.

To study immune responses during sub-lethal pneumococcal lung infection mice were weighed at baseline then anaesthetised by inhalation of isoflurane gas and a 30µl inoculum delivered by pipette slowly to both nostrils, allowing for complete inhalation. Mice recovered from the anaesthetic after a few minutes and were monitored at regular intervals for signs of pre-defined end-points (loss of more than 20% body weight, development of respiratory difficulties, loss of mobility and motility, hunched posture and piloerrection).

Mice were culled at 24 hours for organ harvest: lung and bronchoalveolar lavage (BAL) for bacterial counts and cytokine analysis and lung for RNA. For some of the pilot experiments using this pathogen with and without prior depletion of regulatory T cells (T_{regs}), an earlier endpoint of 8 hours was employed.

*2.4.2 Mouse model of lung bacterial infection (P. aeruginosa, Xen 41) (*Reynolds *et al.,* 2018*)*

Bioluminescent Pseudomonas *aeruginosa* Xen 41 was streaked from glycerol stocks stored at -80°C onto LB agar plates (Section 2.5.2) and incubated overnight (37°C, 5% CO2). The next day, a single colony of Xen 41 was inoculated into a 5ml tube of LB broth (Section 2.5.2)

and incubated overnight (37°C, 5% CO2). The overnight culture was washed in sterile PBS (Gibco, UK – 10010023) by centrifugation at 3000g, 10 min at 4°C. The bacterial pellet was resuspended in 500µl PBS to make an inoculum of approximately 10^8 CFU/30ul inoculum, diluted to make an inoculum of 10^6 CFU/30ul appropriate for infecting the mice. The bacterial inoculum was quantified initially by measurement of OD 600 (prior to infecting the mice) and ultimately by serial plating on to blood agar plates with evaluation of CFU count the following day.

Mice were weighed prior to infection then anaesthetised by inhalation of isoflurane gas and a 30µl inoculum delivered by pipette slowly to both nostrils, allowing for complete inhalation. Mice recovered from the anaesthetic after a few minutes and were monitored at regular intervals for signs of pre-defined end-points (loss of more than 20% body weight, development of respiratory difficulties, loss of mobility and motility, hunched posture and piloerrection). Mice were culled at 24 hours for organ harvest: lung and BAL for bacterial counts and cytokine analysis and lung for RNA.

2.4.3 Collection of Bronchoalveolar lavage (BAL)

Mice were humanely culled by lethal i.p injection of 200 µl Euthatal (Merial Animal Health Ltd., UK) and trachea cannulated. Lungs were lavaged 3 times with 0.4 ml PBS (Gibco, UK – 10010023) using a 1 ml syringe and all BAL fluid for each mouse was pooled. A 30 µl aliquot of BAL fluid per mouse was diluted and plated out for analysis of bacterial count (see below). Remaining BAL fluid was centrifuged at 260 x g for 8 minutes. The supernatant was removed to a new tube and stored at -80 °C for subsequent cytokine analysis by ELISA.

2.4.4 Microbial analysis of infection

To determine the bacterial burden incurred during the lung infection tissue was harvested for bacterial counts. Lungs were dissected out, 5µl sterile PBS per mg tissue was added and the lung sample homogenized. 30µl of 10^2 and 10^3 dilutions of homogenised lung tissue and BAL fluid was plated onto blood agar. Plates were incubated overnight at 37°C, 5% CO2 and

colonies counted the next day. Bacterial counts are expressed as Colony Forming Unit (CFU) per mg or ml of tissue or BAL.

2.4.5 Cell extraction from whole lung lobe

One lung lobe was finely chopped and placed into 5 ml of RPMI 1640 medium (Gibco, UK - 11875093) containing 0.15 mg/ml Collagenase D (Roche, UK - 11088858001) and 25 µg/m1 DNase I (Roche, UK - 11284932001). The lung tissue was incubated with shaking for 1 hour at 37°C and the mixture passed through a 70 µm cell strainer. Cells were washed twice in RPMI 1640 medium before differential cell counting.

2.4.6 Differential cell counting

Following centrifugation of BAL fluid or lung cells, cells were re-suspended in 250µl of RPMI 1640 medium (Gibco, UK - 11875093). A small aliquot of this cell suspension was diluted 1:2 in RBC lysis buffer (Section 2.1.4) to lyse red blood cells, and white blood cells were counted on a haemocytometer. Samples were adjusted to 5 x 10^5 cells / ml and 100 µl (5 x 10^4 cells) spun onto a polysine glass microscope slide (VWR, UK - 6310107). Cytospins were performed by centrifugation (Shandon-Cytospin 3) at 400 rpm for 4 minutes. Slides were allowed to air dry for 5 minutes before fixing in methanol for 3 minutes. Wright Giemsa staining of BAL cytospins allowed different cell types to be distinguished on the basis of morphology. Slides were stained by immersing in Wright Giemsa stain (Sigma, UK - WG16) for 1 minute, followed by distilled water for 6 minutes without agitation. Slides were rinsed briefly in running distilled water to stop stain development and then allowed to air dry before addition of a cover slip and cell counting. A total of 300 cells were counted on each slide and the total number of each cell type recovered calculated by multiplying the percentage of each cell type by the total number of cells isolated in each sample.

2.4.7 Lung homogenate

One lung lobe was weighed and placed into Hank's Balanced Salt Solution (HBSS) without $MgC1_2$ or $CaC1_2$ (Gibco, UK - 14170112), containing a mixture of protease inhibitors (Roche, UK - 11836153001), at a concentration of 100 mg of lung per 1 ml of HBSS solution. The lung was homogenised using an electric homogeniser before centrifugation (1500 x g) and collection of the supernatant for analysis by ELISA. If it was not possible to process the lung

tissue immediately after harvesting, lung lobes were snap frozen using liquid nitrogen and stored at -80 °C.

2.4.8 Cytokine analysis by Enzyme-linked Immunosorbent Assay (ELISA)

Post infection cytokine levels from lung homogenate and BAL were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available kits (Table 2.7). The principle of the process of detection of cytokine of interest is as follows: An immobilized capture antibody first binds the target protein (analyte). Subsequently a detector antibody in liquid phase will bind ("sandwich") the capture antibody immobilized target analyte. Detection of the analyte is achieved with catalysis of a substrate mediated by an enzyme conjugated to the detector antibody. Detection output is proportional to the quantity of analyte in the test sample.

In brief, capture antibody was diluted in the manufacturer's recommended buffer and 100µl added to 96-well immuno-plate (Sigma-Aldrich, UK - M3687-60EA) overnight at 4°C. Next day, the plate was washed twice with 200µl PBS (Gibco, UK – 10010023) before blocking with 200µl 1% BSA/PBS for 1h at RT. The plates were washed twice with 200µl washing buffer (0.05% Tween/PBS) before 100µl of pre-diluted serum samples (1:50 in blocking buffer) added in duplicate. A 2-fold dilution series using recombinant cytokine was also added to the plate starting with the highest concentration as 4000pg/ml and lowest as 15.625pg/ml to form a standard curve to determine the concentration of the unknown samples. A negative control of 100µl blocking buffer was included to determine the background optical density. The plate was incubated overnight at 4°C. The following day, the ELISA plate was rinsed 5 times with washing buffer, before 100µl cytokine detection antibody diluted in blocking buffer was added for 1h at RT. The plate was washed 5 times before 100µl Streptavidin-HRP (R+D systems, Minneapolis, USA-DY988) was added for 30 min at RT. The plate was washed 6 times before 100µl 3,3', 5,5"-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, UK – T0440) was added for detection and the colour change monitored. The enzymatic reaction was stopped by the addition of 100µl 1M H_2SO_4. Optical densities of samples against a recombinant protein standard curve were measured using an ELISA plate reader (µQuant BIO-Tek Instruments, Inc.) and KC Junior software was used to determine the concentration of the cytokines in samples, normalised against the background optical density of the negative control.

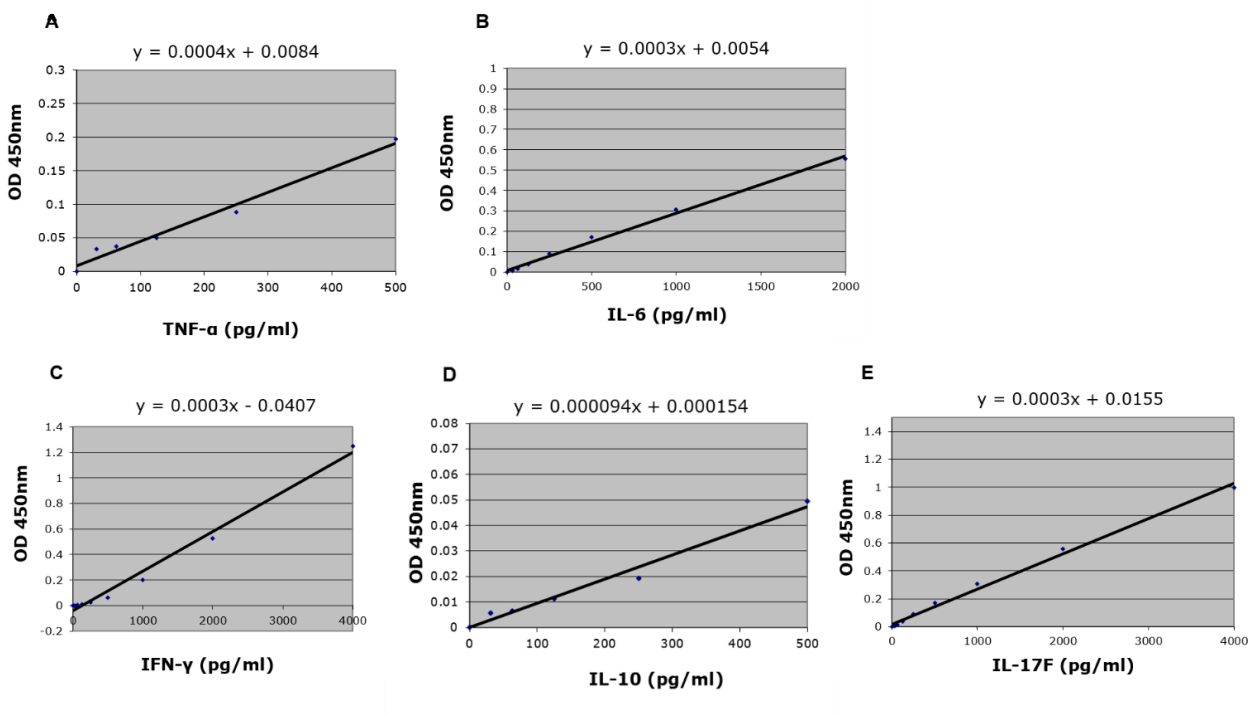


Figure 2.2. Representative standard curves for cytokine analysis by Enzyme-linked Immunosorbent Assay (ELISA). TNF-α (A), IL-6 (B), IFN-γ (C), IL-10 (D), IL-17F (E).

Table 2.7: Antibodies and recombinant proteins

Antibody	Product Details	Catalogue No.	Concentration
ELISA capture antibodies			
Anti mouse IFN-γ	MABTECH AB, Sweden mAb AN18	3321-1H-6	1 µg/ml
Anti mouse IL-10	MABTECH AB, Sweden mAb MT60	3432-1H-6	200 ng/ml
Anti mouse IL-6	Invitrogen, UK - AMCO864D	CMC0063	1.25 µg/ml
Anti mouse TNF-α	R&D systems, USA	DY410	800 ng/ml
Anti mouse IL-17F	R&D systems, USA	DY2057	800 ng/ml
ELISA detection antibodies			
Biotinylated anti mouse IFN-γ	MABTECH, UK - mAb R4-682	3321-1H-6	500 ng/ml
Biotinylated anti mouse IL-10	MABTECH, UK - mAb 51E6	3432-1H-6	200 ng/ml
Biotinylated anti mouse IL-6	Invitrogen, UK - AMCO969D	CMC0063	100 ng/ml
Biotinylated anti mouse TNF-α	R&D systems, USA	DY410	75 ng/ml
Biotinylated anti mouse IL-17F	R&D systems, USA	DY2057	200 ng/ml
Recombinant proteins			
Mouse IFN-γ	MABTECH AB, Sweden	3321-1H-6	15.6–1000 pg/ml
Mouse IL-10	MABTECH AB, Sweden	3432-1H-6	31.2-2000 pg/ml
Mouse IL-6	R&D systems, USA	406-MI	15.6–1000 pg/ml
Mouse TNF-α	R&D systems, USA	DY410	31.2-2000 pg/ml
Mouse IL-17F	R&D systems, USA	DY2057	23.4-1500 pg/ml

2.4.9 Total RNA extraction

To assess the fold change in expression of specific target genes during bacterial lung infection, total RNA from tissue was extracted to make complementary DNA (cDNA) and quantitative Real-Time PCR (qRT-PCR) analysis employed. Harvested lung was stored in 300µl RNALater RNA Stabilisation Reagent (Ambion, US - R0901) at 4°C for 48 hours then removed from RNALater into a clean tube and frozen at -20°C until ready for use.

For tissue samples or samples of greater than 5 x 10^6 cells, total RNA was extracted using the acid phenol method with TRIzol (Invitrogen, UK - 15596018). In brief, tissue was homogenised in 1ml TRIzol. Following cell lysis, 200µl of chloroform was added, the sample mixed by vigorous shaking for 30 seconds and then incubated on ice for 5 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C and the upper aqueous layer removed to a new tube. RNA was precipitated by addition of 500µl of isopropanol. Samples were mixed by inversion and incubated at room temperature for 10 minutes before centrifugation at 12,000 x g to pellet the RNA. RNA pellets were washed once with 75% EtOH, air-dried briefly and dissolved in 20-30µl of RT-PCR Grade Water (Invitrogen, UK – AM9935). RNA concentration and nucleic acid/protein purity ratio were analysed using a NanoDrop spectrophotometer. Total RNA was diluted to 200ng/µl and stored at -80°C until ready for use.

RNA from cell line samples of less than 5 x 10^6 cells was extracted using an RNA binding spin column (Stratagene, UK).

2.4.10 cDNA synthesis

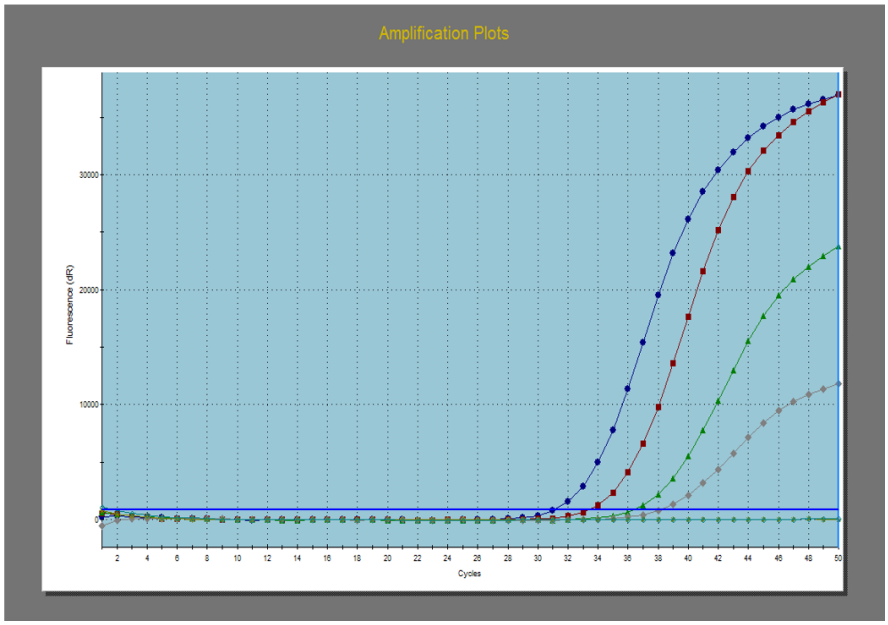
RNA was quantitated by Nanodrop spectrophotometer and 200ng/µl used per cDNA reaction. 1 µl total RNA was reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen, UK - 18080044) in a two-step reaction. In brief, for one reaction mix, 1 µl random primers (250ng/µl; Invitrogen, UK - 48190011), 1µl dNTPs (10mM) and 10 µl RNase-free water was added to 1 µl RNA template and incubated at 65°C for 5 min. The RNA mix was cooled on ice for 1 min before adding 1µl 0.1M DTT (Invitrogen, UK - 707265ML), 1µl Superscript III Reverse Transcriptase, 1µl RNase-out Recombinant

Ribonuclease inhibitor (Invitrogen, UK – 10777019) and 4 µl 5X first strand buffer (Invitrogen, UK - 18080400) to a total volume of 20µl. Reverse transcription was carried out at 50°C for 1h, followed by the final incubation at 70°C for 15 min to inactivate the enzyme. 1 µl of completed cDNA reaction mixture was used as a template for each subsequent PCR reaction. cDNA was stored at -20°C.

2.4.11 Real- time PCR (RT-PCR)

RNA and cDNA was prepared as described. PCR reactions were carried out in a volume of 20 µl containing 1µl of template cDNA, 8 µl of RNase free water (Invitrogen, UK – AM9935), 1 µl of 20x gene specific primer mix (Applied Biosystems - Assays-on-Demand™ Gene Expression Assay) and 10 µl of 2x TaqMan® Universal PCR Master Mix (Applied Biosystems, UK -4352042). Reactions were incubated at 50°C for 2 minutes, 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR reactions were set up in triplicate for each set of primers used, and CT values (the PCR cycle number at which the fluorescence passes a fixed threshold) were obtained using a Stratagene MX3000P real-time PCR machine and software. Variability in the amount of RNA used for each cDNA reaction was controlled for by running a PCR for GAPDH reference gene for each sample. PCR of a dilution series of a single sample showed the reference gene and all other target gene primer sets to have different amplification efficiencies (Figure 2.1). It was therefore not possible to use the $2^{\delta\delta Ct}$ mathematical expression for fold change. CT values for PCR reactions were instead converted to numerical values, using the equation for the appropriate efficiency curve, before normalising each gene of interest value with respect to the GAPDH value for the same sample. The sample with the lowest level of gene expression was assigned a value of 1. Levels of gene expression in all other samples were expressed as a value relative to 1.

A



B

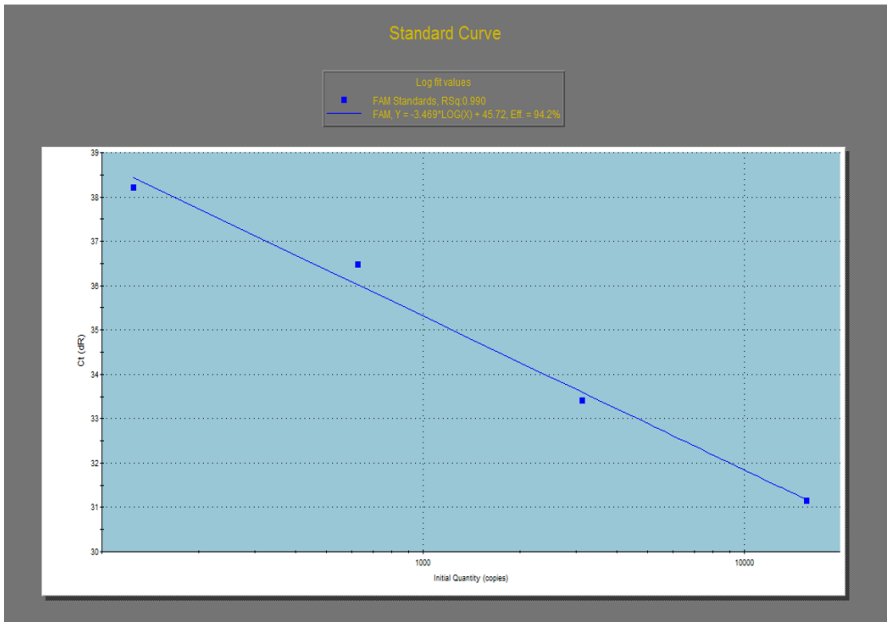


Figure 2.3. Representative RT-PCR primer-hydrolysis probe efficiency standard curve.

A 5-fold serial dilution of stock cDNA was run by RT-PCR for RORγT primer/probe and from the amplification plot (A), Ct values for each cDNA concentration were read at a threshold of 2000. A standard curve of log cDNA concentration versus Ct value (B) was used to calculate the primer/probe efficiency as calculated by MX3000Pro software. (Stratagene, USA).

2.4.12 Inducible depletion of T_{regs} in vivo

Transgenic models can be used to inducibly knockout the T_{reg} population using a targeting construct encoding the human diphtheria toxin receptor (DTR) fused to sequences encoding green fluorescent protein (GFP) and equipped with an internal ribosome entry site (IRES) into the 3' untranslated region of the *Foxp3* gene to produce '*Foxp3*DTR'. These mice undergo ablation of their Foxp3 expressing cells (T_{reg} population) on exposure to diphtheria toxin (Kim *et al*., 2007). To deplete $Foxp3^+$ cells in Foxp3.DTR or HLA-DQ8.Aβ0.DTR mice as described in Chapter 5, Diphtheria Toxin (DT; Sigma-Aldrich, UK – D0564) was given to mice i.p and the presence of $CD4^+CD25^+Foxp3^+$ cells measured by flow cytometry from blood and spleen. A single dose of 100 µl DT (12.5µg/ml) was sufficient to deplete $CD4^+CD25^+Foxp3^+$ cells for 24 hours (Figure 5.1). For infection experiments, Foxp3.DTR or HLA-DQ8. Aβ0.DTR mice were depleted of $Foxp3^+$ cells with 100µl DT daily for 48 h prior to initiation of infection and also on the day of infection in order to maintain full knockdown of target cells.

2.4.13 Flow cytometry and cell sorting

As explained in section 2.4.12 the Foxp3.DTR transgenic mouse has green fluorescent protein (GFP) tagged Foxp3-expressing cells (T_{regs}) which can be identified by flow cytometry and sorted by cell sorter. To investigate transcriptomic differences between systemic T_{regs} (from spleen) and lung resident T_{regs} (from lung) of healthy animals, splenocytes and lung cells were first isolated (as explained in sections 2.4.5 and 2.4.24) and then stained for CD4. Samples were run on a FACSAria II™ flow sorter and $CD4^+$ GFP-expressing cells were sorted directly into lysis buffer (Agilent Technologies, UK – 400-711-13) supplemented with β-Mercaptoethanol (Agilent Technologies, UK – 400-711-111) and stored at -80°C pending RNA extraction for RT-PCR.

2.4.14 Murine models of lung bacterial infection (S. pneumoniae and P. aeruginosa) with T_{reg} ablation.

A 30µl inoculum of bioluminescent *S. pneumoniae* Xen10 or *P. aeruginosa* Xen 41 was prepared as previously described (Section 2.4.1 and 2.4.2). Foxp3.DTR mice received an

intraperitoneal (i.p) injection of 1.25ug diphtheria toxin (DT; Sigma-Aldrich, UK) or PBS day 0, day 1 and day 2. DT and PBS-treated mice were infected intranasally with S. *pneumoniae* or *P. aeruginosa* (day 2) as previously described. The mice mere culled at 24 hours and readouts analysed as for the S. *pneumoniae* or *P. aeruginosa* lung bacterial infection models.

2.4.15 Isolation of bone-marrow derived mesenchymal stem cells (BM-MSCs)

10-12 week old Foxp3.DTR and DQ8.AβO.Foxp3.DTR mice were sacrificed by cervical dislocation. Their femurs and tibiae were carefully stripped of adherent soft tissue, the epiphyses were removed and the marrow was harvested by inserting a 20-gauge needle attached to a syringe into one end of the bone and flushing with Dulbecco's Modified Eagle's Medium (DMEM), high glucose, GlutaMAX (Gibco, UK-10566-016). This flushing process was repeated 5 times to disperse the marrow. Cells were centrifuged (1500 x g, 10 mins), red blood cells (RBCs) lysed with RBC lysis buffer (Section 2.5.4) and remaining cells resuspended in Mesencult Complete Medium (Stemcell Technologies, UK - 05512). Prior to plating in flasks cells were counted and concentration adjusted to approximately 10^6 cells/ml. The cell suspension was transferred to a tissue culture treated flask with vented cap, canted neck (Corning; Cole-Parmer, UK - CLS430641) and placed in a sterile humidified incubator, 37°C, 5% CO_2. Non-adherent cells were removed by changing the culture medium 72 hours later (day 3). Media was changed every 3-4 days until areas of confluent cells appeared.

2.4.16 MSC trypsinisation and passage

Cells were passaged to a new flask when 70 – 80% confluent (day 7-10) and re-plated at a density of $5x10^3/cm^2$. This was achieved by careful trypsinisation. In brief, media was removed from the flask by aspiration and the cell monolayer washed with PBS (Gibco, UK – 10010023) to remove all traces of serum. The PBS was then removed by aspiration and trypsin-ETDA (0.05%), phenol red (ThermoFischer Scientific, UK - 25300062) was dispensed, enough to completely cover the monolayer of cells. The flask was placed in an incubator at

37°C 1-2 minutes, until cells detach from the surface. Progress can be checked by examination with an inverted microscope. The precise time required to remove MSCs from the culture surface is dependent on population density, serum concentration in the growth medium, potency of trypsin, and time since last subculture. Trypsin causes cellular damage so time of exposure was kept to a minimum. When the trypsinisation process had completed, medium containing serum was added to the cell suspension to inhibit further tryptic activity. Cells were resuspended by gently pipetting the cell suspension to break up the clumps, then spun down and replated in a new flask with fresh culture medium. Cells resulting from this replating were designated first-passage cells (P1). At each passage cells were typically diluted 1:2.

2.4.17 Freezing MSCs for later use

Following isolation, expansion and serial passage of a cohort of MSCs isolated from an individual mouse a proportion were frozen down (at passage 2-5) for use at a later date. This involved trypsinisation, washing, centrifugation and resuspension in freezing medium (Section 2.5.6). MSCs were stored in freezing medium for up to 5 days at -80°C then transferred to liquid nitrogen for long term storage.

2.4.18 Isolation of MSCs from compact bone

Femurs and tibias were collected from a 10- 12 week-old mouse and BM cells flushed from bones. Bones were placed in Mesencult complete media (Stem Cell Technologies, UK - 05512), gently crushed, transferred to a tissue culture flask and incubated in a sterile humidified incubator (37°C, 5% CO_2). MSCs migrated out of the bone fragments and adhered to the surface of the flask (visible at 24 hours). MSCs were expanded and passaged as per BM-MSCs.

2.4.19 Isolation of MSCs from adipose tissue

Adipose tissue was collected from around the inguinal lymph nodes and abdominal cavity of a 10-12 week old mouse and digested with Collagenase type A (Roche, UK -10103578001) by incubation at 37°C for 1 hour. The cell suspension was filtered (70µm nylon mesh) to remove tissue fragments and centrifuged (2000 xg, 4 °C, 10min). The upper part of the suspension containing fat/debris was removed and the supernatant discarded. RBC lysis solution (Section 2.1.4) was added to the pellet (5 min, RT) and the cell suspension washed twice with PBS. Cells were resuspended in culture medium: (DMEM; Gibco, UK - 10566-016) + 10% Fetal Bovine Serum (FBS; ThermoFisher Scientific, UK - 16000044) + 1% Penicillin/Streptomycin + gentamicin 10-50ng/ml (ThermoFisher Scientific, UK - 15140148), counted and placed in tissue culture flasks in a sterile humidified incubator, 37°C, 5% CO_2. After 24-48 hours medium was removed from cultures (to eliminate non-adherent cells) and replaced with fresh medium. Feeding continued twice per week. After 5-7 days cells were >70% confluent and ready for passage. They were passaged every 4 days until day 21 when the population stopped expanding.

2.4.20 Characterisation of MSCs isolated from bone marrow / compact bone

Flow cytometry was used to identify specific cell surface markers acknowledged in the literature as characteristically expressed by murine MSCs: MHC class I (constitutively expressed by the majority of MSCs), stem cell antigen-1 (Sca-1) and platelet derived growth factor receptor alpha (PDGRFα) (Morikawa *et al.*, 2009; Holmes *et al.*, 2007). In addition to looking for the positive expression of the named cell surface markers it is common practice to screen for a lack of expression of haematopoietic stem cell markers such as CD45. As MSCs mature and their growth is perpetuated by the culture medium (in preference to other cell types such as the haematopoietic cell lineages) the population becomes predominantly CD45 negative and Sca-1 / PDGFRα double positive.

For each cohort of MSCs, on reaching passage 4-5, an aliquot (approx. $2x10^6$ cells) was assigned to analysis of cell surface marker expression by flow cytometry. MSCs were trypsinised, washed, spun down and resuspended in 2% FBS/PBS for antibody staining. Surface staining was carried out with antibodies listed in Table 2.8 for 20 min at 4°C, before

washing and fixation in 200µl 4% paraformaldehyde (ThermoFisher Scientific, UK - FB002). Fluorescence Minus One (FMO) controls or isotope controls were used to determine positive stained populations and samples were run on a FACSAria II™ flow sorter and analysed using FlowJo software (Treestar, USA). Median fluorescence intensity (MFI) and percentages of MSCs stained for a particular cell surface marker were calculated using FlowJo software. As mentioned earlier, mature MSCs lack expression of CD45, so anti-rat / anti-hamster compensation beads (BD Biosciences, UK - 552845) stained with anti-mouse CD4 antibody conjugated to FITC were used to identify this fluorochrome in order set up appropriate compensation.

Table 2.8: List of antibodies used for MSC characterisation flow cytometry.

Antibody	**Clone**	**Fluorochrome**	**Company**	**Catalogue number**
Anti-mouse CD45	30-F11	FITC	eBioscience (California, USA)	MCD4501
Anti-mouse MHC Class 1 (H-2Kb)	AF6-88.5.5.3	PE	eBioscience (California, USA)	12-5958-82
Anti-mouse Ly6A/E (Sca-1)	D7	PerCP-Cy5.5	eBioscience (California, USA)	45-5981-82
Anti-mouse CD140a (PDGFRα)	APA5	APC	eBioscience (California, USA)	17-1401-81
Anti-mouse CD4 (L3T4)	GK1.5	FITC	eBioscience (California, USA)	11-0041-82

2.4.21 Validation of MSCs isolated from bone marrow / compact bone

MSCs have tri-lineage differentiation potential (into osteocytes, adipocytes and chondrocytes). An important part of the process of validation that the cells in culture are indeed MSCs is demonstration that these cells can differentiate into osteocytes and

adipocytes by culturing them in media with specific supplements. Inducing chondrogenesis in murine MSCs is not as widely used for validation as osteogenesis and adipogenesis so was not attempted as part of my validation process.

For osteogenesis, osteogenic supplement was added to basal culture medium (Osteogenic Proliferation Kit, Stemcell Technologies, UK - 05504). Media was changed twice per week for 3 weeks. Cells were fixed with 10% formalin (Sigma-Aldrich, UK -HT501128-4L) and stained with Alizarin-Red-S (Sigma-Aldrich, UK - 130223) to identify extracellular calcium deposits. For adipogenesis, adipogenic supplement was added to basal culture medium (Adipogenic Proliferation Kit, Stemcell Technologies, UK - 05503). Media was changed twice per week for 3 weeks. Cells were fixed with 10% formalin and stained with 0.5% Oil-red-O (Sigma Aldrich, UK – MAK194) to identify lipid droplets.

2.4.22 MSC labelling

Cells were suspended at a density of 10^6/ml in serum-free culture medium (DMEM; Gibco, UK - 10566-016). 5µl of cell-labelling solution (Molecular Probes' Vybrant Cell- Labeling Solutions; Invitrogen, UK - V22889) was added per ml of cell suspension. The suspension was incubated at 37°C for 20 minutes. The cells were washed and resuspended in culture medium. 10 minutes recovery time was allowed before proceeding with fluorescence measurements by flow cytometry.

2.4.23 Preparation of MSC stimulation factors

For *in vitro* stimulation of MSCs to investigate variation in MSC secreted products (secretome) mouse recombinant IFNγ (R&D systems, USA – 485-MI) or different streptococcal preparations were used. The streptococcal preparations were employed to stimulate murine splenocytes and the supernatant from these splenocyte cultures applied directly to the MSCs in culture. In some instances, heat-killed S. pneumoniae was used. Heat-killed *S. pneumoniae* was prepared from the bacterial inoculum made as described in Section 2.4.1 and heated in a heat block at 80°C for 1h. The resultant heat-killed pathogen

was serially diluted, plated onto CBA plates (VWR, UK – EOLAPP0120-P090) and incubated at 37°C overnight to ensure all bacteria had been killed as evidenced by no bacterial growth.

2.4.24 Preparation of murine splenocytes

Transgenic Foxp3DTR (n=3) mice were humanely sacrificed, spleens dissected out and splenocytes resuspended by repeated injection of the spleen with RPMI 1640 medium (Gibco, UK - 11875093) and collection of the cells. Cells were spun down, resuspended at a density of $3x10^6$ cells/ml in complete RPMI (RPMI + 2ME +10% FCS + L-Glut + P/S) and plated in a 24 well culture plate ready for use as described in section 2.4.25.

2.4.25 MSC stimulation with IFNγ

For *in vitro* stimulation of MSCs to investigate variation in MSC secreted products (secretome) mouse recombinant IFNγ (R&D systems, USA – 485-MI) was used. MSCs were plated in a 24 well tissue culture plate at a density of $2x10^5$/ml in Complete Mesencult culture medium (Stemcell Technologies, UK - 05512) and cultured overnight. The following morning, 4 of the 6 wells of MSCs were stimulated with IFN-γ (200ng/ml) and left in culture for a further 6 hours (2 wells) or 48 hours (2 wells). Unstimulated ('resting') MSCs and MSCs stimulated with IFN-γ as described were harvested for RNA extraction and analysis of target gene expression by RT-PCR.

2.4.26 MSC stimulation with splenocyte supernatant post splenocyte exposure to S. pneumoniae

MSCs were plated in a 24 well tissue culture plate at a density of $2x10^5$/ml in Complete Mesencult culture medium (Stemcell Technologies, UK - 05512) and cultured overnight. Murine splenocytes, plated at a density of $3x10^6$ cells/ml in complete RPMI (Invitrogen, UK) were left unstimulated, or were stimulated with live *S. pneumoniae* (10^5 CFU or 10^6 CFU) or heat-killed *S. pneumoniae* (10^5 CFU or 10^6 CFU) and left in culture for 8 hours. Infected splenocyte cultures were then spun down, supernatant aspirated and added to the MSCs in

culture. At time points 6h, 24h and 48h a cohort of MSCs were trypsinised, washed, spun down and resuspended in lysis buffer (Agilent Technologies, UK- 400-711-13) supplemented with β-Mercaptoethanol (Agilent Technologies, UK – 400-711-111)) and stored at -80°C for subsequent RNA extraction and analysis of target gene expression by RT-PCR.

2.4.27 *IVIS imaging of fluorescently-labelled MSCs in vivo*

Mice (white) were infected i.n with *P. aeruginosa* 10^8 CFU for 60 minutes to induce an inflammatory response in the lung and encourage MSCs to traffic to the site of infection. Mice were then anaesthetised using isoflurane, injected (i.v) with 10^6 MSCs labelled with Molecular Probes' DiD cell labelling solution and fluorescence generated by these labelled MSCs measured using an IVIS Lumina II imaging system (Perkin Elmer). Average radiance for areas of interest in fluorescence images were calculated using Living Image 4.2 software (Perkin Elmer).

2.4.28 IVIS imaging of IFNγ response in vivo

Mice were injected into the peritoneal space (i.p) with 200 µl of 15 mg/ml XenoLight D-luciferin – K^+ Salt (Perkin Elmer, UK - 122799). D-Luciferin is a chemical substance found in the cells of organisms that generate bioluminescence. When luciferin is oxidized under the catalytic effects of firefly luciferase and ATP, light is produced. Luciferin is able to penetrate cell membranes and can be used to monitor activity of cells of interest *in vivo* that have been transduced to express luciferase. Mice were left for 10 minutes prior to induction of anaesthesia using isoflurane and bioluminescence measurement using an IVIS Lumina II imaging system (Perkin Elmer). Average radiance for areas of interest in bioluminescence images were calculated using Living Image 4.2 software (Perkin Elmer).

2.4.29 Statistical analysis

Appropriate statistical tests were carried out according to the nature of the data set using GraphPad Prism software (GraphPad Software Inc, USA) and data presented as mean ± SEM.

Non-parametric Mann Whitney U statistical test was carried out on bacterial counts and percentage weight loss during infection experiments as data points did not follow normal Gaussian distribution. For ELISA readouts of cytokine levels and RT-PCR readouts of gene expression statistical significance was determined using the unpaired student t-test. Significant differences between groups are indicated by asterisks above the bars.

2.4.30 Acknowledgement of experimental training and technical support in this thesis (including data analysis).

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Establishing the mouse model of *P. aeruginosa* lung infection - Dr Kathryn Quigley and Dr. Catherine Reynolds.
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Flow cytometry, cell sorting and IVIS imaging and tracking of the IFN-γ response *in vivo* in the Foxp3.DTR-IFNγ-reporter mouse - Dr Lucas Black (L.B.).

2.5 Non-commercial reagents

2.5.1 Genotyping murine strains

Mammalian tissue lysis buffer

Used for digestion of murine ear clip biopsies to extract genomic DNA for genotyping as described in Section 2.4.2.

6.057g Tris

0.9306g Ethylenediaminetetraacetic Acid (EDTA)

5.844g NaCl

1.0g Sodium Dodecyl Sulfate (SDS)

Resuspend in 500ml ddH2O.

2.5.2 Microbiological culture

Brain Heart Infusion (BHI)

Used for liquid culture of *S. pneumoniae* strains as described in Section 2.5.1

18.5g Brain Heart Infusion (OXOID, UK - CM1135)

500ml ddH_2O

Resuspend in 1L H_2O and autoclave to sterilise.

Luria-Bertani (LB) broth

Used for liquid culture of *P. aeruginosa* strains as described in Section 2.4.2

10g Tryphtone (Merck, Germany)

5g yeast extract (OXOID, UK)

10g NaCl (Sigma-Adrich, UK)

Resuspend in 1L H_2O and autoclave to sterilise.

LB agar

Used for plating out *P. aeruginosa* strains from glycerol stocks as described in Section 2.4.2

200ml ddH20

7.4g LB agar (Merck, Germany)

The solution is autoclaved, cooled to 55̊C then poured into sterile Petri dishes.

2.5.3 Molecular biology

50X TAE buffer

Used for running nucleic acid agarose gels

121.0g Tris

28.6ml 16N acetic acid

50ml 0.5M EDTA pH 8.0

Resuspend in 500ml ddH2O. For pouring and running agarose gels, use 1X TAE.

2.5.4 Flow cytometry

Red blood cell lysis buffer

Used to lyse red blood cells in whole blood and single cell splenocytes preparations

prior to flow cytometric staining as described in Section 2.5.19.

4.14g NH4Cl

0.5g KHCO3

0.018g EDTA

Adjust pH to 7.35 with 0.1M NaOH and make up to 500ml with ddH20. Filter-sterilise before use and store at room temperature until ready for use.

2.5.5 ELISA

ELISA block solution

1% BSA in PBS

ELISA wash buffer

0.05% Tween 20 in PBS

2.5.6 Cell freezing

Dimethyl sulfoxide (DMSO) (Sigma, UK)

Fetal bovine serum (FBS) (ThermoFischer Scientific, UK)

DMSO added to FBS at ratio: 10% DMSO to 90% FBS. Once made up, freezing medium can be stored in the fridge for up to one month.

Chapter 3

3 MSC isolation, characterisation and validation

3.1 Introduction

3.1.1 Murine MSCs differ from human MSCs.

Much of the initial work on MSCs was done using human cells, not murine cells. Murine MSCs (mMSCs) are for more difficult to isolate from the bone marrow (BM) than human MSCs (Phinney *et al*., 1999; Sun *et al*., 2003; Peister *et al*., 2004). There are several reasons for this. Firstly, BM harvests contain low frequencies of MSCs (2-5/10^6 BM nucleated cells) (Koc *et al*., 1999). Secondly, mature haematopoeitic stem cells (HSCs) reside in the center of the BM whereas stromal cells reside near the surface of the bone (Hisha *et al*., 1995). This has been reconfirmed in studies in humans and mice which showed that MSCs reside in the endosteum and also within compact bone (Short *et al*., 2001 Fouillard *et al*., 2003; Zhu *et al*., 2010). It follows that simply flushing the BM out of mouse bones yields a very small number of MSCs and misses the opportunity to isolate the compact bone-resident cohort. MSCs have been harvested in large numbers from compact bone in mice (Zhu *et al*., 2010b) and demonstrated to exhibit immunomodulatory activities *in vivo* (Guo *et al.,* 2006). A third reason for difficulty in mMSC isolation is the heterogeneity of the adherent fraction of the BM cells which is heavily contaminated with HSCs and various fibroblastic cells (Zhao *et al*., 2004). This is in contrast to human marrow adherent cells which are relatively homogeneous and contain a high percentage of MSCs (Prockop, 1997).

Another difficulty faced by researchers wishing to work with MSCs is that there is no single surface marker that defines this cell type. The current approach to characterisation of MSCs is to use a panel of surface markers. This technique has been widely adopted, but is vulnerable to the issue of variability of expression of individual MSC cell surface markers across different species, anatomic source and culture methods. Different studies report cultured MSCs as expressing a variety of subsets of cell lineage-specific antigens, adhesion molecules and growth factor receptors (Jiang *et al*., 2002; da Silva Meirelles *et al*., 2006).

In 2006, in an attempt to reduce variability in the populations of cells being isolated and used as putative MSCs and facilitate the standardization of results across laboratories, the International Society for Cellular Therapy (ISCT) published a position paper stating that human MSCs must meet certain criteria. The first criterion is adherence to plastic when maintained in standard culture conditions. Conventional protocols for the isolation and expansion of MSCs utilise their capacity to adhere to plastic following a 1-2-week period in culture. The second criterion is the expression of specific cell surface markers, identified for human MSCs as CD105, CD73 and CD90, in conjunction with a lack of expression of haematopoietic progenitor (CD45, CD34, CD14), endothelial (CD11b, CD79a, CD19) and HLA-DR surface molecules. Of note, the listed cell surface markers are not specific for MSC, the same markers have been shown to be expressed by dermal fibroblasts (Alt *et al*., 2011; Blasi *et al*., 2011). Lastly, MSCs must demonstrate their tri-lineage differentiation potential into osteocytes, adipocytes and chondrocytes *in vitro* (Dominici *et al*., 2006).

3.1.2 Characterisation of murine MSC

The situation is more complex for MSCs isolated from murine tissues, for which there is no such agreement on criteria for describing bona fide mMSCs. However, some elegant work done by Morikawa and colleagues in the last decade has highlighted some key candidates for consideration in the design of a panel of cell surface markers ascribable to mMSCs (Morikawa *et al*., 2009). These candidate markers have since been adopted by researchers, including Professor Dazzi's research group (Dazzi *et al.*, 2012). Preliminary studies identified several mesenchymal lineage-specific markers in murine BM cells (Koide *et al*., 2007). After initial screening, the group homed in on two possible MSC markers (Morikawa *et al*., 2009), platelet-derived growth factor receptor α (PDGFRα; an early mesodermal marker; Takakura *et al*., 1997) and stem cell antigen-1 (Sca-1; a known stem cell marker; Ortega *et al*., 1986).

Post removal of haematopoetic $CD45^{+}TER119^{+}$ cells they investigated putative MSCs, taken from the $CD45^{-}TER119^{-}$ fraction of freshly prepared BM cells, for the expression of one or both of these markers. Four distinct subpopulations were identified ($PDGFR\alpha^{+}$ / $Sca\text{-}1^{+}$, $PDGFR\alpha^{+}$ / $Sca\text{-}1^{-}$, $PDGFR\alpha^{-}$ / $Sca\text{-}1^{+}$, $PDGFR\alpha^{-}$ / $Sca\text{-}1^{-}$). Each subpopulation was tested by several *in vitro* assays to determine their characteristics. Colony count, as measured by

traditional CFU-F assay, was highest in the PDGFRα^{+} / Sca-1^{+} subpopulation (designated PαS). This subpopulation was the only one to proliferate without senescence, yielding >10^{7} cells from the original 5000 seeded. By day 14 in culture these cells had developed the spindle-shaped morphology indicative of MSC. In contrast, the other subpopulations showed different morphologies, such as the cobblestone appearance typical of endothelial cells seen in the PDGFRα^{-} / Sca-1^{+} group (Morikawa *et al*., 2009).

A key observation relating to the validity of potential MSCs within specific subpopulations was that only the PαS cells differentiated convincingly into adipocytes, chondrocytes and osteocytes. To test this more rigorously, differentiation assays were repeated with expanded PαS cell colonies derived from a single PαS cell. All six clones selected differentiated into osteocytes and chondrocytes and expressed bone and cartilage-related genes as measured by RT-PCR. Four of the six clones also underwent adipogenic differentiation and expressed fat-related genes. The *in vivo* location of this PαS subpopulation was identified as being within the arterial perivascular space, in close proximity to the inner surface of cortical bone and vascular smooth muscle cells in BM, consistent with the perceived role of MSCs as haematopoietic niche cells (Morikawa *et al*., 2009). Indeed, PαS cells were found to express the gene for angiopoeitin-1 (*Ang-1*) by RT-PCR which is known to be an important paracrine mediator in the maintenance of haematopoiesis (Arai *et al*., 2004). The final piece of evidence that this PαS subpopulation of cells constituted bona fide mMSCs came from some carefully constructed *in vivo* experiments in which GFP-labelled PαS cells were demonstrated to repopulate components of the haematopoietic niche. The majority of these cells were located in the perivascular region or adjacent to the inner surface of cortical bone. PαS cells were also located in adipose tissue of recipient mice (Morikawa *et al*., 2009).

3.1.3 MSC labeling for in vivo localisation

The ability of MSCs to migrate or 'home' to specific tissues in order to repopulate a particular subpopulation of cells, or in response to certain environmental cues, is a matter of considerable interest to researchers in the field. For this migration to be visualised there needs to be a system for labelling MSCs. This can be achieved in a variety of ways. MSCs can

be isolated from green fluorescent protein (GFP) transgenic animals, or transfected to express GFP, and tracked by fluorescence using non-invasive biophotonic (IVIS) imaging (Xiang *et al*., 2009). MSCs can be isolated from luciferase-expressing transgenic animals, or transfected to express the luciferase reporter gene and tracked by bioluminescence using IVIS imaging. Following i.v injection of MSCs and i.p injection of luciferin substrate MSCs can be visualized *in vivo* by IVIS imaging of the bioluminescent signal (Westrich *et al*., 2010; Jang *et al*., 2010).

The two approaches can be combined to create MSC-GFP-luci cells which are both fluorescent upon appropriate excitation and bioluminescent post exposure to luciferin substrate (Xiang *et al*., 2009; Cao *et al*., 2016). In a mouse model of fibrosarcoma, Xiang and colleagues showed that xenogeneic MSC-GFP-luci selectively homed to and engrafted into the tumour stroma. Interestingly, high luciferase activity was evident in the lungs, as well as the tumour, at 24 hours post injection, suggestive of some degree of 'lung trapping' of MSCs. Lung luciferase activity decreased gradually back down to baseline levels over the course of 1 week. Tumour bioluminescence was detectable up to 17 days post MSC administration. In this and other studies, using fluorescent and bioluminescent signal tracing as a means of monitoring cell migration and localization, IVIS imaging data was validated using Western blots and immunofluorescent staining for GFP (Xiang *et al*., 2009; Jang *et al*, 2010).

An alternative method of MSC labeling is the use of iron oxide nanoparticles which can be visualized with magnetic resonance imaging (MRI). This has been demonstrated *in vivo* in a metastatic lung cancer model. MSCs, labeled with magnetic nanoparticles and delivered by intravenous injection were tracked to pulmonary metastases using MRI (Loebinger *et al*., 2009). In this study MSCs were double-labeled with iron particles and DiI fluorescent dye to facilitate the co-localisation of these markers within tumour cells and dispel concerns of ectopic iron detection being wrongly attributed to MSC localization. DiI labelling has previously been validated as a technique for tracking human cells transplanted into mice (Schormann *et al*., 2008).

3.1.4 MSC distribution following systemic administration

An issue for consideration when working with MSCs is what some researchers refer to as "the lung barrier" or "pulmonary first-pass effect". Studies have shown that potential MSC migration to damaged organs post systemic administration is impeded by cell trapping within the lung (Xiang *et al*., 2009; Schrepfer *et al*., 2007). Schrepfer and colleagues investigated the size of murine MSCs in culture and the dimensions of murine pulmonary capillaries. They found that the diameter of suspended MSCs *in vitro* (15-19 µm) exceeded capillary diameter as measured *in vivo* by fluorescently labelled microspheres. Interestingly, the numbers of trapped 10-15 µm microspheres were significantly decreased by prior treatment with vasodilator sodium nitroprusside (Schrepfer *et al*., 2007). Similar observations were made in rats given syngeneic radioactively-labeled MSCs by both intra-arterial (i.a) and intravenous (i.v) roots. Following both methods of infusion, radioactivity associated with MSCs was detected primarily in the lungs (for as long as 48h post injection) and then secondarily in the liver and other organs. When sodium nitroprusside was used, more labeled MSCs cleared the lungs, resulting in a larger proportion being detected in the liver (Gao *et al*., 2001).

Whilst the primary factor attributable to MSCs lodging in the lungs is likely to be cellular diameter, cellular attachment potential (MSCs attaching to the vessel wall and MSCs adhering to each other) may well be contributory. BM MSCs have been demonstrated to be attachment-competent cells, utilising fibro-nectin for attachment in *in vitro* culture conditions; it has been suggested that MSCs may attach to fibronectin-rich endothelial domains *in vivo* (Gao *et al*., 2001). However, despite these observations of lung trapping, researchers have gone on to achieve successful MSC systemic delivery and distribution to target organs without the need for prior vasodilatation. A recent study by Johnson et al showed that in a murine model of chronic wound infection intravenous MSC lodged in the lungs initially, but *in vivo* imaging studies revealed MSC migration from the lungs to the wound site over the next 24–72 hours (Johnson *et al*., 2017). Of course, if the target organ for MSC treatment is the lung, then a degree of sequestration in the lung, provided there is not significant capillary blockage and consequent pulmonary embolism, might be of some benefit. The ability to deliver cellular therapy to the lung via a simple intravenous approach

could be regarded as a major advantage. Of note, retained MSCs appear to target areas of injured lung in preference to healthy lung (Yan *et al*., 2007).

3.1.5 The MSC secretome

There are several approaches to validating MSCs, some of which are indispensable for proof of validity, such as demonstration of differentiation potential into cells of mesodermal lineage, and others that are probably less critical and can be pursued at the investigator's discretion. After demonstrating MSC differentiation capabilities I briefly investigated the MSC secretome, as published studies using Transwell insert culture systems to separate MSCs from target cells have suggested that many of the immunomodulatory effects of MSCs are likely mediated through paracrine mechanisms as opposed to direct cell-cell contact (Di Nicola *et al*., 2002; Tse *et al*., 2003; Djoud *et al*., 2003). Indeed, MSC-conditioned media has been shown to replicate the treatment effects of MSCs themselves (Aslam *et al*., 2009). One study comparing the phenotypic and functional features of human adipose-derived MSCs (AD-MSCs) with normal human dermal fibroblasts (NHDFs) concluded that AD-MSCs cannot be distinguished from NHDFs *in vitro* by evaluating their phenotypic profile or differentiation potential, but only through the analysis of their anti-inflammatory and angiogenic properties, including evaluation of secreted soluble mediators pre- and post-priming with TNF-α. Of note, stimulation with TNF-α enhanced the anti-inflammatory potential of the MSCs whilst the fibroblasts acquired pro-inflammatory activity (Blasi *et al.*, 2011).

The paracrine mediators implicated in MSC immunomodulation include growth factors, cytokines and chemokines, some of which are geared towards dampening down an established inflammatory response, others are more relevant to tissue regeneration and repair. The small collection chosen for investigation in this thesis (TGF-β, IL-6 and IL-10) is postulated to be involved in a variety of ways in MSC impact on immune response to bacterial infection (Kadioglu *et al*., 2004; Rafaghello *et al*., 2008; Nemeth *et al*., 2009; Neill *et al*., 2012). MSC-mediated TGF-β secretion has been shown to result in potent inhibition of T-cell proliferation (Ahuja *et al*., 1993; Fox *et al*., 1993; Di Nicola *et al*., 2002). In corroboration with this is the observation that the proliferative capacity of T cells co-

cultured with MSCs can be restored by the addition of anti-TGF-β neutralizing antibody (Di Nicola *et al*., 2002). Of relevance to lung infection, CBA/Ca mice deficient in TGF-β succumbed much more readily to invasive pneumonia than BALB/c mice with intact TGF-β signaling. The quite profound difference in pathogen handling was attributed to the protective effects of TGF-β-mediated Treg expansion seen in the resistant BALB/c mice, which was lacking in the susceptible CBA/Ca mice (Neill *et al*., 2012). In animal models of asthma, it has been demonstrated that administration of murine BM-MSCs reduces bronchial inflammation, airway hyper responsive and mucus secretion. MSC anti-inflammatory effect is attributed to a variety of mechanisms including production of TGF-β, which recruits Tregs to the lungs (Goodwin *et al*., 2011; Nemeth *et al*., 2010; Kavanagh and Mahon, 2011).

The secretome of activated MSCs also includes IL-6, which appears to play a part in halting the differentiation of monocytes into mature DCs, and thus impacts on the efficiency of antigen presentation (Jiang *et al*., 2005). Some studies suggest that IL-6 is secreted constitutively by MSCs (Kim *et al*., 2005b; Wagner *et al*., 2007) and inhibits neutrophil apoptosis (Asensi *et al*., 2004). Indeed, MSC/neutrophil co-culture studies demonstrate an IL-6-mediated MSC-protective effect against neutrophil apoptosis and inhibition of excessive oxidative metabolism which may well contribute to observed MSC anti-inflammatory effects (Rafaghello *et al*., 2008).

IL-10, like TGF-β, is a known immunosuppressive cytokine that can inhibit pro-inflammatory cytokine secretion by APCs and can induce T_{regs} (Asseman and Powrie, 1998). When used at concentrations that reflect those seen in MSC culture, IL-10 has been shown to suppress T cell proliferation in allogeneic MLRs. However, the addition of neutralizing monoclonal antibody directed against IL-10 to MLR containing MSC failed to prevent MSC-mediated suppression (Ryan *et al*., 2007), suggesting that there are a number of factors at play in determining MSC suppressive effects and a degree of redundancy exists in the mechanisms of immunosuppression. Nakajima and colleagues recently published some interesting work looking at neuroprotective effects of MSCs. They investigated the therapeutic benefit of adeno-associated virus (AAV)-mediated IL-10 overexpression in MSCs transplanted during the acute phase of ischemic stroke. Results showed that IL-10-overexpressing MSCs

significantly reduced infarct volume and enhanced motor functional recovery at 72 hours post transient middle cerebral artery occlusion (Nakajima *et al*., 2017).

In the final part of this chapter I explore how IFN-γ-stimulated MSCs differ from unstimulated MSCs in their secretome and the time course of this response to stimulation. Human and murine MSCs have been shown to constitutively express low levels of immunomodulatory factors such as PGE-2, TGF-β1 and HGF (Di Nicola *et al*., 2002; Ryan *et al*., 2007). It has been demonstrated that stimulation by pro-inflammatory cytokines IFN-γ (Krampera *et al*., 2006) and TNF-α (Wang *et al*., 2007) alters the MSC secretome, inducing the secretion of immunosuppressive molecules such as TGF-β and HGF (Krampera *et al*., 2006; Wang *et al*., 2007; Ryan *et al*., 2007). This is relevant for clinical usage, as allogeneic MSC must remain immune-regulatory in an inflamed environment if they are to be therapeutic. It is therefore of fundamental importance to understand the impact of pro-inflammatory stimuli, such as IFN-γ and TNF-α, on MSC secretion of soluble factors involved in the suppression of alloantigen-driven T cell proliferation (Ryan *et al*., 2007).

In addition to the effects of the inflammatory environment on MSC function, there is evidence that MSC exposure to pathogens can stimulate changes in their biological function: proliferation, differentiation, migration and apoptosis (Fiedler *et al*., 2013; Ward *et al*., 2015). MSCs also exhibit variations in their secretome, increasing the secretion of cytokines and chemokines that they make constitutively (TGF-β, IL-6 and PGE2), which can enhance their immunomodulatory effects (Kol *et al*., 2014; Josse *et al*., 2014). This may not be a long-term phenomenon however, pathogen-induced changes in MSC behaviour may ultimately lead to demise of the MSC (Holban *et al*., 2014). Holban and colleagues investigated the role of *P. aeruginosa* quorum sensing molecules on the viability and cytokine profile of hMSCs. They found that the initial response of MSCs was to increase cytokine production, but this was only transient, they then underwent apoptosis (Holban *et al*., 2014).

3.2 Mesenchymal stem (stromal) cells can be isolated from a combination of murine bone marrow and compact bone.

MSCs were isolated from murine BM and compact bone as described in sections 2.5.15 and 2.5.18 of the Methods chapter. The initial cell population from BM, consisting of a small number of MSCs in amongst other stromal cells, fibroblasts and HSCs, was cultured in specialised culture media designed to support the proliferation and expansion of MSCs; Mesencult complete media (Stem Cell Technologies). Post flushing of BM, bones were gently crushed and placed in culture with the cells. Pilot experiments, investigating the isolation of MSCs from compact bone alone, showed that MSCs migrate out of the bone fragments and adhere to the surface of the flask, visible in very small numbers at 48 hours (data not shown). The technique of combining BM cells with compact bone in culture was thus adopted to maximize the efficiency of MSC isolation from each mouse sacrificed for this purpose.

Non-adherent cells were removed by changing the culture medium every 3-4 days until areas of confluent cells appeared. At each culture medium change cell density and appearance was checked by phase contrast microscopy to verify the presence of adherent cells with spindle-shaped morphology, consistent with MSCs. After 3-5 days, adherent, spindle-shaped cells appeared as individual cells or clusters of only a few cells (Figure 3.1A). By day 7-10, the number of colonies had increased and a few large colonies could be seen. Macrophages remained as a contaminant at this early stage in culture and could be distinguished from MSCs by their different shape. They exhibited stronger adherence to plastic at the first attempt at passage of the cells to a new culture flask, and thus were not as easily trypsinized, and could be left behind, so as not to contaminate the MSC population any further. By the end of the fourth passage cultures had very low observed numbers of cells suggestive of macrophages and consisted of sheets of spindle-shaped cells consistent with MSC morphology (Figure 3.1B).

Initial pilot experiments growing MSCs from different anatomic niches (BM, compact bone, BM and compact bone in combination and adipose tissue) showed most promise in terms of reliable proliferation and expansion in the bone marrow / compact bone preparation so this was taken forward for use in the infection and MSC treatment experiments. Attempts were

also made to isolate MSCs from murine lung, but this was not successful owing to the scarcity of the desired cell type in lung tissue (data not shown).

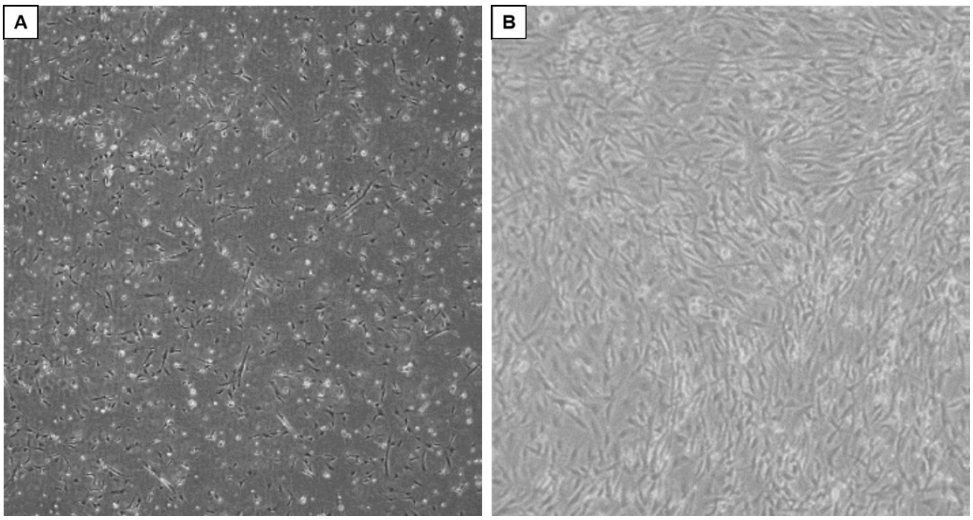


Figure 3.1 Morphological characteristics of MSCs.

A) Phase contrast image of MSCs in culture, 4 days post isolation, at passage 0 (P0). A few spindle-shaped cells are visible amongst the HSC and fibroblast contaminants (magnification = 10x).

B) Phase contrast image of passage 4 (P4) MSCs which have been in culture for 3-4 weeks. The majority of cells are spindle-shaped bipolar fibroblast-like cells; the population appears considerably more homogeneous than the P0 cells (magnification = 10x).

Assisted by L. L. and L. W.

3.3 Phenotypic Characterisation of MSCs

For each preparation of MSCs isolated from a specific mouse, on reaching passage 4-5, an aliquot (approx. $2x10^6$ cells) was assigned to analysis of cell surface marker expression by flow cytometry. MSCs were analysed for the expression of a small panel of antigens: MHC class I, stem cell antigen-1 (Sca-1), platelet derived growth factor receptor alpha (PDGRFα) and CD45. As explained in the introduction to this chapter, the choice of cell surface markers to look for in mMSC remains a matter for debate. Professor Francesco Dazzi's research team advised on choice of markers for use during MSC characterization. The Dazzi lab employs the approach inspired by Morikawa (Morikawa *et al*., 2009) and analyses MSCs for the positive expression of MHC class I, Sca-1 and PDGRFα and the lack of expression of CD45 and TER-119.

Results show that 99% of cells within the selected MSC gate were strongly stained with monoclonal antibodies directed against MHC-1, which is regarded as being constitutively expressed by the majority of MSCs (Holmes *et al*., 2007) and were negative for the expression of CD45, a pan-haematopoetic marker. Within this MHC-1 +ve CD45 –ve population, more than 85% stained for both Sca-1, a hallmark of haematopoeitc and mesenchymal stem/progenitor cells, and PDGFRα, an early mesodermal marker (Figure 3.2).

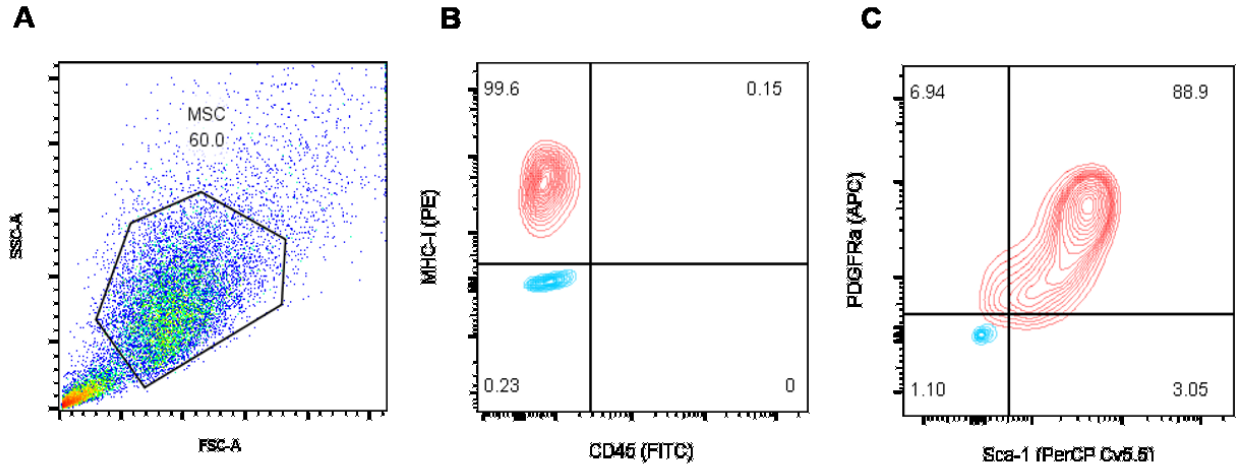


Figure 3.2. Characterisation of mature (passage 5) MSCs as CD45 negative, MHC-I expressing and PDGFRα and Sca-1 double positive.

MSCs were isolated from murine bone marrow, expanded in culture in sterile conditions at 37°C, 5% CO_2 and passaged when confluent. Cell surface expression of MHC class I, stem cell antigen-1 (Sca-1), platelet derived growth factor receptor alpha (PDGRFα) and CD45 was measured on passage 5 (P5) MSCS by flow cytometry to confirm maturation.

(A) MSC gating strategy. The majority of cells are (B) CD45 negative and express MHC-I and (C) Sca-1/ PDGRFα double positive.

Assisted by L. L. and J. R.

3.4 Validation of MSCs

MSCs have at least tri-lineage differentiation potential (into osteocytes, adipocytes and chondrocytes). An important part of the process of validation of MSCs in culture is the demonstration that these cells can differentiate into osteocytes and adipocytes by culturing them in media with specific supplements.

MSC cultured with adipogenic-inductive medium retained their spindle-shaped morphology but spread out over the culture surface, appearing less densely distributed than the control cohort by the end of the first week. From day 10, intercellular lipid vacuoles were visible (observed in between MSCs and newly generated adipocytes), reaching a peak in frequency and distribution around day 14. Detection was enhanced by staining with Oil-Red-O (Figure 3.3B). No lipid vacuoles were detected by Oil-Red-O staining in control MSC cultures (Figure 3.3A). When cultured in osteo-inductive medium MSC underwent a noticeable change in cellular morphology from spindle-shaped to cuboidal. By day 28 this morphological change was accompanied by a substantial calcium deposition, as measured by increased staining with Alizarin-Red-S (Figure 3.3C). No calcium deposition was detected by Alizarin-Red-S staining in control MSC cultures.

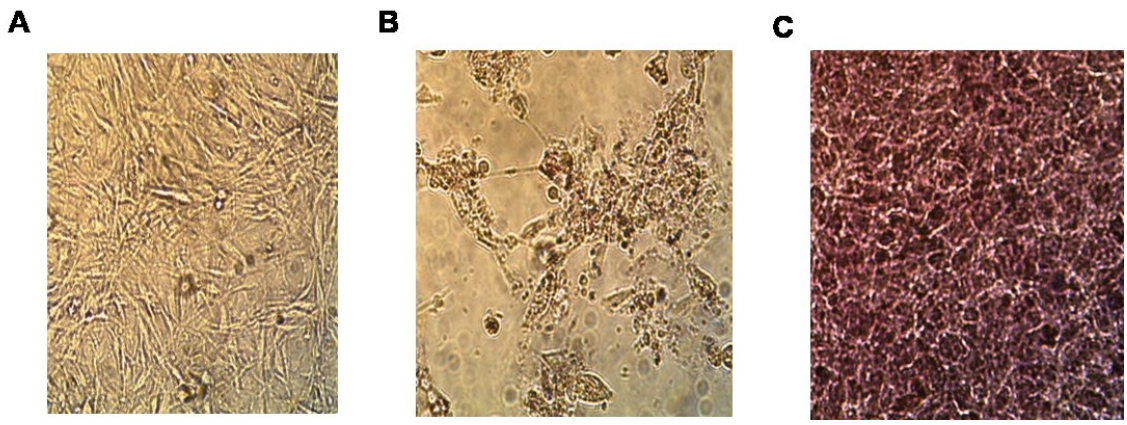


Figure 3.3. Validation of mature (passage 5) MSCs by demonstration of their differentiation capacity into adipocytes and osteocytes.

Murine MSCs were cultured in supplemented media. After 3 weeks, adipocytes were fixed with 10% formalin and stained with 0.5% Oil-red-O to identify lipid droplets and osteocytes stained with Alizarin-Red-S to identify extracellular calcium deposits.

(A) Undifferentiated passage 5 (P5) BM-MSCs cultured in standard media.

(B) P5 BM-MSCs cultured in adipogenic media, stained with Oil-Red-O to identify lipid droplets.

(C) P5 BM-MSCs cultured in osteogenic media, stained with Alizarin- Red-S to identify extracellular calcium deposits.

Assisted by L. L. and L. W.

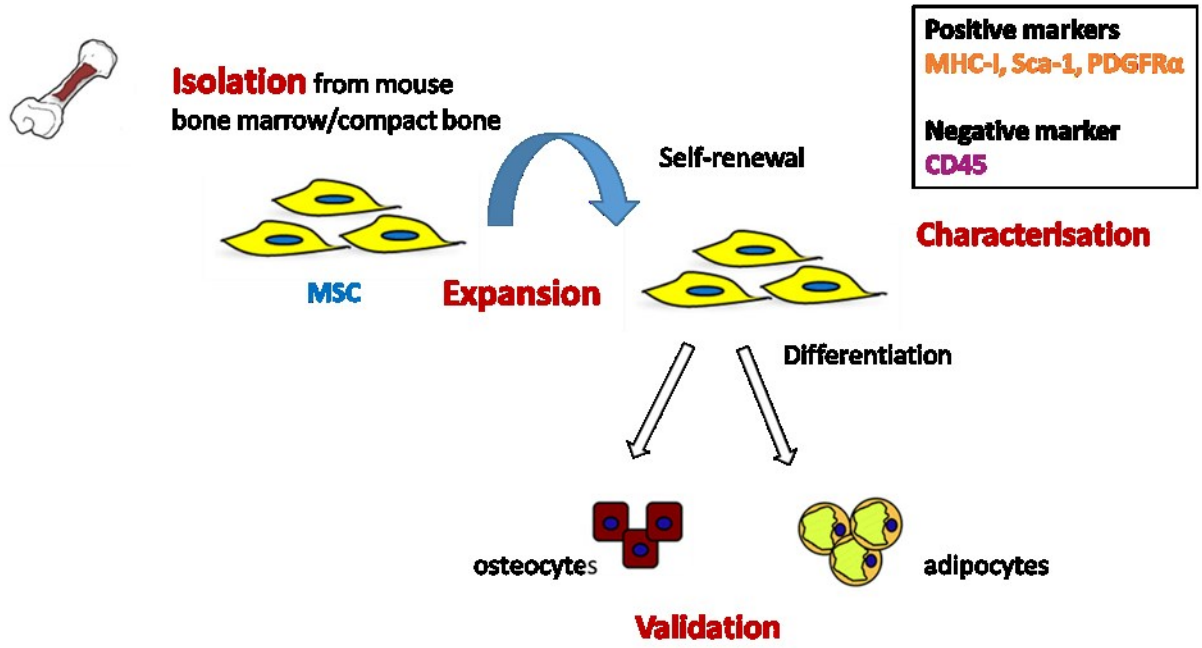


Figure 3.4. MSC isolation, expansion, characterisation and validation.

3.5 Labelled MSCs can be tracked *in vivo* by IVIS imaging and home to infected lung.

Following validation of the MSCs in culture, direct visualisation of MSCs *in vivo* was an exciting prospect to demonstrate where MSCs go following intravenous administration, whether or not they are trapped in lung, and for how long they reside at their final destination. I devised an approach that encompassed labelling MSCs with a fluorescence marker, and detecting the signal post i.v administration using IVIS imaging, thus allowing tracking of MSCs *in vivo* in a live animal.

My starting point was to select an appropriate fluorescent marker for MSC labelling. Molecular Probes' Vybrant dyes DiD, DiI and DiO cell-labelling solutions were trialed alongside each other to establish which of them had optimal characteristics for potential *in vivo* detection. Each dye was added to aliquots of MSCs to uniformly label cells prior to identification by flow cytometry over a 2-day period. Of the 3 labelling solutions trialed, Vybrant dye DiD was chosen for MSC labelling owing to its brightness, longevity of staining and consequent tissue penetrating capabilities (Figure 3.5).

Intravenous injection of MSCs in mice is notoriously difficult because of the high incidence of lethal pulmonary embolism (PE), even with relatively low numbers (Krampera *et al*., 2013). I addressed this problem by adopting a meticulous approach to MSC suspension preparation, ensuring careful separation of cells by repeated aspiration/expulsion from the injecting syringe through the injecting needle prior to i.v administration. Infusion over 20-30 seconds was favoured over bolus injection. DiD-labelled MSCs were injected into white mice initially, to maximise the chance of visualisation of fluorescent signal using IVIS technology. Prior to injection, these mice were infected intranasally with *P. aeruginosa,* to create an inflammatory environment locally in the lung towards which MSCs would preferentially migrate. Immediately following tail vein injection (t=0) a fluorescent signal was visible at the injection site. 20 minutes later (t=20) fluorescence was detectable in the thoracic region, where it remained, though reduced in intensity, at the 40-minute time point (t=40) (Figure 3.6 A-C).

In a separate experiment, this time conducted in black mice and utilizing an alternative lung pathogen (*S. pneumoniae*), comparison was made in the dynamics of MSC distribution between infected and uninfected mice. The mouse infected i.n with *S. pneumoniae* and

treated 3h later with i.v MSCs exhibited increased fluorescence in the lung compared with liver and spleen, 40 mins post MSC administration, as determined by flow cytometric analysis of fluorescent cells in *ex vivo* organs. Fluorescent cells, present at lower numbers than in lung, were also detectable in liver and spleen. A healthy, uninfected mouse injected with MSCs exhibited a far smaller increase in lung and liver cell fluorescence at the 40 minute time point. No fluorescence was detected in cells isolated from the organs of a PBS-treated control mouse (Figure 3.7). These observations are based on very small n numbers, but suggest that MSCs are not be routinely trapped in the lung, and can migrate to inflamed lung in the context of bacterial lung infection.

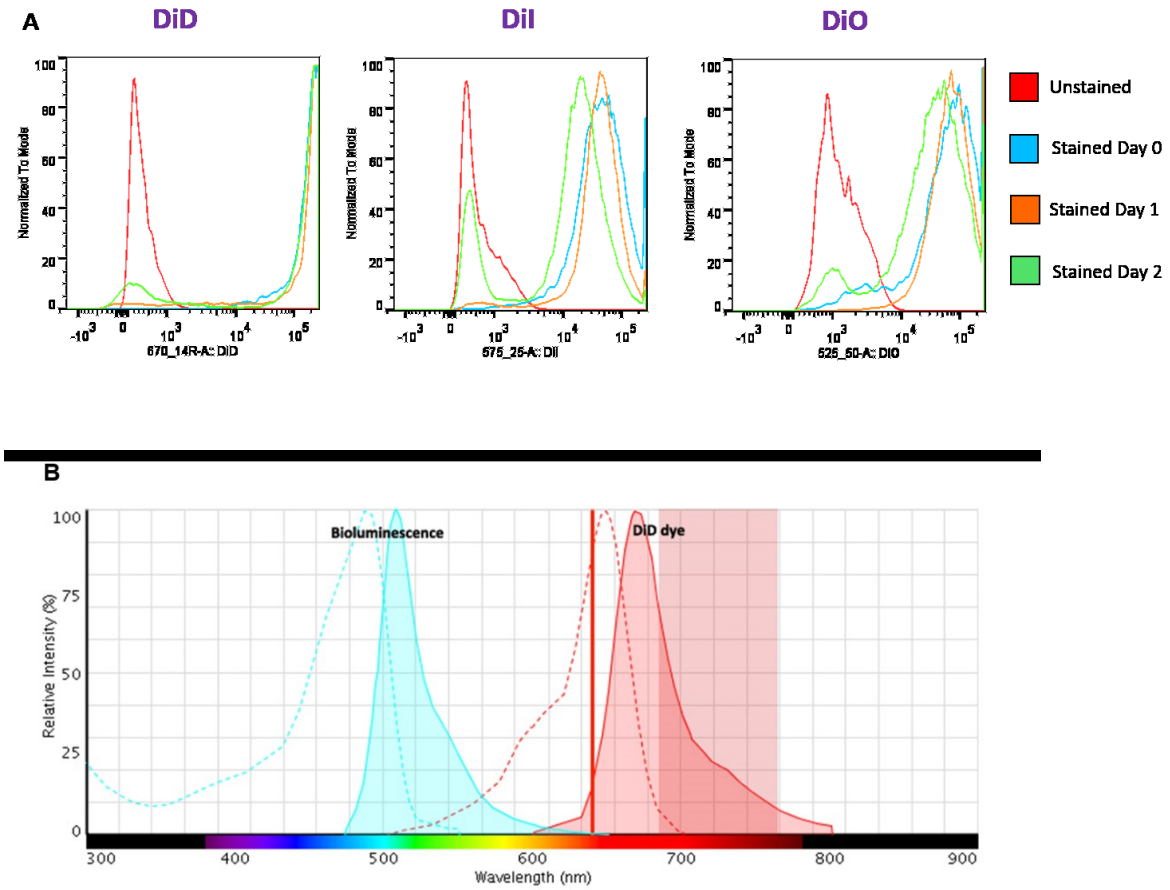


Figure 3.5. Murine MSC labelling with fluorescent dye DiD was the brightest and most long lasting with the majority of MSCs remaining stained after 2 days in culture.

Molecular Probes' Vybrant dyes DiD, DiI and DiO cell-labelling solution was added to MSCs *in vitro* to uniformly label cells and facilitate identification using flow cytometry over a 2-day period (A). The fluorescent wave length at which DiD dye can be visualized is in the red region of the spectrum which equates to good tissue penetration (B).

Assisted by J. M.

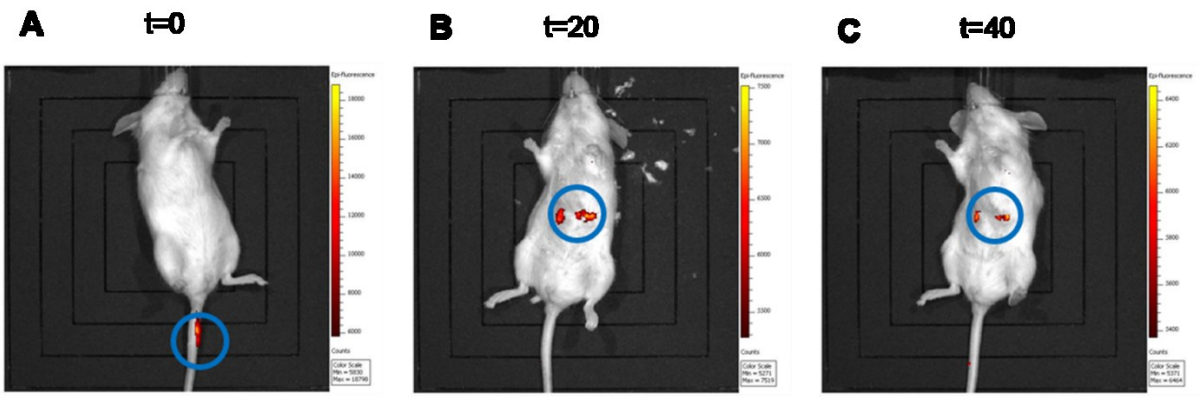


Figure 3.6. MSCs traffic to lung infected with *P. aeruginosa*

NODE mice (white fur and reduced skin pigmentation) were infected i.n with *P. aeruginosa* 10^8 CFU to generate a local inflammatory environment in the lung. Sixty minutes post infection, mice were injected intravenously with 10^6 DiD-labelled mMSC and imaged by IVIS (A) immediately post MSC injection (t=0), (B) 20 (t=20) and (C) 40 (t=40) minutes post MSC treatment.

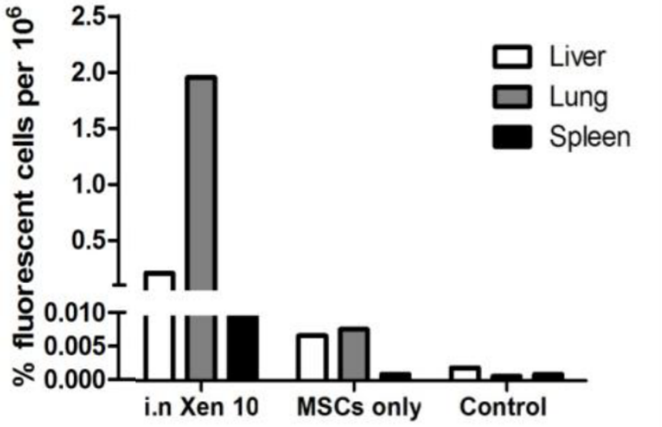


Figure 3.7. MSCs traffic to lung infected with *S. pneumoniae*

C57BL/6 mice were infected i.n. with *S. pneumoniae* 10^7 CFU (n=1) or given PBS (n=2). After 3 hours, 10^6 DiD-labelled MSCs were injected via the tail vein. Organs were harvested 40 minutes after DiD-labelled MSC treatment and cells extracted from liver, lung and spleen analysed for flouresence by flow cytometry. The graph illustrates the percentage of fluorescent cells per million in each organ for the 3 groups: i) infection with *S. pneumoniae* (Xen 10) and MSC treatment, ii) MSC treatment only (no infection), and iii) PBS control (no infection and no MSC). Assisted by J. M.

3.6 Differences in MSC secretome induced by stimulation ('licensing') with IFN-γ and exposure to supernatant from splenocytes infected with *S. pneumoniae*.

The final part of this chapter investigates a small selection of paracrine mediators which MSCs are known to secrete and thus constitute a proportion of the 'MSC secretome'. It addresses differences in the secretome of unstimulated 'resting' MSCs compared with stimulated MSCs. I was interested to explore if *in vitro* stimulation with IFN-γ would influence the secretome of the MSCs I had in culture. This is particularly relevant to the next phase of my work which utilizes these cultured MSCs in mouse models of bacterial lung infection, where they are exposed to an inflammatory microenvironment at the point of administration. Two timepoints post IFN-γ treatment, 6h and 48h, were selected for assessment of the effect of IFN-γ stimulation on the MSC secretome. Differences in the secretome of unstimulated, 6h and 48h stimulated MSCs were determined by analysis of target gene expression by RT-PCR. The target genes chosen, *tgf-β1*, *il-6* and *il-10*, have been shown by other investigators to be part of the secretome of validated MSCs (Jiang *et al*., 2005; Ryan *et al*., 2007).

Unstimulated MSCs constitutively expressed low levels of TGF-β, IL-6 and IL-10. At the 6h time point post stimulation with IFNγ, TGF-β expression increased significantly to approximately 4x the expression levels of resting MSCs. By 48h, TGF-β expression had returned almost to baseline levels, similar to that of resting MSCs. In contrast, IL-6 expression levels increased more gradually over the 48h experiment and peaked later than TGF-β, at the 48h endpoint. Again, there was an approximately 4-fold difference in IL-6 expression between unstimulated MSCs and MSCs 48h post stimulation. IL-10 expression levels also increased gradually over the 48h period, but to a lesser extent, approximately 2-fold in 48h stimulated MSCs compared with unstimulated MSCs (Figure 3.7). These findings are consistent with a model in which MSC can be activated by inflammatory signals and that such activation supports potent immunosuppressive activity.

The MSC secretome also appears to be influenced by exposure to pathogen, specifically supernatant from splenocytes infected with live and heat-killed *S. pneumoniae*. Unstimulated MSCs constitutively expressed low levels of TGF-β and this was unaffected by exposure to supernatant from uninfected 'healthy' splenocytes. At the 6h time point post stimulation with supernatant from splenocytes infected with live S. pneumoniae, TGF-β

expression increased significantly, and this continued to the 24 hour time point. This response was even more pronounced in the MSC cohort exposed to supernatant from splenocytes treated with heat-killed *S. pneumoniae,* where TGF-β expression levels reached nearly 3 times that of unstimulated MSCs (Figure 3.8). This data is interesting because it suggests that MSCs are acutely sensitive to their environment and respond to an encounter with *S. penumoniae*-infected cells by upregulating TGF-β expression. If this occurs *in vivo*, it may drive T_{reg} recruitment, which may be beneficial to host response to this pathogen.

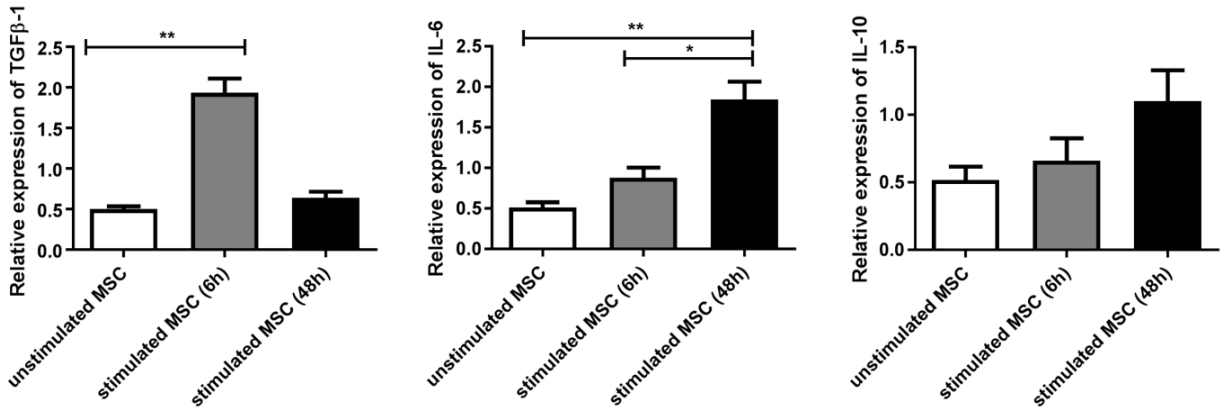


Figure 3.8. Relative expression of genes encoding TGF-β1 and IL-6 by MSCs is enhanced by stimulation with IFN-γ *in vitro*.

Passage 5 MSCs (Foxp3DTR background) seeded at $2x10^5$ cells/ml were cultured overnight in 24 well tissue culture plates. Unstimulated 'resting' MSCs and MSCs stimulated with IFN-γ (200ng/ml) for 6h or 48h were harvested for RNA extraction and expression of TGF-β1, IL-6 and IL-10 measured by RT-PCR. The experiment was repeated 3 times with consistent results achieved. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*$p<0.05$ **$p<0.01$).

Assited by C. R.

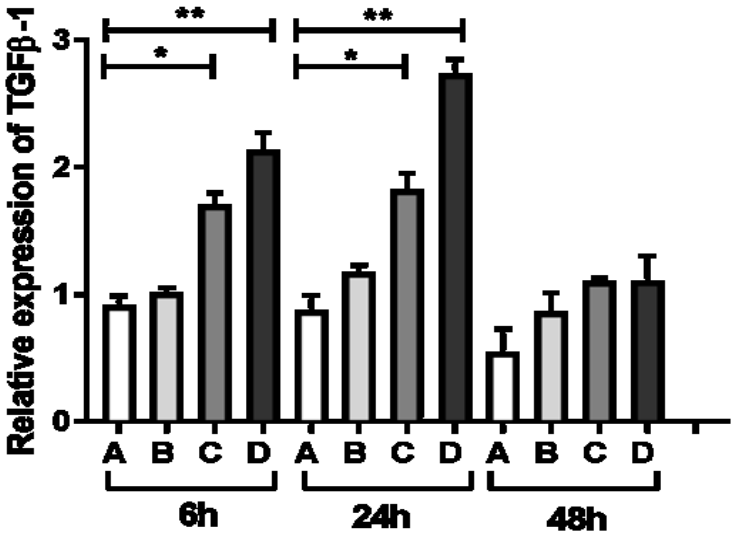


Figure 3.9. Relative expression of gene encoding TGF-β1 by MSCs is enhanced by stimulation with supernatant from splenocytes exposed to live and heat-killed *S. pneumoniae in vitro*.

Passage 5 MSCs (Foxp3DTR background) seeded at $2x10^5$ cells/ml were cultured overnight in 24 well tissue culture plates. Unstimulated 'resting' MSCs (condition A) and MSCs stimulated with supernatant from: uninfected splenocytes (condition B), splenocytes infected with 10^5 CFU live *S. pneumoniae* (condition C) or 10^5 CFU heat-killed *S. pneumoniae* (condition D), for 6h, 24h or 48h were harvested for RNA extraction and expression of TGF-β1 by RT-PCR.

Assisted by C. R.

Chapter 4

4 MSC and models of bacterial lung infection

4.1 Introduction

4.1.1 Mouse models of bacterial lung infection

There are relatively few studies of specific bacterial infection in murine models, the majority use LPS-induced lung injury as a model of gram-negative sepsis, or the CLP model of polymicrobial sepsis. Clinically relevant lung bacterial infection can be modelled in C57BL/6 mice by infecting with *S. pneumoniae*, a virulent gram-positive lung pathogen commonly encountered in humans as a cause of pneumonia. The gram-negative pathogen *P. aeruginosa* is also important in lung pathology; acute lung infection with this pathogen can be readily investigated in a mouse model. Chronic infection and colonization with *P. aeruginosa* is more difficult to model in the mouse. It is a well recognised problem for patients with cystic fibrosis and non-CF bronchiectasis, and it is plausible that MSC therapy may, in time, be employed to facilitate pathogen eradication in these patients.

The experiments described in this thesis have used *S. pneumoniae* parent strain A66.1 transformed with a lux transposon plasmid pAUL-A Tn4001 luxABCDE (Kmr) which confers expression of bioluminescence at wavelength 600nm, reported to be detectable for at least 48 hours *in vivo* (Francis *et al*., 2001). *P. aeruginosa* Xen 41 (transformed from parent strain PA01) is similarly bioluminescent (at wavelength 490nm) and can be administered intranasally to generate lung bacterial infection (Francis *et al*., 2001). One reason for choosing to use bioluminescent labelled bacteria was so that bacterial load in the lung could be imaged and measured longitudinally using IVIS technology. Unfortunately, this was not achievable because the transgenic lines used were on a C57BL/6 (black fur) background and the bioluminescent signals generated by Xen 10 and Xen 41 too weak to penetrate the thoracic cage and overlying tissue.

4.1.2 Mouse models assessing MSC impact on bacterial lung infection

Most *in vivo* experimental models assessing MSC immunomodulation are based on rodent species. Mouse models have been used to assess the impact of MSC treatment on a range of pathologies, including sepsis (Nemeth *et al*., 2009; Mei *et al*., 2010). Many of the published studies using mouse models employ murine MSCs (mMSCs). As discussed in Chapter 3, mMSC differ from hMSC in several ways, including culture conditions and time required for expansion *in vitro*. There are also differences in the molecular pathways of immunosuppression utilised by murine versus human MSCs. hMSCs use IDO whereas mMSCs use iNOS in immunosuppression (Meisel *et al*., 2011). These two enzymes are modulated by different inflammatory molecules, meaning that the murine microenvironment may not influence the activity of hMSCs in a manner equivalent to the human system, if hMSCs are used in a mouse model. Human MSCs have been shown to have protective effects in mouse models, but the issue remains that most murine cytokines with MSC 'licensing' activity do not cross-react with the analogous human receptors, which has the potential to impair MSC impact and weaken the data obtained (Krampera *et al.,* 2013).

4.1.3 MSCs as potential candidates to treat bacterial lung infection

Appropriate antibiotic treatment and supportive measures such as haemodynamic resuscitation and protective lung ventilation are the mainstays of treatment for severe pneumonia which may be complicated by sepsis and/or acute lung injury (ALI). Inadequate pathogen clearance and unbridled inflammation, which promotes cellular apoptosis and tissue injury, contribute to the end organ damage observed in these patients (Hudson *et al*., 1995; Ware and Matthay, 2000). Following the early report by Ringden and colleagues describing a protective effect of MSC treatment following colonic perforation and peritoneal sepsis, several studies have been published that support the notion that MSCs are not simply immunosuppressive, but can exert their own antimicrobial effects *in vivo*, which serve to augment baseline host response to pathogen (Ringden *et al*., 2007; Nemeth *et al*., 2009; Mei *et al*., 2010). Proposed mechanisms include direct antibacterial effects via the production of anti-microbial peptides/proteins (Krasnodembskaya *et al*., 2010; Meisel *et al*., 2011) and indirect effects via stimulation of host cell phagocytosis (Mei *et al*, 2010; Krasnodembskaya *et al*., 2012).

The results of the study conducted by Duncan Stewart's group, which demonstrated MSC-mediated enhancement of bacterial clearance in the context of suppression of the host inflammatory response, stimulated much interest, as it introduced the concept of MSC-mediated antimicrobial activity working in synergism with MSC immunoregulatory effects. (Mei *et al*., 2010). MSC-mediated inhibition of inflammatory cytokine/chemokine production impairs the migration of additional immune effector cells responsive to microbial invasion, and inhibits their production of pro-inflammatory mediators and free radicals. In concert with these activities, MSCs participate directly in microbial eradication and stimulate monocyte/macrophage phagocytosis. The outcome is enhanced pathogen clearance in the context of reduced inflammation which facilitates the preservation of tissue integrity and organ function (Nemeth *et al*., 2009; Mei *et al*., 2010; Mezey and Nemeth, 2015).

This chapter explores the impact of mMSC derived from BM/compact bone on bacterial clearance and host inflammatory response in mouse models of *S. pneumoniae* and *P. aeruginosa* lung infection.

4.2 Optimisation of mouse models of *S. pneumoniae* and *P. aeruginosa* lung infection

My starting point for investigating the impact of MSCs on lung bacterial infection with *S. pneumoniae* and *P. aeruginosa* was to optimise mouse models of these infections, ensuring reproducibility of factors such as dose of infecting inoculum and pathogenicity of this inoculum as determined by weight loss and specific markers of disease over time. For *S. pneumoniae* lung infection, pilot studies using a range of doses (CFU) for infection were carried out. To execute reliable and reproducible infection experiments, a system was devised whereby an accurately determined CFU of bacterial preparation was prepared ahead of infection and defined aliquots could be used in repeat experiments.

Optical density, measured in a spectrophotometer, was used as a measure of the concentration of bacteria in suspension. As visible light passes through a cell suspension the light is scattered. In a suitably prepared bacterial suspension, where cultivating broth has been removed by washing, greater scatter indicates that more bacteria are present. The optical density at 600nm (OD600) of serial dilutions of bacterial preparations (prepared as described in sections 2.4.1 and 2.4.2) was recorded and respective CFU counts determined by plating out on suitable agar. This enabled a growth curve to be generated for each pathogen: OD versus CFU, which could then serve as a guide for prospective determination of infecting dose from OD600 value (Figure 4.1).

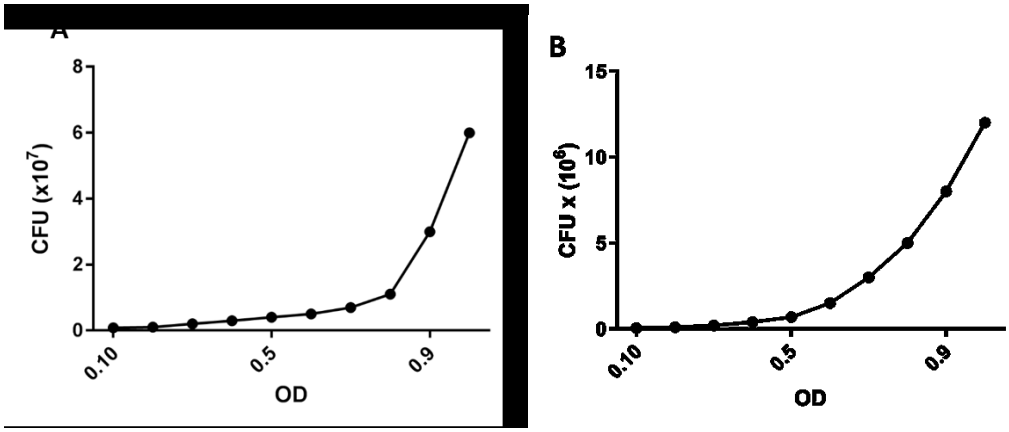


Figure 4.1. CFU generated from serial dilutions of *S. pneumo* (A) and *P. aeruginosa* (B) corresponding to optical density (OD) readings 0-1.

Cultured bioluminescent *S. pneumoniae,* Xen10, was washed and resuspended in 2ml PBS to make an inoculum of approximately 10^8 CFU/30 µl. Optical density (OD) was used to inform the dilution factor required to make a final working preparation consistent with an inoculum of $5x10^7$ CFU/30 µl for use in the mouse model of *S. pneumonia*e lung infection.

Cultured bioluminescent *P. aeruginosa,* Xen41, was washed and resuspended in 500 µl PBS to make an inoculum of approximately 10^{10} CFU/30 µl. Optical density (OD) was used to inform the dilution factor required to make a final working preparation consistent with an inoculum of 10^6 CFU/30 µl for use in the mouse model of *P. aeruginosa* lung infection.

Assisted by K. Q.

Following determination and standardisation of a range of infection inoculums for each pathogen, an appropriate infecting dose was selected for each infection model. For *S. pneumoniae,* infecting doses of 10^8, 10^7 and 10^6 CFU were tested over a 20-hour period in Foxp3.DTR mice. Animals were checked regularly for evidence of respiratory difficulties, loss of mobility, hunching and piloerection and reweighed 4 hourly to ensure that any weight loss did not exceed 20% of their starting body weight. Mice receiving 10^8 CFU exhibited laboured breathing and hunched posture at 6 hours. The experiment was ended (for this group only) at the 6-hour time point. Animals receiving 10^7 and 10^6 CFU were monitored until 20 hours post infection when all animals were humanely sacrificed and BAL and lung tissue harvested for analysis of bacterial burden.

Experimental mice receiving 10^8 CFU, and culled at 6 hours post infection exhibited less than 3% weight loss, but had a detectable pathogen load in lung tissue and BAL fluid. Mice receiving 10^7 CFU, culled at 20 hours post infection, exhibited between 5 and 10% weight loss and had a reduced pathogen load in lung tissue and BAL fluid compared to the mice given 10^8 CFU. Mice receiving 10^6 CFU, culled at the 20-hour endpoint, did not lose weight and appeared to have almost completely cleared the pathogen by 20 hours post infection (Figure 4.2). Based on this data, a dose of 5 x 10^7 CFU *S. pneumoniae* was selected for initial experiments involving MSC treatment.

Using published studies of MSC impact on host response to infection as a guide, it seemed reasonable that MSC treatment studies in the infection models should last at least 24 hours in duration, if MSCs were to be given sufficient opportunity in the time frame involved to have an effect. 10^8 CFU *S. pneumoniae* was likely to be too potent an inoculum for this time period. It seemed likely that 10^6 CFU would be cleared by host immunity without the need for MSC therapy. Thus, it was postulated that between 10^7 and 5 x 10^7 would be optimal for detection of bacterial burden and measurable weight loss, which may be amenable to demonstrate a treatment effect by MSC. The experiment was repeated in DQ8.Abo mice to check that this transgenic line responded similarly to dosing with this inoculum of *S. pneumoniae*. The mice behaved in a comparable manner to Foxp3.DTR mice in their handling of this inoculum as measured by weight loss and bacterial load (data not shown).

For the *P. aeruginosa* mouse model of lung infection, using data produced previously in the lab (Dr Kathryn Quigley, PhD thesis) outlining calculated survival curves for different doses

of *P. aeruginosa* in a number of transgenic lines on a C57BL/6 background, it was confirmed that a dose of 10^6 CFU *P. aeruginosa* given to Foxp3.DTR transgenics resulted in detectable numbers of bacteria in lung tissue and BAL fluid, associated with weight loss of 5-10% over a 24 hour period (data shown in the context of *P. aeruginosa* lung infection and MSC treatment, Figure 4.7).

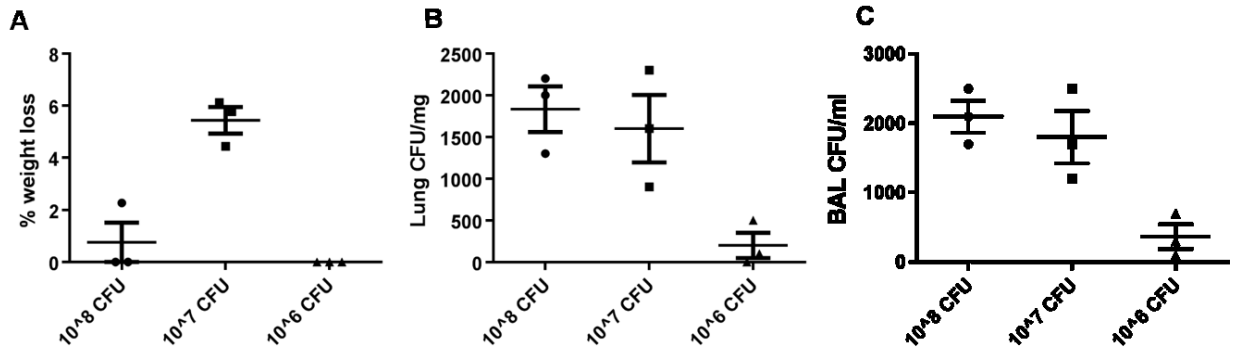


Figure 4.2. Dose response to lung infection with *S. pneumoniae*.

Foxp3.DTR male and female mice aged 8-12 weeks were weighed prior to infection with bioluminescent *S. pneumoniae, Xen 10* i.n. at inoculums of 10^8 CFU/30 µl, 10^7 CFU/30 µl or 10^6 CFU/30 µl (n=3 per group). Mice were monitored at 2 hourly intervals by clinical observation (for development of respiratory difficulties, loss of mobility and motility, hunched posture and piloerection) and reweighed every 4 hours to ensure that weight loss did not exceed 20% of starting body weight. The experimental endpoint for each group was determined by clinical observation and weight loss parameters. For the group receiving 10^8 CFU/30 µl the experiment was ended at 6 hours. For groups receiving 10^7 and 10^6 CFU/30 µl the experiment was ended at 20 hours and BAL and lung tissue harvested for analysis of bacterial burden.

(A) Percentage weight loss. (B) CFU/mg in lung tissue. (C) CFU/ml in BAL fluid. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*$p<0.05$ **$p<0.01$). This experiment was repeated 3 times with consistent results obtained.

Assisted by K. Q. and D. C.

4.3 Optimisation of MSC treatment in a mouse model of *S. pneumoniae* lung infection

The optimised mouse model of *S. pneumoniae* lung infection could now be used to investigate the impact of systemic MSC treatment on host response to infectious challenge with this pathogen.

Firstly, the dose of MSCs to be intravenously injected needed to be established. Published studies, employing a variety of animal models, vary widely in the number of cells injected per treatment. The majority of those utilising mouse models document injections of between 10^5 and 10^6 MSCs in 100-200 µl PBS. Using this as a starting point, my initial study of MSC treatment effect used doses of 10^5, 5 x 10^5 and 10^6 MSCs to see if i) MSCs had any impact on weight loss and bacterial clearance in this mouse model of lung bacterial infection and ii) if there was any evidence of dose response.

Secondly, the timing of MSC administration needed to be determined. Having consulted the literature, it was apparent that there was a substantial variation in published studies in the timing of MSC injection with respect to infectious challenge or injurious insult. This is illustrated in two seminal papers which focus on MSC treatment for sepsis. Nemeth and colleagues studied the effect of pretreatment; MSCs (10^6 per injection) were delivered 24 hours before CLP-induced polymicrobial sepsis. An MSC protective effect was attributed to MSC-mediated reprogramming of macrophages to produce increased amounts of IL-10 (Nemeth *et al*., 2009). In contrast, Mei and colleagues delivered MSCs (2.5 x 10^5 per injection) 6 hours post CLP. They demonstrated both MSC-mediated antibacterial and anti-inflammatory effects which they attributed to enhanced antigen presentation, phagocytosis and bacterial killing (Mei *et al*., 2010). Based on accumulating evidence that MSCs require 'licensing' by exposure to an inflammatory environment in order to exert their immunomodulatory effects, a delay of 3 hours was introduced into the study protocol from the induction of infection to the injection of MSCs. MSC treatment delivery a few hours following infectious insult is also more clinically relevant than a pre-treatment approach.

DQ8.Abo mice infected with a pre-defined inoculum of *S. pneumoniae* (5x10^7 CFU) were treated 3 hours post infection with 10^5, 5 x 10^5 or 10^6 MSCs injected via the tail vein. All MSC treatment groups exhibited reduced weight loss and bacterial burden in lung tissue and BAL fluid. Level of pro-inflammatory cytokine IL-6 was also reduced in the BAL fluid of MSC-

treated mice. Reduction in weight loss, bacterial counts and BAL IL-6 levels was statistically significant in mice treated with 5 x 10^5 MSCs compared to PBS-treated controls (Figure 4.3). This dose and timing of MSC injection was taken forwards into larger scale experiments investigating the MSC protective effect in *S. pneumoniae*.

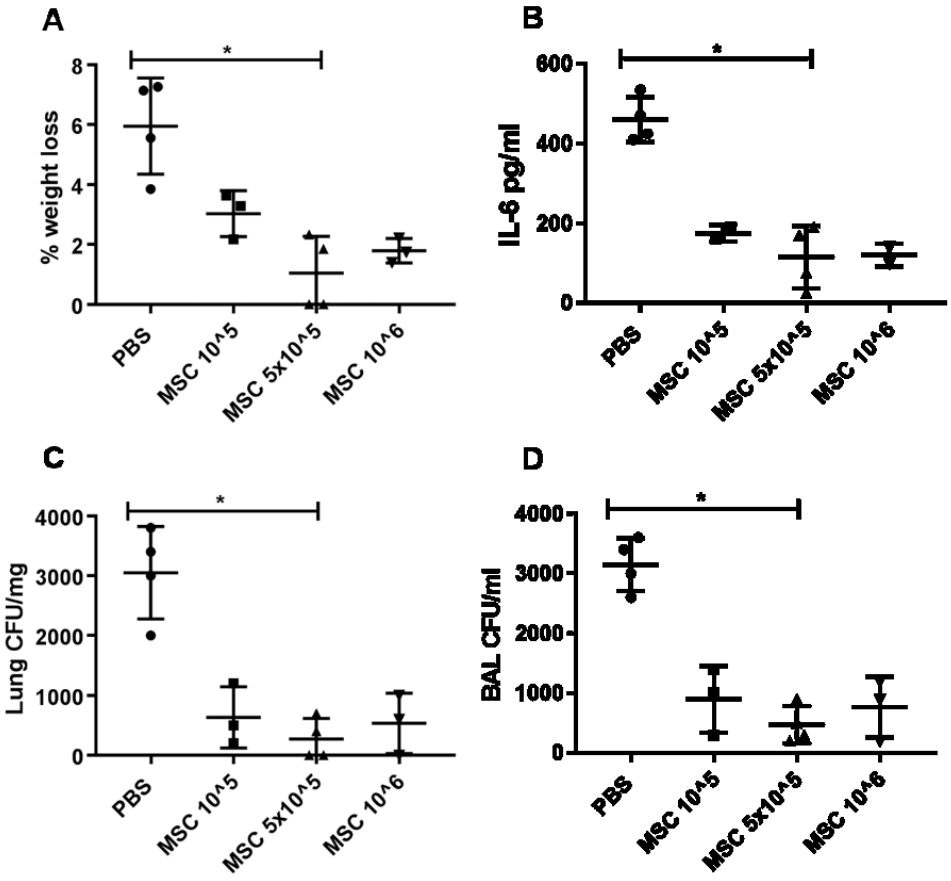


Figure 4.3. Reduction in weight loss, IL-6 levels in BAL fluid and bacterial burden in the lung and BAL of mice infected with *S. pneumoniae* and treated with a dose of 5x10^5 MSCs compared to PBS-treated controls.

DQ8.Abo male and female mice aged 8-12 weeks were weighed prior to infection with 5x10^7 CFU *S. pneumoniae, Xen 10* i.n. 3 hours post infection mice were treated with 10^5 mMSCs (DQ8.Abo background, P5) (n=3), 5x10^5 mMSCs (n=4), 10^6 mMSCs (n=3) or PBS (n=4) i.v. Mice were re-weighed prior to humane sacrifice at 24 hours and BAL and lung tissue harvested.

(A) Percentage weight loss. (B) IL-6 in BAL fluid. (C) CFU/mg in lung tissue. (D) CFU/ml in BAL fluid. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

4.4 The impact of MSC treatment in a mouse model of *S. pneumoniae* lung infection

To further investigate MSC protective effects in lung bacterial infection, Foxp3DTR mice were infected with *S. pneumoniae* and treated 3 hours later with i.v MSCs. Mice receiving MSC treatment exhibited reduced weight loss and bacterial burden in lung tissue and BAL fluid compared to PBS-treated control mice (Figure 4.4).

Following confirmation of MSC-mediated antibacterial effects in this experimental system, MSC impact on the levels of pro- and anti-inflammatory cytokines in lung tissue and BAL fluid was investigated. MSC treatment had a predominantly anti-inflammatory effect, decreasing levels of pro-inflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-17F in lung tissue and IFN-γ, and IL-6 in BAL fluid. Reduced IL-10 was also observed in lung and BAL, though statistical significance was not attained (Figure 4.5). This is in keeping with the results of Mei's study (where MSCs were administered post septic insult) in which IL-10 levels in blood and BAL were decreased by MSC treatment, and differs from Nemeth's study (utilising MSC pre-treatment) in which IL-10 was significantly increased in the MSC treated group (Mei *et al*, 2010; Nemeth *et al.*, 2009).

MSC impact on the expression in lung tissue of genes encoding these cytokines was investigated. Results were equivocal for gene expression. MSC treatment had a predominantly anti-inflammatory effect with suppression of genes encoding pro-inflammatory cytokines: TNF-α, IL-6 and IL-17F, although statistical significance was not achieved (Figure 4.6A). Real-time PCR analysis of the relative expression of genes encoding transcription factors T-bet, GATA-3, RORγT and Foxp3 did not reveal any significant differences induced by MSC treatment (Figure 4.6B).

Considering this data collectively, it appears that in this model of *S. pneumoniae* lung infection, MSC treatment 3 hours post infection reduces weight loss, enhances bacterial clearance and dampens down the expression of pro-inflammatory cytokines in lung tissue and BAL. Whether or not this MSC protective effect is specific to Gram positive bacterial lung infection, or reproducible following infection with a Gram-negative lung pathogen, is the subject of the final set of experiments in this chapter.

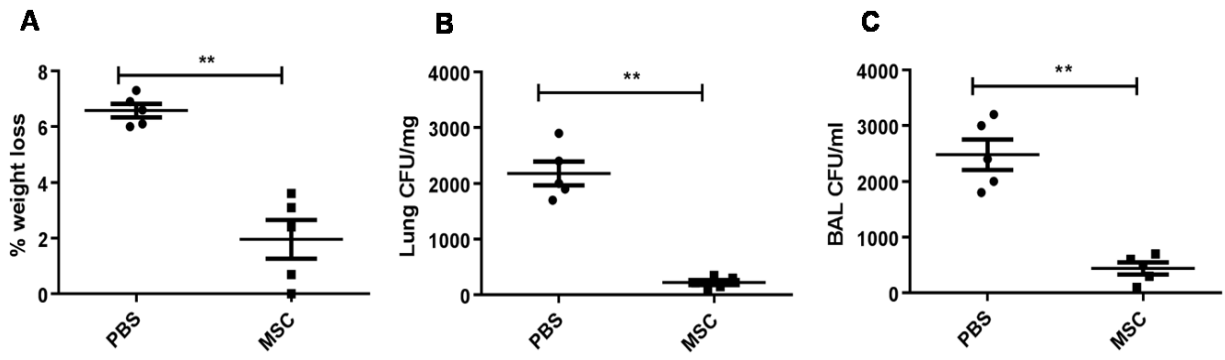


Figure 4.4. Reduced weight loss and bacterial burden in lung and BAL of MSC treated mice infected with *S. pneumoniae*.

Male Foxp3DTR mice aged 8-12 weeks were weighed prior to infection with $5x10^7$ CFU *S. pneumoniae, Xen 10,* i.n. 3 hours post infection, 1 group (n=5) was treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.; the other group (n=5) received PBS i.v. Mice were re-weighed prior to humane sacrifice at 24 hours and BAL and lung tissue harvested.

(A) Percentage weight loss. (B) CFU/mg in lung tissue. (C) CFU/ml in BAL fluid. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

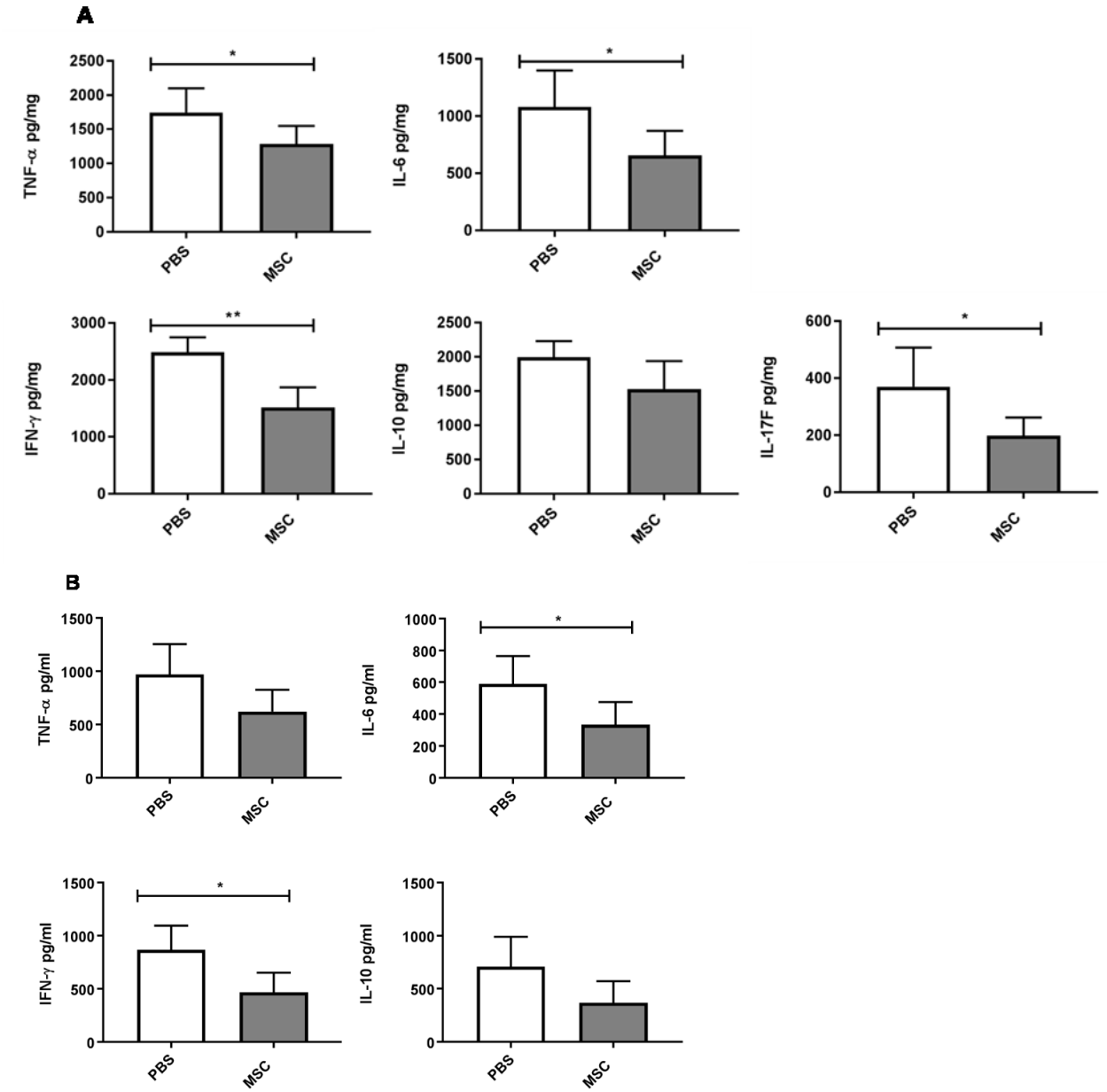


Figure 4.5. Cytokine analysis by ELISA from lung homogenate and BAL 24 hours post *S. pneumoniae* lung infection.

Male Foxp3DTR mice aged 8-12 weeks were weighed prior to infection with $5x10^7$ CFU *S. pneumoniae, Xen 10,* i.n. 3 hours post infection 1 group (n=5) was treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.

(A) Cytokines in lung homogenate. (B) Cytokines in BAL. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

Assisted by K. Q. and C. R.

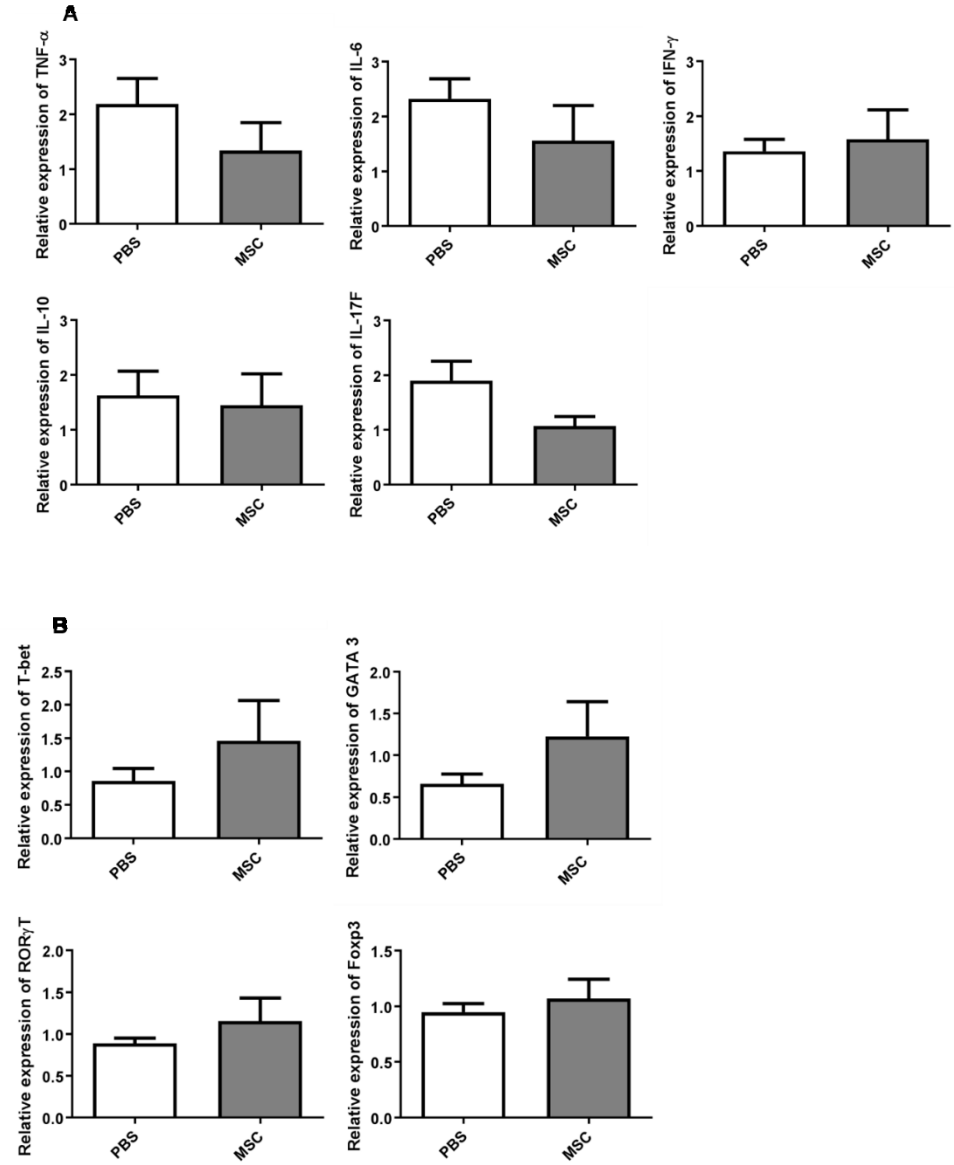


Figure 4.6. Real-time PCR analysis of cytokine and transcription factor gene expression in lung 24 hours post *S. pneumoniae* lung infection.

Male Foxp3DTR mice aged 8-12 weeks were weighed prior to infection with $5x10^7$ CFU *S. pneumoniae, Xen 10,* i.n. 3 hours post infection 1 group (n=5) was treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.

(A) Cytokine gene expression. (B) Transcription factor gene expression. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

4.5 The impact of MSC treatment in a mouse model of *P. aeruginosa* lung infection

MSC antimicrobial effects have been demonstrated against a range of pathogens (*E. coli, P. aeruginosa, S. aureus*) (Gupta *et al*., 2012; Kradnobenskaya *et al*., 2012, Yuan *et al*., 2014).

Next, the impact of MSC treatment on bacterial clearance and inflammatory response in a mouse model of Gram negative bacterial lung infection, specifically *P. aeruginosa* was investigated. Foxp3DTR mice were infected with *P. aeruginosa* and treated 3 hours later with i.v MSCs. As observed in the *S. pneumoniae* infection and MSC treatment study, mice receiving MSC treatment exhibited reduced weight loss and bacterial burden in lung tissue and BAL fluid compared to PBS-treated control mice (Figure 4.7).

MSC impact on the levels of pro- and anti-inflammatory cytokines in lung tissue and BAL fluid was investigated. MSC treatment had a discernible anti-inflammatory effect, decreasing levels of pro-inflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-17F in lung tissue and BAL fluid. IL-10 was also significantly reduced in lung and BAL of MSC treated mice. (Figure 4.8).

MSC impact on the expression in lung tissue of genes encoding these cytokines was investigated. Again, MSC treatment had an anti-inflammatory effect, reducing expression of genes encoding pro-inflammatory cytokines: TNF-α, IFN-γ and IL-17F. Possible suppression of gene expression for IL-6 and IL-10 was observed, although statistical significance was not achieved (Figure 4.9A). Real-time PCR analysis of the relative expression of genes encoding transcription factors T-bet, GATA-3, RORγT and Foxp3 revealed possible increased expression of all transcription factors in the MSC treated group, although statistical significance was not achieved (Figure 4.9B).

Considering this data collectively, it appears that in this model of *P. aeruginosa* lung infection, MSC treatment 3 hours post infection reduces weight loss, enhances bacterial clearance and dampens down the expression of pro-inflammatory cytokines in lung tissue and BAL. The mechanism(s) by which the observed MSC-mediated anti-inflammatory effects promote pathogen clearance of both Gram-positive and Gram-negative organisms required further investigation.

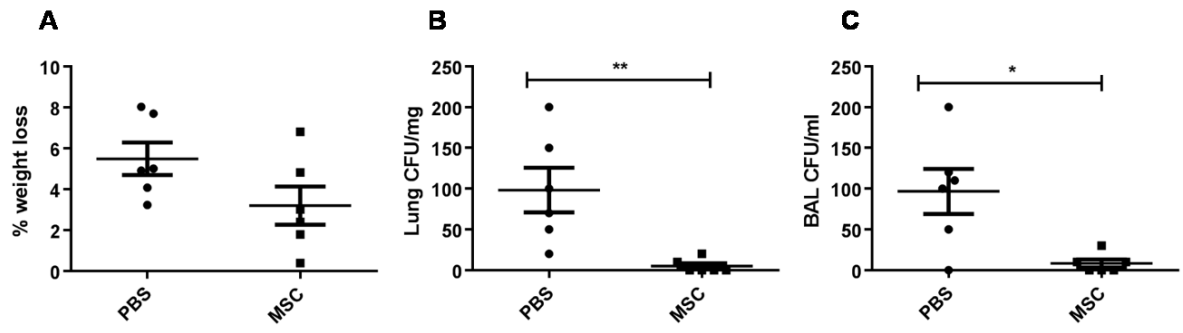


Figure 4.7. Reduced weight loss and bacterial burden in lung and BAL in MSC treated mice infected with *P. aeruginosa*.

Female Foxp3DTR mice aged 8-12 weeks were weighed prior to infection with 10^6 CFU *P. aeruginosa, Xen 41,* i.n. 3 hours post infection, 1 group (n=6) was treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.; the other group (n=6) received PBS i.v. Mice were re-weighed prior to humane sacrifice at 24 hours and BAL and lung tissue harvested.

(A) Percentage weight loss. (B) CFU/mg in lung tissue. (C) CFU/ml in BAL fluid. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*$p<0.05$ **$p<0.01$). This experiment was repeated 3 times with consistent results obtained.

Assisted by K. Q. and C. R.

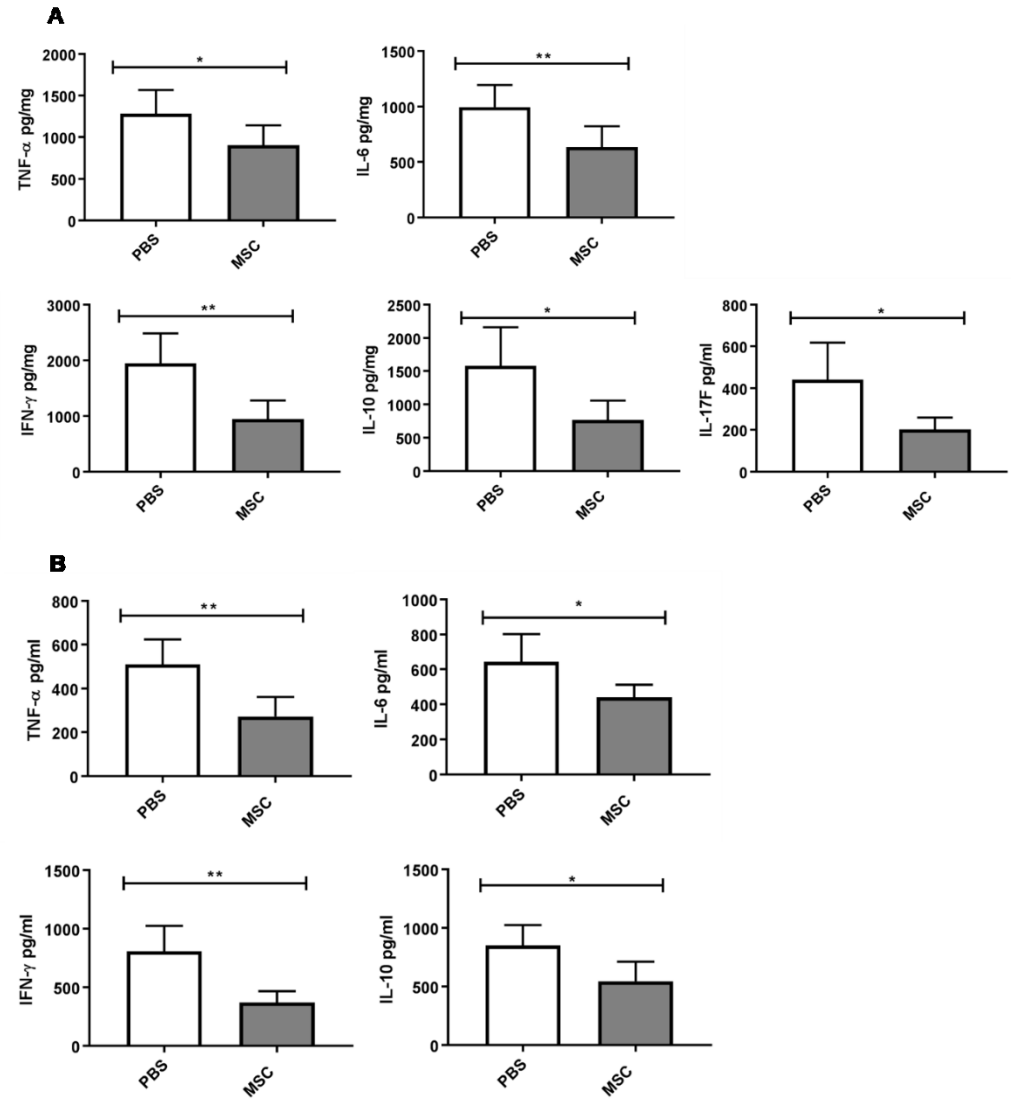


Figure 4.8. Cytokine analysis by ELISA from lung homogenate and BAL 24 hours post *P. aeruginosa* lung infection.

Female Foxp3DTR mice aged 8-12 weeks were weighed prior to infection with 10^6 CFU bioluminescent *P. aeruginosa, Xen 41,* i.n. 3 hours post infection 1 group (n=6) was treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.

(A) Cytokines in lung homogenate. (B) Cytokines in BAL. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

Assisted by K. Q. and C. R.

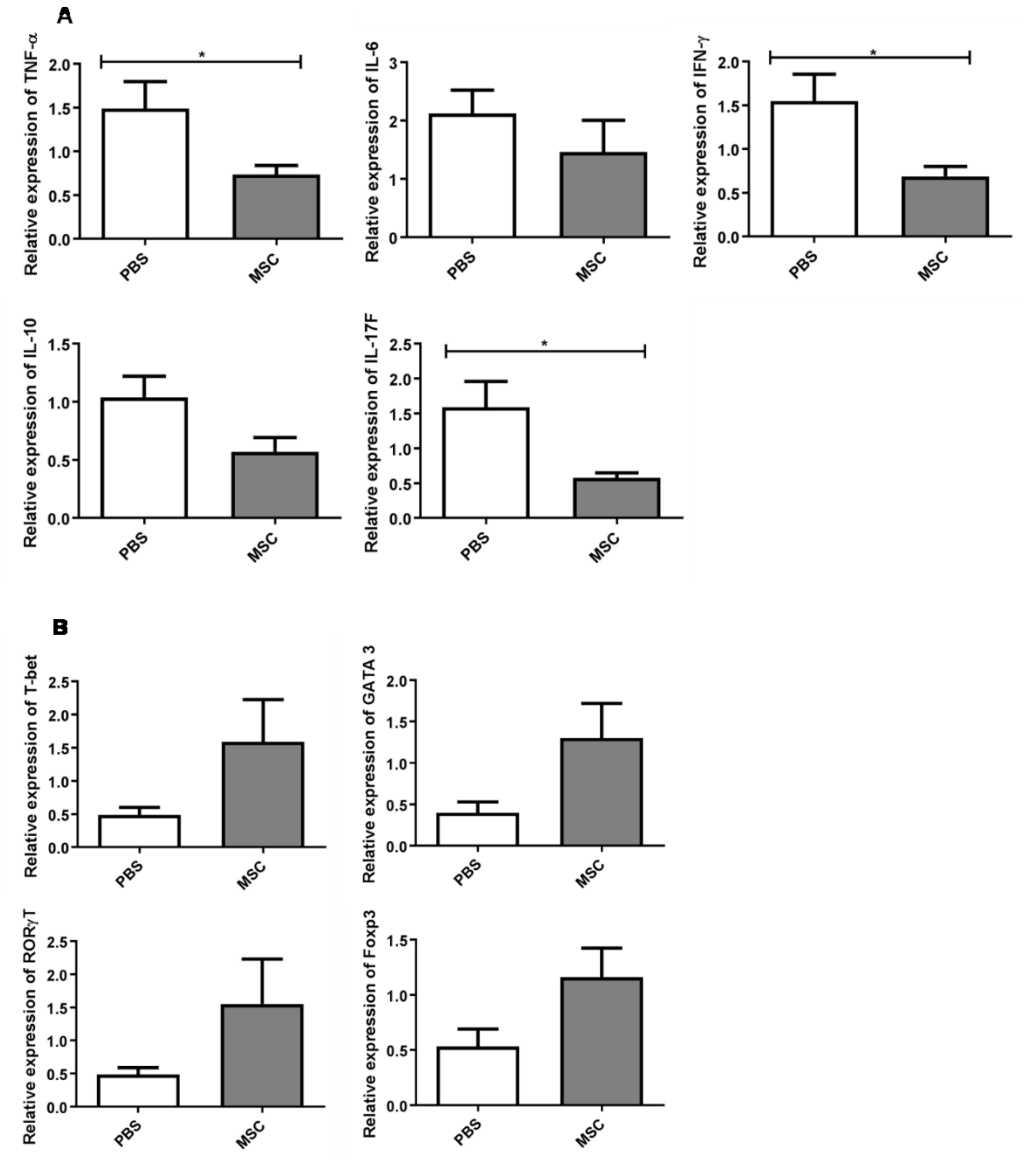


Figure 4.9. Real-time PCR analysis of cytokine and transcription factor gene expression in lung 24 hours post *P. aeruginosa* lung infection.

Female Foxp3DTR mice aged 8-12 weeks were weighed prior to infection with 10^6 CFU *P. aeruginosa, Xen 41,* i.n. 3 hours post infection 1 group (n=6) was treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.

(A) Cytokine gene expression. (B) Transcription factor gene expression. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

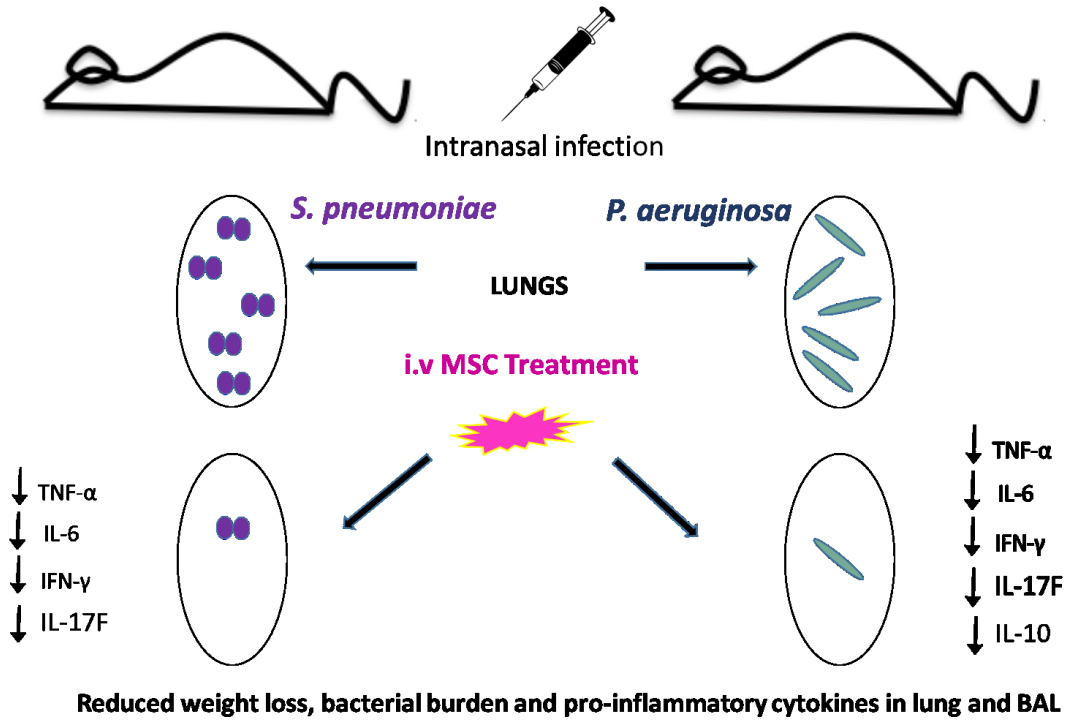


Figure 4.10. The impact of MSC treatment on weight loss, lung bacterial load and inflammatory cytokines post infection with *S. pneumoniae* and *P. aeruginosa.*

MSC treatment is effective in reducing weight loss and bacterial load in mice infected with Gram positive lung pathogen *S. pneumoniae* and Gram-negative lung pathogen *P. aeruginosa.* This was associated with a reduction in pro-inflammatory cytokines (TNF-α, IL-6, IFN-γ and IL-17F) in lung tissue and BAL fluid.

IL-10 was also significantly reduced in lung and BAL of MSC treated mice infected with *P. aeruginosa.*

Chapter 5

5 Treg effects in lung infection and their interaction with MSC

5.1 Introduction

5.1.1 Investigating the role of T_{regs} in bacterial lung infection

Various techniques have been employed to investigate the role of T cells during infectious diseases. Early studies focused on the use of antibodies to specifically deplete a chosen cell population *in vivo* (Vignali *et al.*, 1989; Müller *et al.*, 1987). This has both advantages and disadvantages; for example, most antibodies used for depletion are specific and will only remove the chosen cell type. However, the efficiency of antibody-mediated depletion *in vivo* can vary depending on antibody affinity with the target. Thus, over the past 20 years popularity has risen for the use of transgenic murine models with subsets of T cells or cytokines genetically removed (Bettelli *et al.*, 1998; Moon and McSorley, 2009). Their use carries the confidence that any results attained will be due to complete depletion, without the fear of partial and transient effects as observed through the use of neutralising antibodies. This field has been augmented by the creation of conditional and inducible transgenic knockout models which allow researchers to study cell types or transcription factors that severely affect development or even prove fatal to the animal if knocked out in conventional transgenic animals. An example of a conditional inducible knockout transgenic is used in experiments described in this thesis and is discussed in more detail in Section 5.2.

Specific to infection with *S. pneumoniae*, Preston et al showed that, in a mouse model of OVA induced allergic airways disease, intra tracheal infection with S*. pneumoniae* increased both the numbers and percentages of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in mediastinal lymph nodes compared to uninfected, allergic controls. The investigators suggested that this *S. pneumoniae*-induced increase in T_{regs} and the subsequent suppression of T_H2 cell and eosinophil proliferation by these T_{regs} may be responsible for the observed attenuation in allergic airways disease in infected allergic mice (Preston *et al*., 2011). Other investigators have shown that the development and mobilisation of $CD4^+$ $CD25^+$ T_{regs} is induced by *S. pneumoniae* infection to a greater extent than by other Gram-positive bacteria, and that

T_{regs} are critical regulators of responses *to S. pneumoniae* infection, functioning to inhibit the overly exuberant inflammatory responses that can occur (Kadioglu *et al*., 2004; Thorburn *et al*., 2010). Bacterial toxin pneumolysin and type-3-polysaccharide have been identified as components of the pneumococcus which not only induced the generation of T_{regs} but also suppressed iNKT cell accumulation, resulting in a reduction in development of airway hyper-responsiveness (Thorburn *et al*., 2012).

Several studies have suggested that lymphocytes play a role in host defense against *P. aeruginosa* infection, but very few have investigated the contribution of T_{regs} specifically. Koh et al infected mice deficient in Rag2 ($Rag2^{-}/^{-}$) and therefore lacking functional T cells, with a series of doses of *P. aeruginosa* (PA01) intranasally. $Rag2^{-}/^{-}$ animals exhibited increased mortality compared to WT controls, but, interestingly only at one bacterial dose. The survival difference between the two groups was lost with inocula 2-fold higher or lower (Koh *et al*., 2009). O. Carrigan and colleagues investigated the impact of T_{reg} depletion on host response to *P. aeruginosa* lung infection and found no difference in survival outcome between anti-CD25 (T_{reg} depleted) and control Ab-treated (T_{reg} replete) mice. Lung infection with *P. aeruginosa* induced an increase in nT_{regs} in the spleen of mice, but no differences in *P. aeruginosa*-induced NF-κB activation and cytokine or chemokine production were observed between the two groups. Furthermore, no difference in airway lung infiltration or lung histology was observed. The investigators concluded that despite evidence of T_{reg} expansion, nT_{regs} do not contribute significantly to the host response to P. aeruginosa lung infection (O. Carrigan *et al*., 2008).

5.1.2 Tissue resident T_{regs}

Tregs have been identified as resident in non-lymphoid tissues including adipose tissue (Feuerer *et al*., 2009; Vasanthakumar *et al*., 2015; Cipolleta *et al*., 2015), skeletal muscle (Burzyn *et al*., 2013) and lung (Arpaia *et al*., 2015). Mackay et al recently showed that tissue resident memory $CD8^{+}$ T cells express high levels of transcription factor HOBIT (homologue of BLIMP1 in T cells; also referred to as ZFP683), and also BLIMP1 (B lymphocyte-induced maturation protein 1; also referred to as PRDM1). In mixed bone-marrow chimeric mice infected with herpes simplex virus (HSV), both HOBIT and BLIMP1 had to be present for

development of these cells, but not for their circulating counterparts. The investigators concluded that BLIMP1 and HOBIT cooperate to specifically promote memory $CD8^+$ T cell development. They designated BLIMP1 and HOBIT as "universal regulators of tissue residency" in lymphocytes (Mackay *et al*., 2016).

Expression of these transcription factors is not restricted to memory $CD8^+$ T cells, but does appear to be a feature of effector-type lymphocytes within the $CD8^+$ T cell, NK and NKT cell lineages predominantly. Analysis by qPCR of HOBIT expression in a panel of leukocyte subsets revealed that HOBIT was expressed only at low levels, or not at all, in total $CD4^+$ T cells, T_{regs} and DCs (Vieira Braga *et al*., 2015). Consistent with a role in tissue residency, liver-resident NK cells but not conventional NK cells expressed HOBIT. Furthermore, deficiency in BLIMP1 and HOBIT was associated with significantly reduced numbers of NKT cells in the liver but not the spleen (Mackay *et al.,* 2016).

It appears that the molecular pathways responsible for driving tissue residency are conserved between lymphocyte populations expressing HOBIT and BLIMP1. The expression of a set of 192 genes was found to be altered in circulating versus tissue resident memory $CD8^+$ T cells. Interestingly, many of the genes showing variability in expression, were also associated with tissue-resident NK cells and NKT cells. Genes required for tissue egress, such as S1pr1 (which encodes sphingosine 1-phosphate receptor 1) were downregulated in the tissue-resident populations. HOBIT was the single transcription factor found to be upregulated in this gene set. Combined deficiency of BLIMP1 and HOBIT appears to inhibit tissue residency through de-repression of the tissue residency signature genes such as S1pr1 in $CD8^+$ T cells (Mackay *et al*., 2016). Thus, it appears that these novel transcription factors work together to promote tissue residency of certain subsets of innate and adaptive lymphocytes.

5.1.3 T_{reg} interactions with MSCs

The final part of this chapter investigates the impact of MSC treatment on T_{reg} replete and T_{reg} deficient mice infected with *S. pneumoniae* and *P. aeruginosa.* There is a considerable amount of evidence, *in vitro* and *in vivo*, that MSC can activate, expand and recruit T_{regs} in a variety of circumstances (Selmani *et al*., 2008; Casiraghi *et al*, 2008; Sun *et al*., 2011; Lee *et*

al., 2017). There remain some unanswered questions however, specifically whether or not T_{regs} are pivotal for MSC effect? There exists some data from *in vitro* studies that suggest this may not be the case. Krampera and colleagues found that MSC-mediated inhibition of both naïve and antigen experienced T cells required the presence of MSCs in culture and MSC–T cell contact. The MSC inhibitory effect observed did not require the presence of APCs and was not mediated through $CD4^+CD25^+$ T_{regs} (Krampera *et al*., 2003). It may be the case *in vivo* also that MSCs do not rely on T_{regs} to exert their immunomodulatory and antibacterial effects.

It is plausible though that T_{reg} depletion prior to infective or inflammatory insult may impact in some way on MSC protective effect, if only to heighten the initial immune response, owing to the absence of T_{reg}-mediated control of inflammation. A study of MSC therapy in a murine model of acute kidney injury demonstrated that MSCs increased the percentage of T_{regs} in the spleen and ischaemic kidney. Antibody depletion of T_{regs} blunted (but did not completely abolish) the therapeutic effect of MSCs, while co-culture of splenocytes with MSCs promoted T_{reg} expansion. Splenectomy abrogated both MSC protective effect and the expansion of T_{regs}, suggesting that in this model, MSCs exert their protective effect, at least in part, through T_{reg} induction via interactions with splenocytes. Interestingly, MSCs were also shown to reduce IFN-γ production by $CD4^+$ and $CD8^+$ T cells infiltrating the ischaemic kidney, without any change in the numbers of these T cells as measured by flow cytometry. This T cell effect was also abrogated by splenectomy, leading the investigators to suggest that T_{regs} might be involved in the downregulation of IFN-γ production of local T cells (Hu *et al*., 2013).

5.2 Specific depletion of $Foxp3^+$ cells during lung infection.

Conditional inducible Foxp3 knockout mice (Foxp3.DTR) have previously been shown to exhibit specific depletion of $Foxp3^+$ cells (Kim *et al.*, 2007). A targeted construct containing cDNA encoding Diphtheria Toxin Receptor (DTR), followed by IRES and GFP sequences, is inserted into the 3' UTR of *foxp3*. This results in the expression of a fully functional GFP-Foxp3 fusion protein. Consequently, T_{regs} can be easily identified via flow cytometry. Cells

expressing Foxp3 also express DTR and upon the administration of Diphtheria Toxin (DT) specific depletion of $Foxp3^+$ cells is achieved (Kim *et al.*, 2007).

Foxp3.DTR mice were used to investigate the role of $Foxp3^+$ regulatory cells in host response to bacterial lung infection. The starting point was demonstration that T_{reg} depletion in this transgenic animal was sufficient. The dosing regimen of DT administration had previously been optimised to achieve full $Foxp3^+$ cell knockdown in this strain of mice. $Foxp3^+$ cell depletion was achieved after 1 dose of 100 µl DT (12.5µg/ml) given by intraperitoneal (i.p) injection. T_{reg} depletion was maintained by repeat dosing every 48 hours. To be confident that T_{reg} knockdown was achieved prior to infecting the animals for analysis of differences in bacterial clearance between T_{reg} replete and T_{reg} deficient cohorts, the following protocol was devised (represented by the schematic below; Figure 5.1A). Mice were DT-treated at 48 hours (-48h) and at 24 hours (-24h) prior to infection, and also on the day of infection (0h). Mice were infected for 24 hours before humane sacrifice and organ harvest (24h). Percentage of Foxp3-GFP^+ cells of the $CD4^+$ population was analysed by flow cytometry from mice culled following 2 doses of DT, prior to infection, to ensure that efficient knockdown had been achieved prior to embarking on the infection component of the experiment (Figure 5.1B).

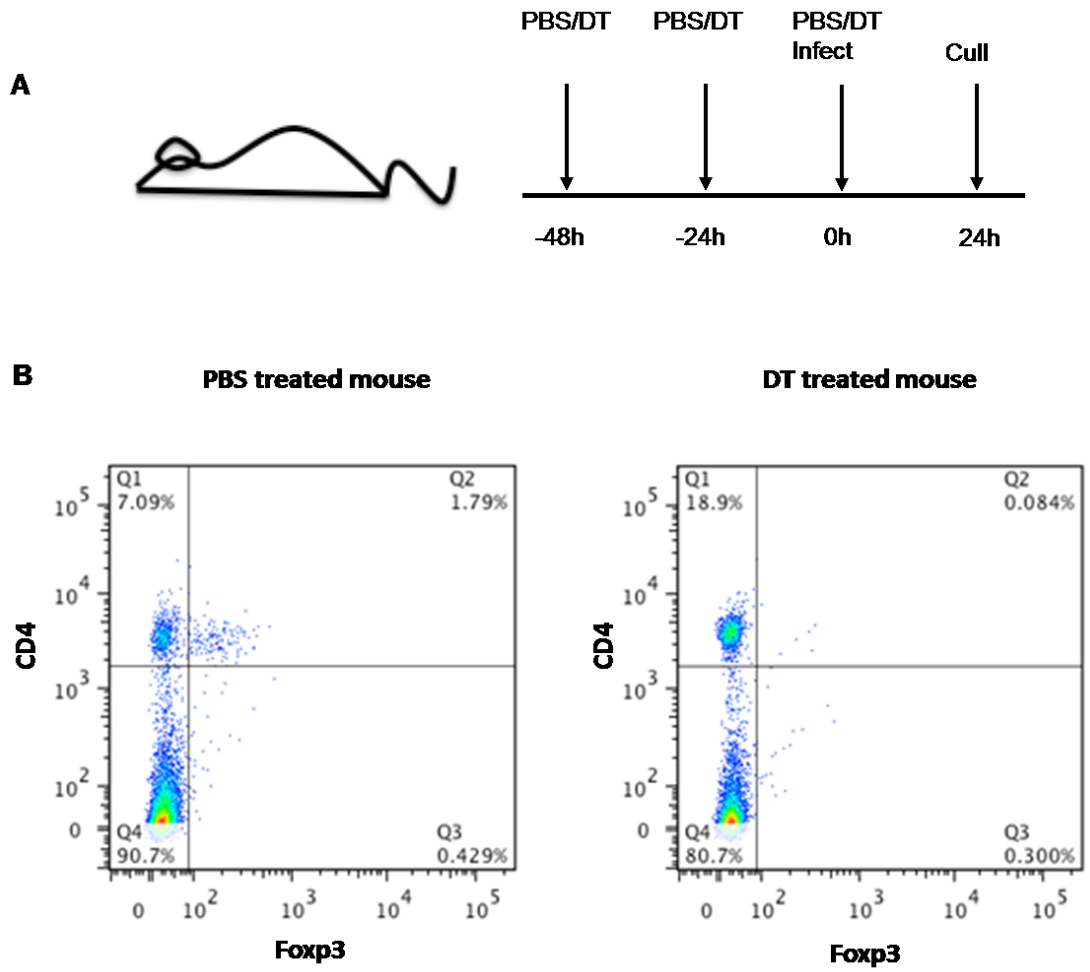


Figure 5.1. Diphtheria Toxin (DT) mediated Foxp3$^+$ cell depletion and infection induction.

A. Schematic representing timing of DT (or PBS) treatment, infection induction and experimental end point.

To evaluate T_{reg} depletion Foxp3.DTR males and females aged 8-12 weeks were given 100 µl DT (12.5µg/ml) (n=3) or PBS (n=3) i.p daily for 2 consecutive days. Spleens were harvested and splenocytes isolated for CD4-staining and analysis of CD4$^+$ GFP-expressing cells.

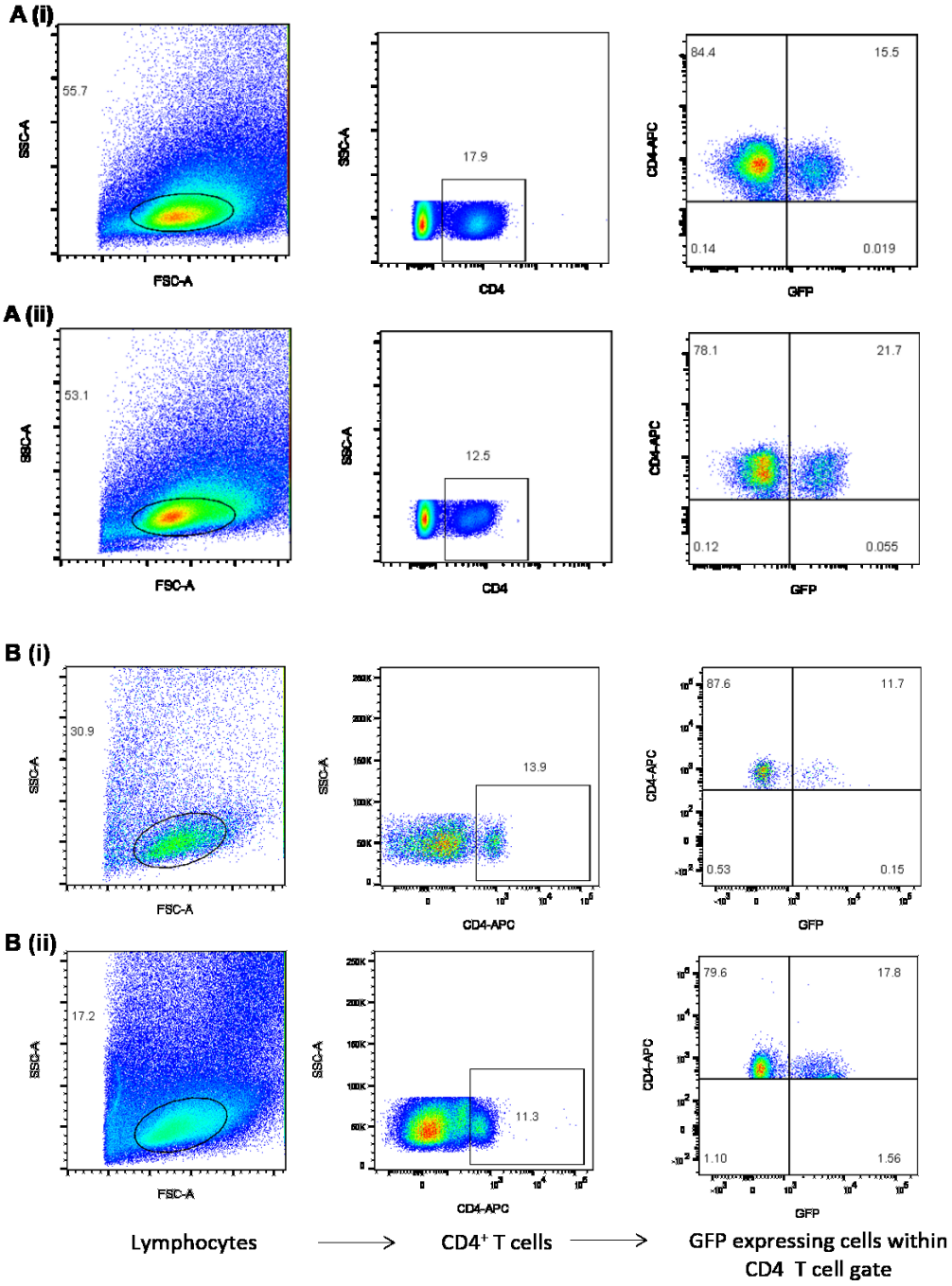
B. Representative staining of Foxp3-GFP$^+$ and CD4$^+$ cells (PE-stained) in the spleen of untreated (left) and DT treated mice (right).

Assisted by C. R.

5.3 Regulatory T cells are increased in spleen and lung after infection with *S. pneumoniae.*

One of the advantages of using the Foxp3.DTR transgenic mouse to study the role of T_{regs} in lung infection is the GFP tag associated with $CD4^+$ $CD25^+$ $Foxp3^+$ T_{regs} which facilitates their identification *ex vivo* by flow cytometry. Cells were stained with CD4 antibody and $CD4^+$ T cells co-expressing GFP were identified as T_{regs} (Figure 5.2 A and B).

There have been several reports of bacterial infection promoting an expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{regs} in peripheral blood, spleen and draining lymph nodes (Okeke *et al*, 2013; Preston *et al*, 2011, O. Carrigan et al., 2008). The Foxp3.DTR model of *S. pneumoniae* infection was investigated to establish if T_{reg} expansion was a feature of host response. Foxp3.DTR transgenic mice were infected with *S. pneumoniae* or given PBS. After 24 hours spleen and lung was harvested and splenocytes and lung cells isolated for CD4-staining and flow cytometry analysis of $CD4^+$ GFP^+ cells. Gating on $CD4^+$ cells within the initial lymphocyte gate and then looking within that population for GFP^+ cells (T_{regs}) revealed that infection with *S. pneumoniae* increased the percentage of T_{regs} within the $CD4^+$ T cell population in lung and spleen, compared with the PBS-treated control group (Figure 5.2 C).


A (i)
55.7
SSC-A
FSC-A
17.9
SSC-A
CD4
84.4
15.5
0.14
0.019
CD4-APC
GFP
A (ii)
53.1
SSC-A
FSC-A
12.5
SSC-A
CD4
78.1
21.7
0.12
0.055
CD4-APC
GFP
B (i)
30.9
SSC-A
FSC-A
13.9
SSC-A
CD4-APC
87.6
11.7
0.53
0.15
CD4-APC
GFP
B (ii)
17.2
SSC-A
FSC-A
11.3
SSC-A
CD4-APC
79.6
17.8
1.10
1.56
CD4-APC
GFP
Lymphocytes
CD4+ T cells
GFP expressing cells within
CD4 T cell gate

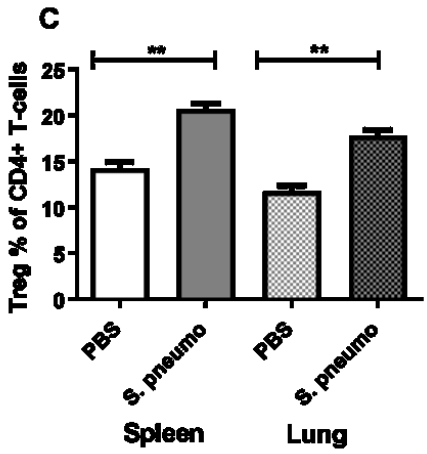


Figure 5.2. Lung infection with *S. pneumoniae* increases the percentage of T_{regs} in spleen and lung.

Foxp3.DTR transgenic male and female mice aged 8-12 weeks were infected with $5x10^7$ CFU bioluminescent S. *pneumoniae, Xen 10* i.n. (n=3) or given PBS i.n (n=3). After 24 hours spleen and lung was harvested and splenocytes and lung cells isolated for CD4-staining and flow cytometry analysis of $CD4^+$ GFP^+ cells. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*$p<0.05$ **$p<0.01$). This experiment was repeated 3 times with consistent results obtained.

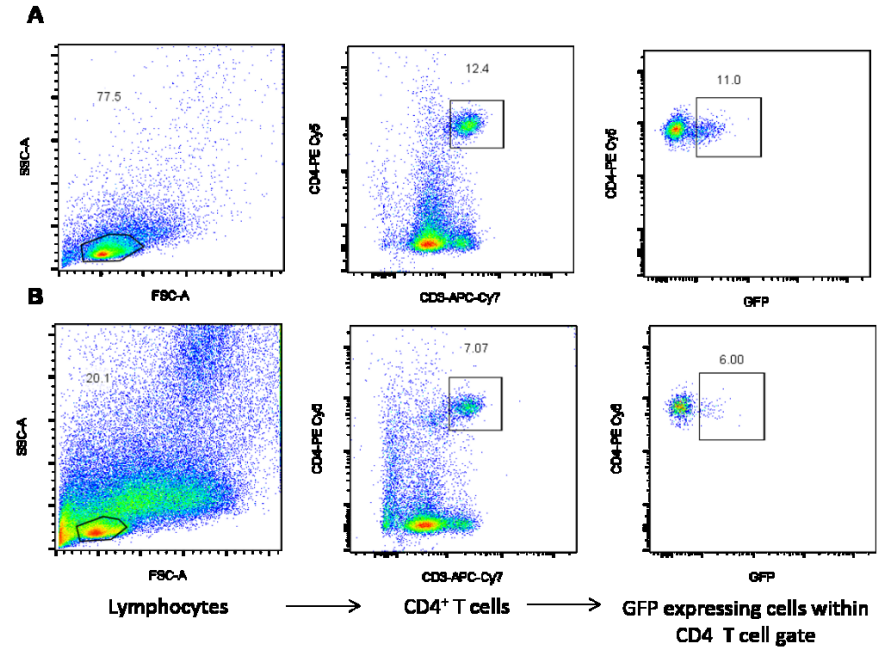
Representative staining of Foxp3-GFP^+ and $CD4^+$ cells (APC-stained) in the spleen (A) and lung (B) of uninfected (i) and *S. pneumoniae*-infected (ii) mice.

(C) Percentage of T_{regs} within the $CD4^+$ T cell populations of spleen and lung in uninfected and infected cohorts.

Assisted by C. R. and J. R.

5.4 Transcriptomic differences between splenic and lung-resident T_{regs}.

T_{regs}, as a percentage of $CD4^+$ T cells, are increased in spleen and lung post infection with *S. pneumoniae.* There are a few possible mechanisms by which this T_{reg} expansion may occur, including the contribution of tissue resident T_{regs}. T_{regs} isolated from spleen and lung of uninfected healthy mice were interrogated to see if they differed from each other in the transcriptomic expression of selected target genes: BLIMP-1, HOBIT, S1pr1 and CCR4. Of these, the gene encoding CCR4 was the only one present at high enough levels for a signal to be obtained by RT-PCR. Perhaps surprisingly, given the established importance of CCR4 expression for the preferential migration of T_{regs} to the lung and ongoing lung tropism, there was no significant difference in the relative expression of *ccr4* in T_{regs} isolated from spleen and lung (Figure 5.3).



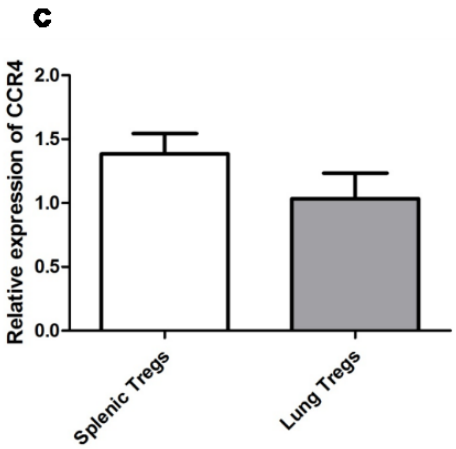


Fig 5.3 RT-PCR of transcription factor gene expression in T_{regs} isolated by cell sorting from spleen and lung of uninfected Foxp3.DTR transgenic mice.

Spleen and lung was harvested from Foxp3DTR transgenic male and female mice aged 8-12 weeks (n=3) and splenocytes and lung cells isolated for CD4-staining and sorting of $CD4^+$ GFP^+ cells. RNA was isolated for RT-PCR analysis of transcription factor gene expression.

Gating strategy for cell sorting of $CD4^+$ GFP^+ T_{regs} from spleen (A) and lung (B) cells. RT-PCR analysis of difference in CCR4 gene expression between splenic and lung T_{regs} (C).

Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t test (*p<0.05). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R. and L. B.

5.5 Diphtheria toxin (DT) treated Foxp3DTR transgenic mice have increased numbers of neutrophils in the lung in the first 8 hours of *S. pneumoniae* lung infection.

The observed expansion in T_{regs} observed in mice infected with *S. pneumoniae* discussed in Section 5.3 raised the question as to whether or not T_{regs} are beneficial to host defense of this important lung pathogen, and, if depleting them prior to infection would impact on pathogen clearance and lung inflammation. Initial investigation utilised an 8-hour model of *S. pneumoniae* lung infection. Foxp3.DTR mice were given two daily doses of DT to deplete $Foxp3^+$ cells prior to, and on the day of, infection with *S. pneumoniae.* Mice were closely monitored for signs of sickness for 8 hours before humane culling, organ harvest and analysis of weight loss, bacterial counts and cell numbers in lung tissue and BAL fluid.

Upon depletion of $Foxp3^+$ cells there was a trend to increased weight loss, bacterial burden in lung tissue and inflammatory cell infiltrate in lung and BAL. Of note, T_{reg}-deficient mice had an increased number of neutrophils in the lung compared to PBS-treated controls (Figure 5.4). The combined observations that *S. pneumoniae* lung infection induces T_{reg} expansion and that T_{reg} depletion may impede pathogen clearance implied that T_{regs} are indeed important in host defense of this pathogen.

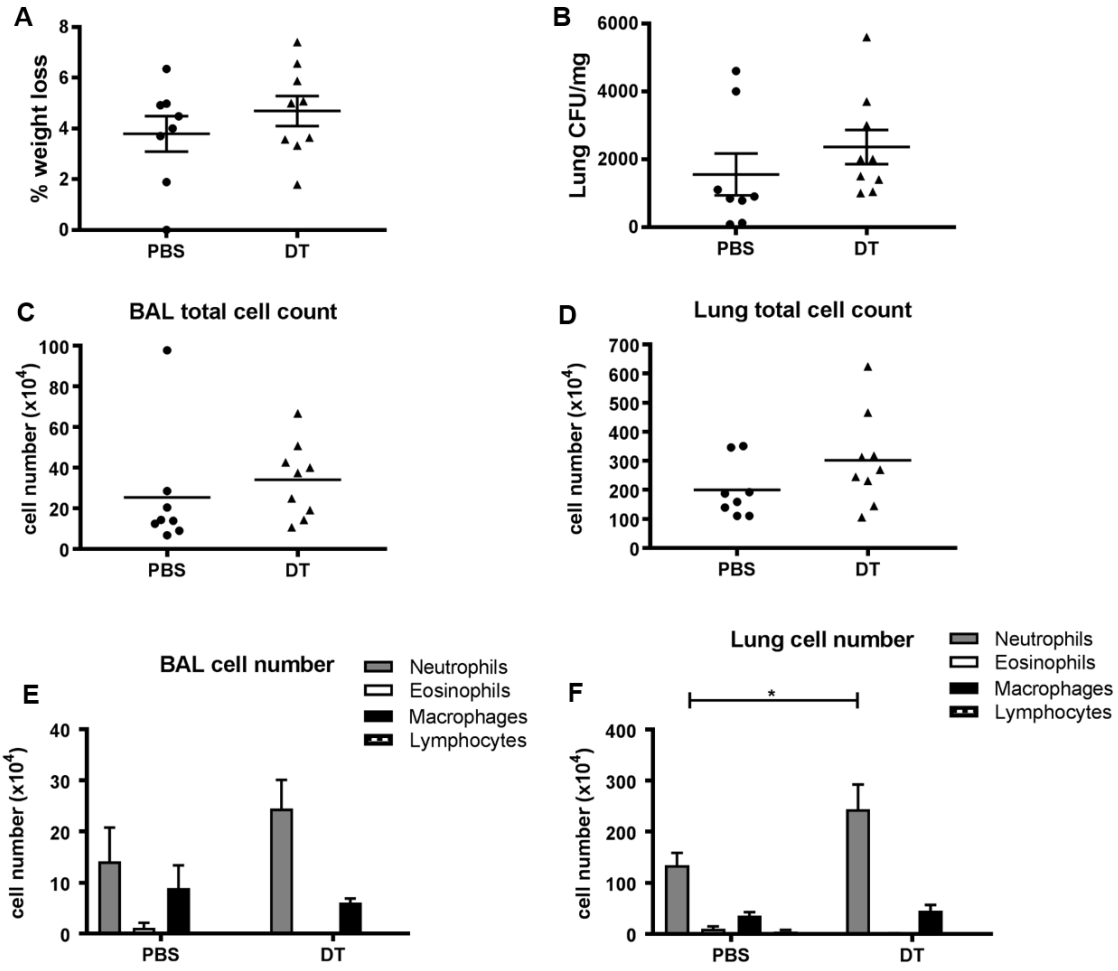


Figure 5.4. 8 hours post S. *pneumoniae* infection, diphtheria treated (DT) T_{reg} deficient mice had an increased number of neutrophils in the lung compared to PBS-treated controls. DT treated T_{reg} deficient mice (n=9) and PBS treated control mice (n=8) aged 12 weeks were weighed prior to infection with 10^7 CFU bioluminescent S. *pneumoniae, Xen 10* i.n. Mice were re-weighed prior to humane sacrifice at 8 hours and BAL and lung tissue harvested.

Percentage weight loss (A). CFU/mg in lung tissue (B). Total cell count in BAL (C) and lung (D). Total cell number (neutrophils, eosinophils, macrophages, lymphocytes) in BAL (E) and lung (F). Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*$p<0.05$). This experiment was repeated 3 times with consistent results obtained. Experiments conducted in collaboration with C. R. and K. Q.

5.6 Diphtheria toxin (DT) treated Foxp3DTR transgenic mice exhibit increased bacterial burden 24 hours post infection with *S. pneumoniae*.

A 24-hour model of T_{reg} depletion and *S. pneumoniae* lung infection was developed to investigate further the notion that depleting T_{regs} may impede pathogen clearance. A similar approach to T_{reg} depletion and lung infection was adopted as for the 8-hour *S. pneumoniae* lung infection model, but a range of alternative readouts of DT effect were employed. Foxp3.DTR mice were given two daily doses of DT to deplete $Foxp3^+$ cells prior to, and on the day of, infection with *S. pneumoniae.* After 24 hours mice were humanely culled and BAL and lung tissue was harvested. Percentage weight loss and bacterial counts in lung tissue and BAL fluid was determined. Upon removal of $Foxp3^+$ cells mice exhibited a significant increase in weight loss and bacterial burden in lung tissue and BAL fluid (Figure 5.5).

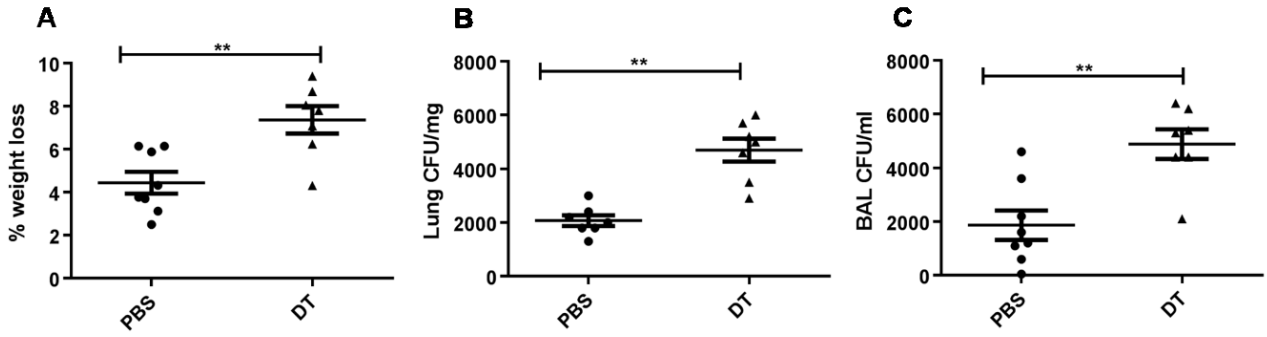


Fig 5.5. 24 hours post S. *pneumoniae* infection, diphtheria treated (DT) T_{reg} deficient mice had increased weight loss and bacterial burden compared to PBS-treated controls. DT treated T_{reg} deficient mice (n=7) and PBS treated control mice (n=7) aged 8-12 weeks were weighed prior to infection with $5x10^7$ CFU S. *pneumoniae* i.n. Mice were re-weighed prior to humane sacrifice at 24 hours and BAL and lung tissue harvested.

(A) Percentage weight loss. (B) CFU/mg in lung tissue. (C) CFU/ml in BAL fluid. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained. Assisted by C. R.

This increase in weight loss and bacterial burden in DT-treated mice was accompanied by changes in cytokine gene expression as determined by RT-PCR (Figure 5.6 A). DT treatment to deplete T_{regs} invoked a general increase in the expression of genes encoding inflammatory cytokines; TNF-α was significantly elevated in the DT-treated group. The removal of $Foxp3^+$ cells also resulted in a possible increase in KC (the murine equivalent of IL-8) though statistical significance was not achieved. Elevation of this important neutrophil chemoattractant would fit with the observed increase in lung neutrophils in the DT-treated group of the 8-hour model of T_{reg} depletion and *S. pneumoniae* lung infection (Figure 5.4).

Efficient T_{reg} knock down was confirmed by the relative lack of *foxp3* gene expression in DT-treated mice compared with PBS-treated controls (Figure 5.6 B).

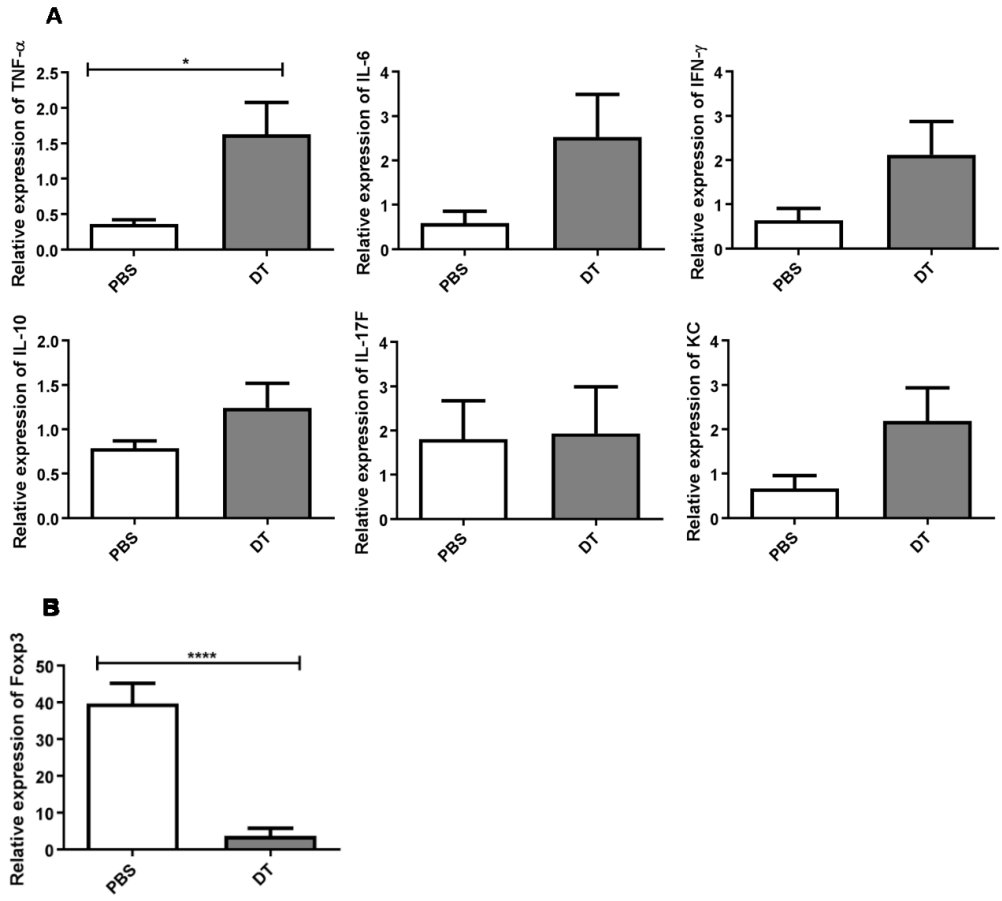


Fig 5.6. RT-PCR analysis of cytokine and Foxp3 gene expression in lung 24 hours post *S. pneumoniae* lung infection in DT-treated mice and PBS-treated controls.

DT treated T_{reg} deficient mice (n=7) and PBS treated control mice (n=7) aged 8-12 weeks were weighed prior to infection with $5x10^7$ CFU S. *pneumoniae* i.n. Mice were re-weighed prior to humane sacrifice at 24 hours and lung tissue harvested.

(A) Cytokine gene expression. (B) Foxp3 gene expression. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

5.7 IFN-γ reporter-Foxp3.DTR mice treated with DT to deplete T_{regs} prior to infection with *S. pneumoniae* express higher levels of IFN-γ than PBS-treated controls.

DT-treatment prior to a 24-hour lung infection with *S. pneumoniae* impairs bacterial clearance and appears to increase inflammatory cytokine gene expression for IL-6, IFN-γ and TNF-α, although TNF-α was the only one to achieve statistical significance.

To investigate this, the IFN-γ reporter mouse (made in the Boyton lab), was crossed onto the Foxp3.DTR transgenic line. The IFN-γ reporter transgenic reports IFN-γ expression by producing bioluminescence following luciferin substrate injection which is detectable by IVIS imaging. The IFN-γ reporter-Foxp3.DTR transgenic, in addition to reporting IFN-γ expression, is an inducible knockout of $Foxp3^{+}GFP^{+}$ cells (T_{regs}). Thus, following DT treatment for 2 consecutive days and infection with *S. pneumoniae* as previously described, the IFN-γ reporter-Foxp3.DTR transgenic could be imaged with IVIS to investigate differences in the IFN-γ-response of DT treated (T_{reg} deficient) mice compared with PBS-treated (T_{reg} replete) controls.

The starting point in using the IFN-γ reporter-Foxp3.DTR transgenic was the establishment of sufficient T_{reg} knock down following DT treatment. Mice receiving daily DT treatment for 2 consecutive days had reduced GFP^{+} cells as a percentage of their $CD3^{+}$ $CD4^{+}$ T-cell populations in spleen and lung as measured by flow cytometry (Figure 5.7).

Once the DT mediated depletion of $Foxp3^{+}$ T cells was confirmed, mice were infected with *S. pneumoniae* to develop a 24-hour model of *S. pneumoniae* lung infection in T_{reg} replete and T_{reg} deficient mice in this transgenic line. IFN-γ reporter-Foxp3.DTR mice were imaged by IVIS at baseline, prior to any intervention. They were re-imaged 3 days later, post treatment with DT (or PBS) and infection with *S. pneumoniae* for 24 hours. As expected the mice showed an increase in IFN-γ response post lung infection with *S. pneumoniae*; interestingly this was enhanced by DT treatment to deplete T_{regs} prior to infection (Figure 5.8 A and B). This result implies that depletion of T_{regs}, in the context of *S. pneumoniae* lung infection, increases expression of IFN-γ as suggested by the RT-PCR data on cytokine gene expression in the Foxp3.DTR transgenic depleted of T_{regs} and infected with *S. pneumoniae* for 24 hours (Figure 5.6 A).

Following completion of the live animal IVIS imaging, mice were humanely sacrificed, lungs and spleen removed and *ex vivo* imaging of bioluminescence undertaken to evaluate IFN-γ response in these organs. Bioluminescence was detectable in similar amounts in the spleens of PBS and DT-treated infected mice. The lungs showed a trend for increased bioluminescence in the DT-treated group though this did not reach statistical significance (Figure 5.8 C and D).

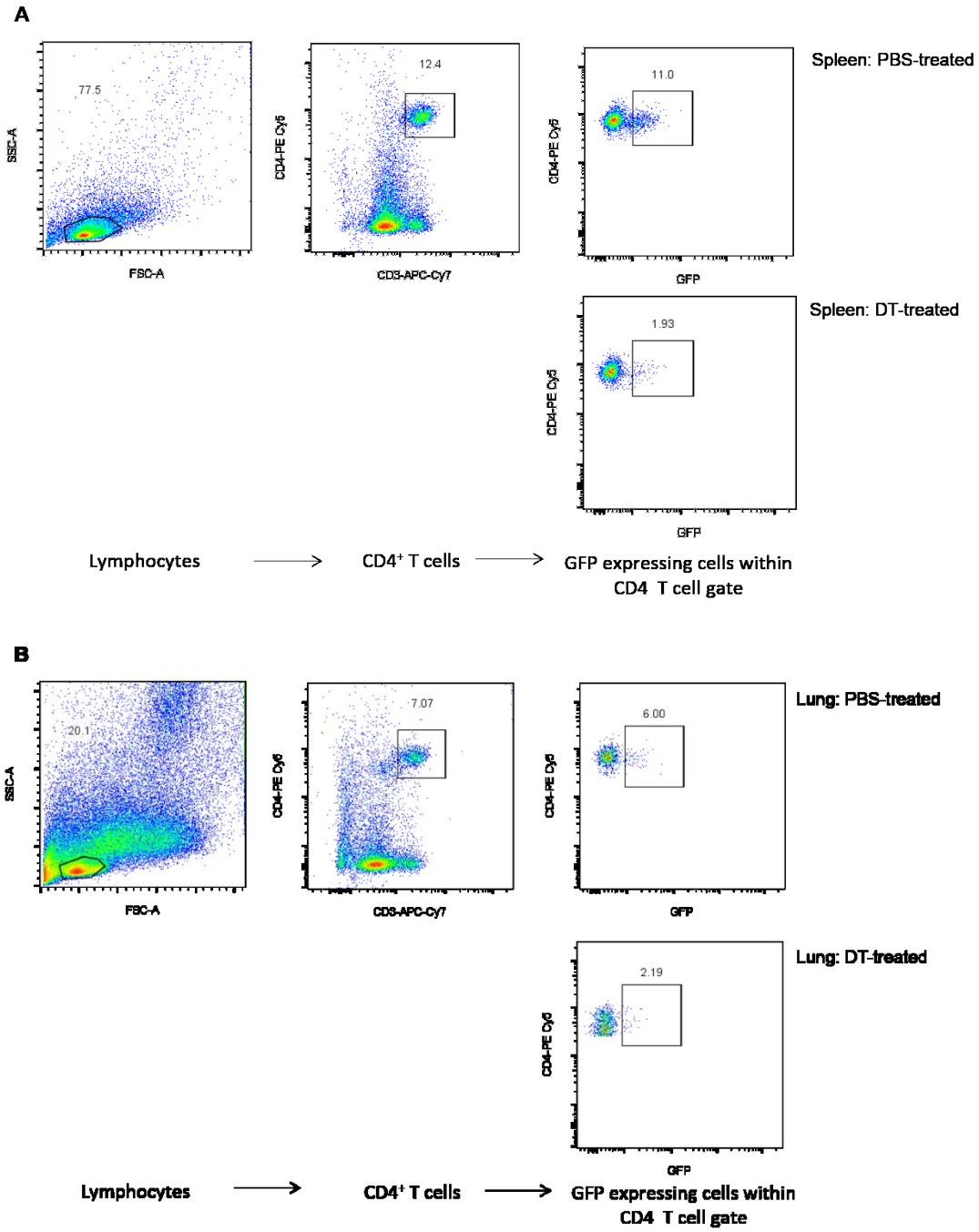


Figure 5.7. Depletion of T_{regs} in spleen and lung following DT treatment administered to the IFN-γ reporter mouse crossed onto the Foxp3 DTR transgenic line.

IFN-γ reporter mice that had been crossed onto the Foxp3 DTR transgenic line were treated with daily administration (i.p) of DT (n=3) or PBS (n=3) for 2 consecutive days prior to humane sacrifice and organ harvest for subsequent analysis of $CD4^+$ GFP^+ cells by flow cytometry.

Representative staining of Foxp3-GFP$^+$ and CD4$^+$ cells (PE Cy5-stained) in the spleen (A) and lung (B) of untreated (top panel) and DT treated mice (bottom panel). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R. and L. B.

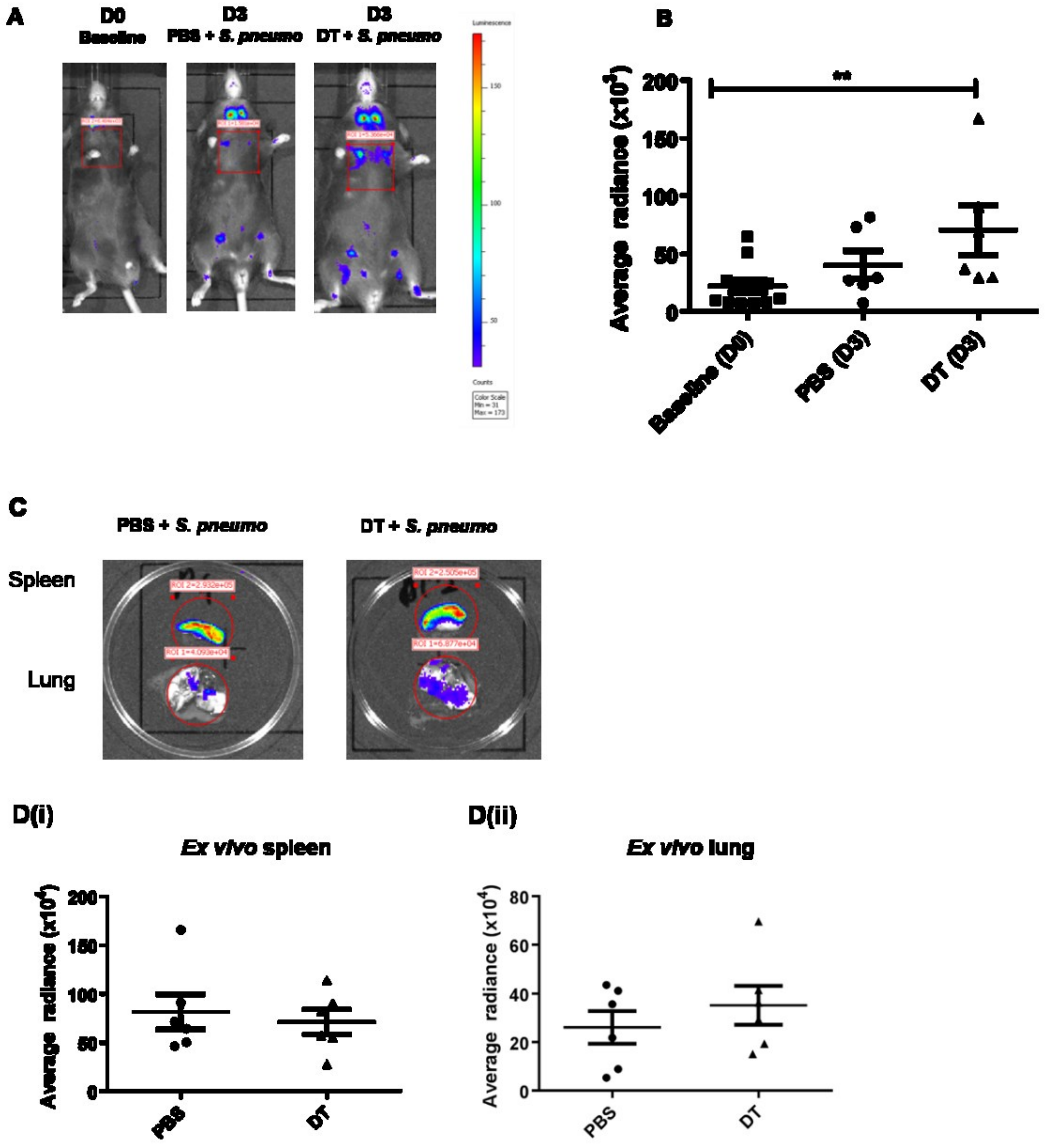


Figure 5.8. IFN-γ reporter-Foxp3.DTR mice show an increase in IFN-γ response post lung infection with *S. pneumoniae*, which is enhanced by DT treatment to deplete T_{regs} prior to infection.

IFN-γ reporter mice that had been crossed onto the Foxp3 DTR transgenic line were injected (i.p) with 200 µl of 15 mg/ml XenoLight D-Luciferin – K+ Salt. After 10 minutes mice were imaged by IVIS to obtain a baseline reading of IFN-γ response prior to any intervention (day 0). Mice were then treated with daily administration (i.p) of DT (n=6) or PBS (n=6) for 2 consecutive days and then infected (i.n) with $5x10^7$ CFU bioluminescent *S. pneumoniae, Xen 10.* 24 hours post infection mice were imaged again by IVIS (day 3) prior to humane sacrifice and organ harvest for subsequent *ex vivo* IVIS imaging of spleen and lung.

(A) Representative IVIS image of baseline (D0) and post infection (D3) IFN-γ response in PBS and DT-treated mice showing selected region of interest (ROI) in the thorax. (B) Average radiance in selected ROI at D0 and D3 in PBS and DT-treated mice. (C) Representative IVIS image of *ex vivo* spleen and lung from PBS and DT-treated infected mice. (D) Average radiance of *ex vivo* spleen and lung. This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R. and L. B.

5.8 Impact of Treg depletion on MSC treatment effect post infection wih *S. pneumoniae*

Following the demonstration of an MSC protective effect in a 24h model of *S. pneumoniae* lung infection, and the subsequent discovery that depleting T_{regs} prior to infection with this pathogen impedes bacterial clearance, it was of interest to see if T_{reg} depletion prior to infection and MSC treatment impacted on MSC protective effect. DT-treated T_{reg} deficient Foxp3.DTR mice and PBS-treated T_{reg} replete mice were infected with *S. pneumoniae* and treated 3 hours later with i.v MSCs. Mice receiving MSC treatment post T_{reg} depletion (DT + MSC group) exhibited increased weight loss and bacterial burden compared to the T_{reg} replete mice (MSC group), however, this observation could be explained by the baseline increase in weight loss and bacterial burden in T_{reg} depleted animals (Figure 5.9).

Experiments earlier in this thesis have demonstrated MSC impact on the expression in lung tissue of genes encoding a select few pro- and anti-inflammatory cytokines 24h post infection with *S. pneumoniae.* MSC treatment had a predominantly anti-inflammatory effect, suppressing the expression of genes encoding pro-inflammatory cytokines: IFN-γ, TNF-α, IL-6 and IL-17F. In contrast, DT treatment to deplete T_{regs} prior to infection (in the absence of MSC treatment) resulted in an increase in the gene expression of these cytokines. This pattern was maintained in MSC-treated mice with and without T_{reg} depletion, though statistical significance was not achieved. Mice infected with *S. pneumoniae* and treated with DT prior to MSC injection trended towards increased expression levels of genes encoding IFN-γ, TNF-α, IL-6, IL-17F and KC compared with MSC treated T_{reg} replete mice (Figure 5.10 A). Efficient T_{reg} knock down was confirmed by the relative lack of *foxp3* gene expression in DT-treated mice compared with PBS-treated controls (Figure 5.10 B).

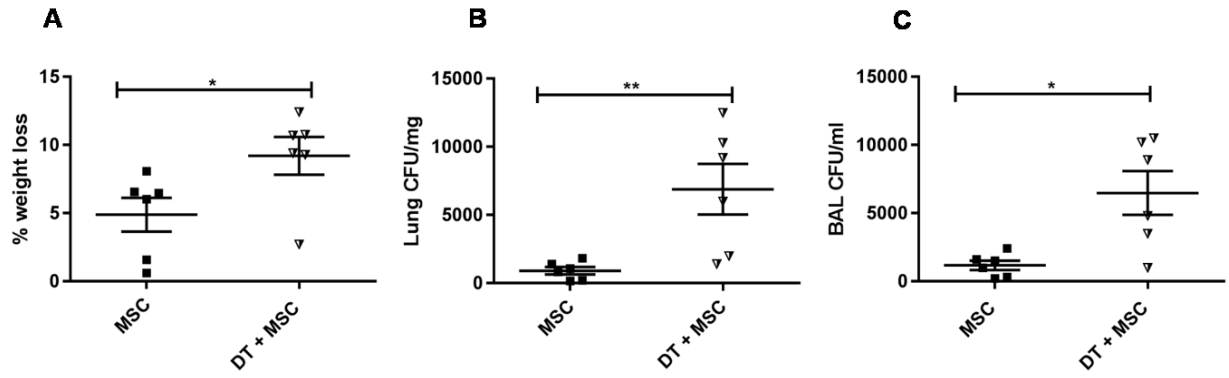


Figure 5.9 Increased weight loss and bacterial burden in DT-treated mice infected with *S. pneumoniae* and treated with MSCs compared to T_{reg} replete controls.

DT-treated T_{reg} deficient Foxp3DTR mice aged 8-12 weeks (n=6) and PBS treated T_{reg} replete mice (n=6) were weighed prior to infection with $5x10^{7}$ CFU bioluminescent *S. pneumoniae, Xen 10* i.n. 3 hours post infection both groups were treated with $5x10^{5}$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v. Mice were re-weighed prior to humane sacrifice at 24 hours and BAL and lung tissue harvested.

(A) Percentage weight loss. (B) CFU/mg in lung tissue. (C) CFU/ml in BAL fluid. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*$p<0.05$ **$p<0.01$). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

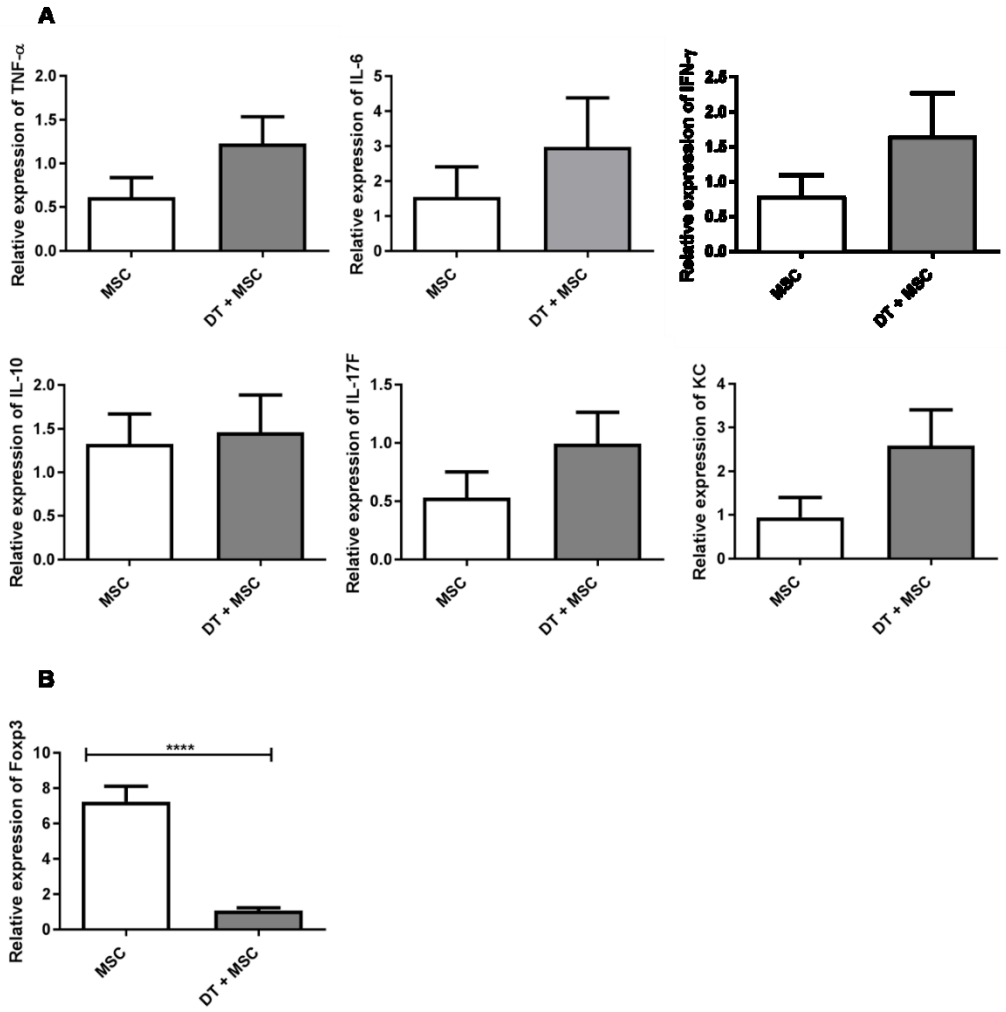


Figure 5.10. RT-PCR analysis of cytokine and Foxp3 gene expression in lung 24 hours post *S. pneumoniae* lung infection.

DT-treated T_{reg} deficient Foxp3DTR mice aged 8-12 weeks (n=6) and PBS treated T_{reg} replete mice (n=6) were weighed prior to infection with $5x10^{7}$ CFU bioluminescent *S. pneumoniae, Xen 10* i.n. 3 hours post infection both groups were treated with $5x10^{5}$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v. Mice were re-weighed prior to humane sacrifice at 24 hours and BAL and lung tissue harvested.

(A) Cytokine gene expression. (B) Foxp3 gene expression. This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001). This experiment was repeated 3 times with consistent results obtained.

To examine, more robustly, the impact of T_{reg} depletion on MSC protective effect in *Strep pneumoniae* lung infection, an experiment was designed to incorporate 4 separate treatment groups. Mice were treated with DT (2 groups) or PBS (2 groups) prior to infection with *Strep pneumoniae,* followed by i.v MSC (2 groups) or PBS (2 groups) 3h post infection. This enabled comparisons to be made between T_{reg} replete infected mice (Foxp3+ (PBS)); T_{reg} replete, MSC treated, infected mice (Foxp3+ (MSC)); T_{reg} deficient infected mice (Foxp3- (PBS)) and T_{reg} deficient, MSC treated infected mice (Foxp3- (MSC)).

In line with the results from initial MSC treatment studies, infected, T_{reg} replete mice treated with MSCs (Foxp3+ (MSC)) exhibited a significant reduction in weight loss and bacterial burden compared to PBS-treated controls (Foxp3+ (PBS)). Also in agreement with previous results, DT-treated T_{reg} deficient mice (Foxp3- (PBS)) exhibited a significant increase in weight loss and bacterial load compared to the T_{reg} replete cohort (Foxp3+ (PBS)). Mice receiving MSC treatment post T_{reg} depletion (Foxp3- (MSC)) exhibited reduced weight loss and reduced bacterial burden compared to the DT treated mice not given MSCs (Foxp3- (PBS)). The difference in bacterial burden between MSC treated T_{reg} replete (Foxp3+ (MSC)) and T_{reg} depleted groups (Foxp3- (MSC)) is complex to interpret as it is difficult to gauge to what extent the increased baseline bacterial burden and heightened inflammatory state associated with T_{reg} depletion influences the efficacy of MSC therapy.

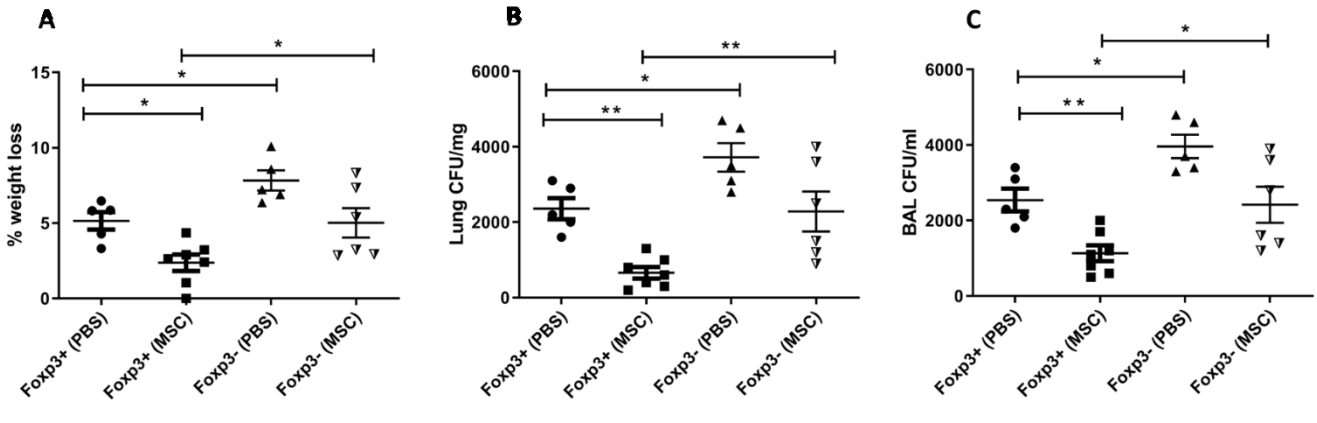


Figure 5.11. Reduced weight loss and bacterial burden in MSC treated mice infected with *S. pneumoniae* compared to PBS-treated controls; increased bacterial burden in DT treated mice.

DT-treated T_{reg} deficient Foxp3DTR mice aged 8-12 weeks (n=11) and PBS treated T_{reg} replete mice (n=12) were weighed prior to infection with $5x10^{7}$ CFU bioluminescent *S. pneumoniae, Xen 10* i.n. 3 hours p.i. 2 groups (n=13) were treated with $5x10^{5}$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v. Mice were re-weighed prior to humane sacrifice at 24 hours and BAL and lung tissue harvested.

(A) Percentage weight loss. (B) CFU/mg in lung tissue. (C) CFU/ml in BAL fluid. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

Cytokine analysis by ELISA from lung homogenate and BAL fluid revealed a significant reduction in levels of TNF-α, IL-6, IFN-γ and IL-10 in the MSC treated versus untreated T_{reg} replete mice. There was a general trend in reduction in the levels of these cytokines in T_{reg} deficient MSC-treated versus untreated mice, but results were not significant. T_{reg} depletion increased levels of TNF-α in lung tissue but did not impact significantly on the levels of the other cytokines investigated. Depletion of T_{regs} prior to treatment with MSCs was associated with increased levels of TNF-α and IFN-γ in lung tissue and BAL fluid and IL-6 in BAL fluid compared with MSC treatment alone (Figure 5.12).

The results of RT-PCR analysis of lung cytokine gene expression correlated closely with the data on cytokine protein levels as measured by ELISA. The relative expression of genes encoding TNF-α, IL-6, IFN-γ, IL-10 and IL-17F was reduced in the MSC treated versus untreated T_{reg} replete mice. The trend observed in reduction in levels of these cytokines in T_{reg} deficient MSC-treated versus untreated mice was again evident, however, the relative expression of genes encoding TNF-α, IL-6 and IFN-γ was significantly lower in the MSC treated T_{reg} deficient group compared with T_{reg} deficient mice not receiving MSCs. T_{reg} depletion increased gene expression levels of IL-6 but did not impact significantly on gene expression of the other cytokines investigated. Depletion of T_{regs} prior to treatment with MSCs promoted an increase in the expression of genes encoding TNF-α, IL-6, IFN-γ (Figure 5.13 A).

RT-PCR analysis of the relative expression of genes encoding transcription factors T-bet, GATA-3, RORγT and Foxp3 revealed some interesting findings. Firstly, a significant reduction in *t-bet* (a key transcription factor for Th1 cells) and *rorγt* (a key transcription factor for Th17 cells) was observed in MSC-treated T_{reg} replete mice compared to PBS-treated controls, in line with the reduction in levels of IFN-γ and IL-17F seen post MSC treatment. Secondly, the relative expression of *gata-3* (a key transcription factor for Th2 cells) was significantly increased in MSC-treated T_{reg} replete mice compared to PBS-treated controls. Efficient T_{reg} knock down was confirmed by the relative lack of *foxp3* gene expression in DT-treated mice compared with PBS-treated controls (Figure 5.13 B). MSC-mediated modifications in the relative expression of genes encoding transcription factors which are important for the differentiation and function of different subsets of effector T cells are interesting and require further investigation.

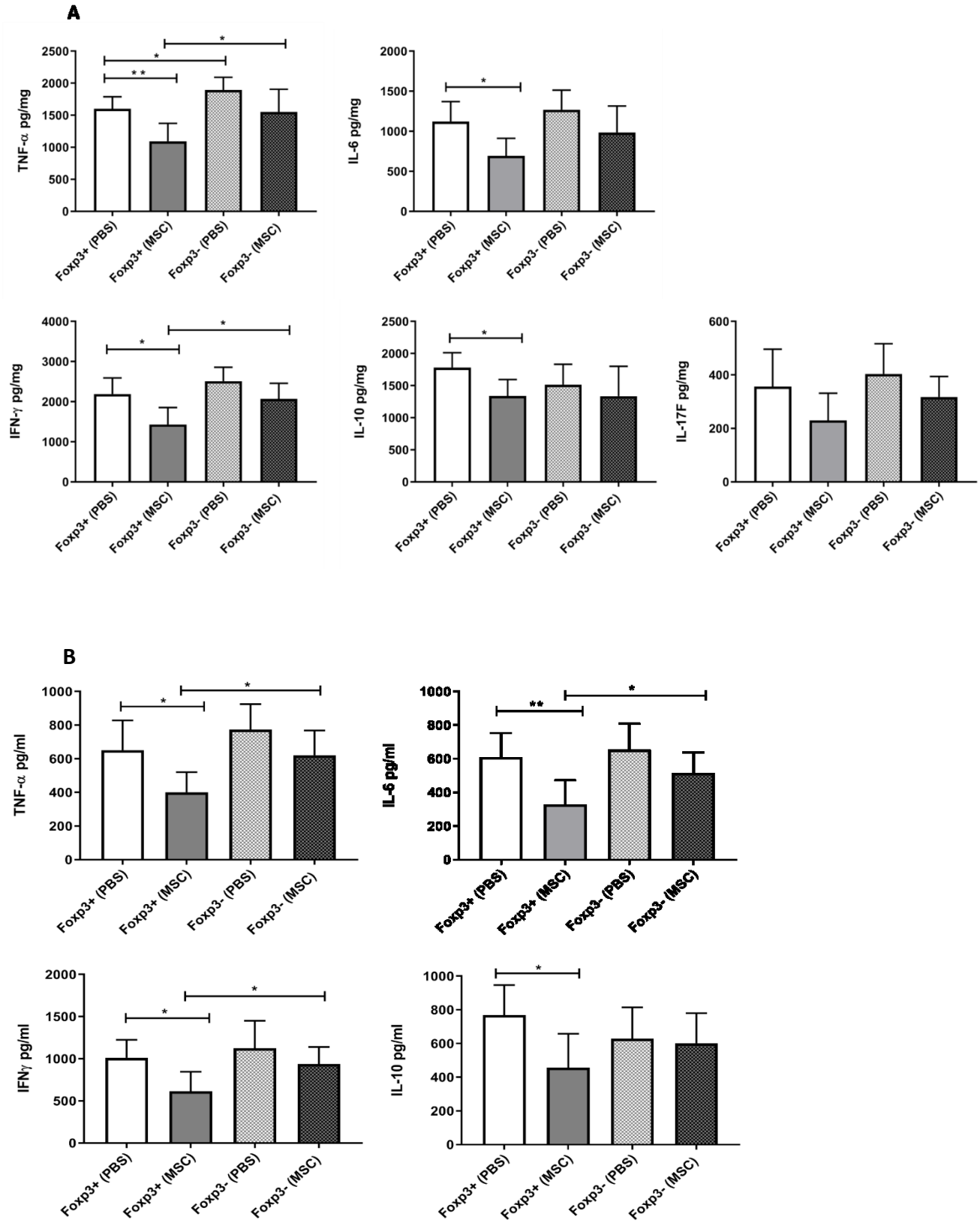


Figure 5.12. Cytokine analysis by ELISA from lung homogenate and BAL 24 hours post *S. pneumoniae* lung infection.

DT-treated T_{reg} deficient Foxp3DTR mice aged 8-12 weeks (n=11) and PBS treated T_{reg} replete mice (n=12) were weighed prior to infection with $5x10^{7}$ CFU bioluminescent *S. pneumoniae, Xen 10* i.n. 3 hours p.i. 2 groups (n=13) were treated with $5x10^{5}$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.

(A) Cytokines in lung homogenate. (B) Cytokines in BAL. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*$p<0.05$ **$p<0.01$). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.

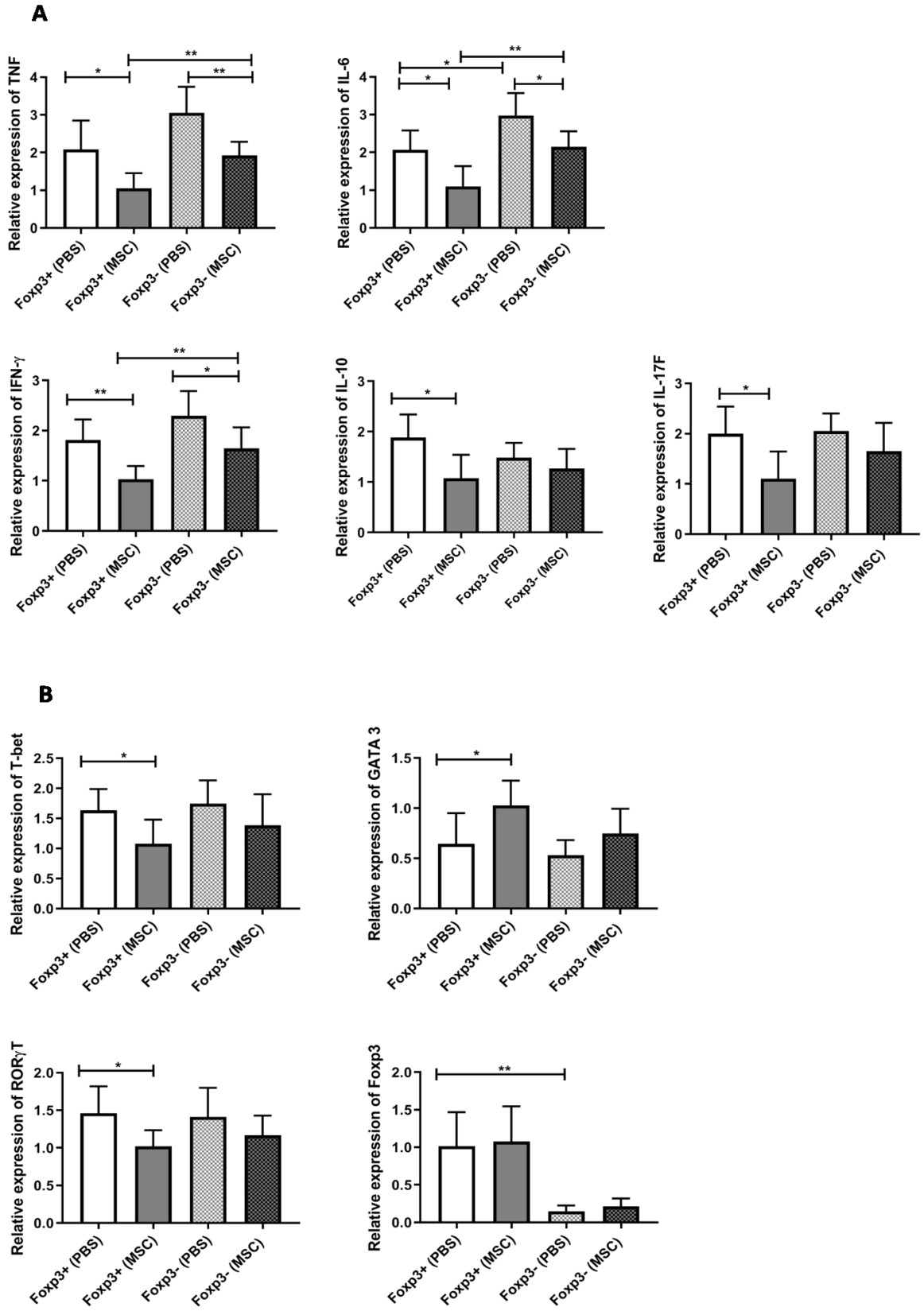


Figure 5.13. RT-PCR analysis of cytokine and transcription factor gene expression in lung 24 hours post *S. pneumoniae* lung infection.

DT-treated T_{reg} deficient Foxp3DTR mice aged 8-12 weeks (n=11) and PBS treated T_{reg} replete mice (n=12) were weighed prior to infection with $5x10^7$ CFU bioluminescent *S. pneumoniae, Xen 10* i.n. 3 hours p.i. 2 groups (n=13) were treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.

(A) Cytokine gene expression. (B) Transcription factor gene expression. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained. Assisted by C. R.

5.9 DT treatment to deplete T_{regs} does not reduce clearance of *P. aeruginosa* and MSC protective effect is maintained.

Chapter 4 of this thesis demonstrates an MSC protective effect following intranasal infection with 2 separate lung pathogens, *S. pneumoniae* and *P. aeruginosa*. For both pathogens, MSC protective effect was associated with a reduction in pro-inflammatory cytokines, suggesting that MSCs exert anti-inflammatory effects in these models of bacterial lung infection. Subsequent experiments in a mouse model of *S. pneumoniae* lung infection have shown that treatment with DT to deplete T_{regs} prior to infection impedes bacterial clearance and is associated with heightened inflammation as dictated by increased levels of pro-inflammatory cytokines in lung tissue and BAL fluid.

The impact of T_{reg} depletion on bacterial clearance and inflammatory response in a mouse model of *P. aeruginosa* lung infection was investigated using IFNγ-reporter.Foxp3.DTR mice. Utilising the 4-group approach to experimental design, previously described for the *S. pneumoniae* mouse model, the impact of T_{reg} depletion on MSC protective effect was also explored. Mice were treated with DT (2 groups) or PBS (2 groups) prior to infection with *P. aeruginosa,* followed by i.v MSC (2 groups) or PBS (2 groups) 3h post infection. This enabled comparisons to be made between T_{reg} replete infected mice (Foxp3+ (PBS)); T_{reg} replete, MSC treated infected mice (Foxp3+ (MSC)); T_{reg} deficient infected mice (Foxp3- (PBS)) and T_{reg} deficient, MSC treated infected mice (Foxp3- (MSC)).

In line with the results from initial MSC treatment studies, infected, T_{reg} replete mice treated with MSCs exhibited a significant reduction in weight loss and bacterial burden compared to PBS-treated controls. In contrast to the results obtained from the *S. pneumoniae* infection model, DT treated T_{reg} deficient mice did not exhibit any significant change in weight loss or bacterial burden compared to the T_{reg} replete cohort. In fact, there was a trend towards reduced weight loss and bacterial burden in the T_{reg} deficient mice. MSC protective effect was preserved following T_{reg} depletion. A trend of reduced weight loss and bacterial burden in MSC treated Treg deplete mice (Foxp3- (MSC)) compared to T_{reg} deplete mice not given MSCs (Foxp3- (PBS)) was observed, though statistical significance in bacterial counts was achieved for lung tissue only (Figure 5.14).

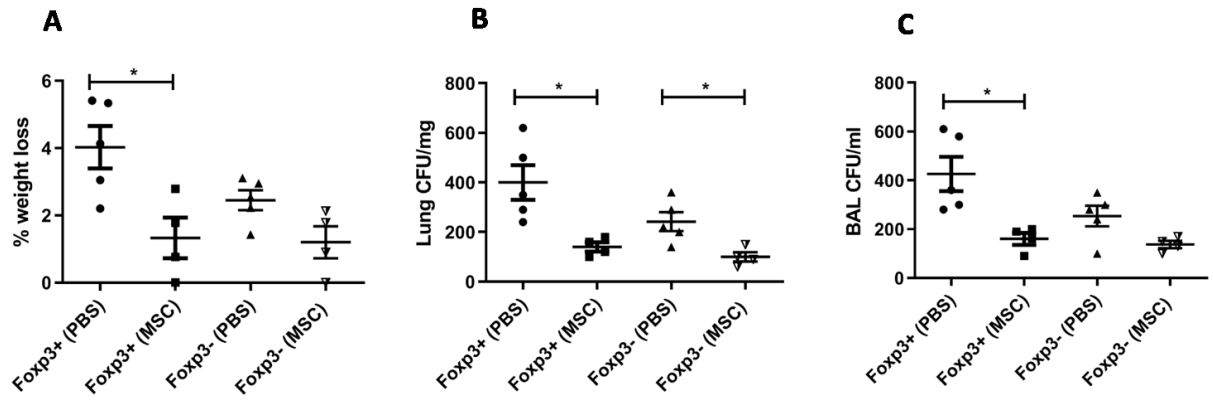


Figure 5.14. Reduced weight loss and bacterial burden in MSC treated mice infected with *P. aeruginosa* compared to PBS-treated controls and in the DT-treated mice receiving MSCs compared to the DT only cohort.

IFN-γ.Foxp3DTR reporter male and female mice aged 8-12 weeks were weighed prior to injection with 50µg/Kg DT (n=10) or PBS (n=10) i.p. daily for 48 hours and infection with 10^6 CFU bioluminescent *P. aeruginosa, Xen 41.* 3 hours after infection 2 groups (n=10) were treated with $5x10^5$ mMSCs (DQ8.Abo-Foxp3DTR background P5). Mice were weighed at 24 hours, BAL and lung tissue harvested. CFU/mg in lung tissue and CFU/ml in BAL fluid was measured.

(A) Percentage weight loss. (B) CFU/mg in lung tissue. (C) CFU/ml in BAL fluid. Data are presented as mean +/- SEM. Statistical significance was determined using Mann Whitney U statistical test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

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Cytokine analysis from lung homogenate revealed a significant reduction in protein levels of TNF-α, IL-6, IFN-γ and IL-17F in MSC treated versus untreated T_{reg} replete mice. A similar pattern was observed in BAL fluid, but the overall result was more conclusive; all cytokines investigated, including the anti-inflammatory cytokine IL-10, were significantly reduced in these mice. There was a general trend of reduction in the levels of these cytokines in T_{reg} deficient MSC treated versus untreated mice; significance was achieved for TNF-α, IL-6 and IL-17F in both lung tissue and BAL fluid. DT treatment to deplete T_{regs} (in the absence of MSC administration) had no effect on the levels of the cytokines measured (Figure 5.15).

The results of RT-PCR analysis of lung cytokine gene expression correlated closely with the data on cytokine protein levels as measured by ELISA. The relative expression of genes encoding TNF-α, IL-6, IFN-γ and IL-17F was reduced in the MSC treated versus untreated T_{reg} replete mice. The trend observed of reduction in levels of these cytokines in Treg deficient MSC treated versus untreated mice was again evident; the relative expression of genes encoding TNF-α, IL-6 and IL-17F was significantly lower in the MSC treated T_{reg} deficient group compared with T_{reg} deficient mice not receiving MSCs. T_{reg} depletion (in the absence of MSC treatment) had no impact on gene expression levels of any of the cytokines investigated. (Figure 5.16 A).

RT-PCR analysis of the relative expression of genes encoding transcription factors T-bet, GATA-3, RORγT and Foxp3 revealed a significant reduction in *rorγt* in MSC-treated T_{reg} replete mice compared to PBS-treated controls, in line with the reduction in levels of IL-17F seen post MSC treatment. This reduction in *rorγt* was reproduced in the MSC treated T_{reg} deficient group compared to the T_{reg} deficient group not given MSCs. No significant differences were found in the relative expression of *t-bet* or *gata 3* in MSC treated versus untreated groups. Efficient T_{reg} knock down was confirmed by the relative lack of *foxp3* gene expression in DT-treated mice compared with PBS-treated controls (Figure 5.16 B).

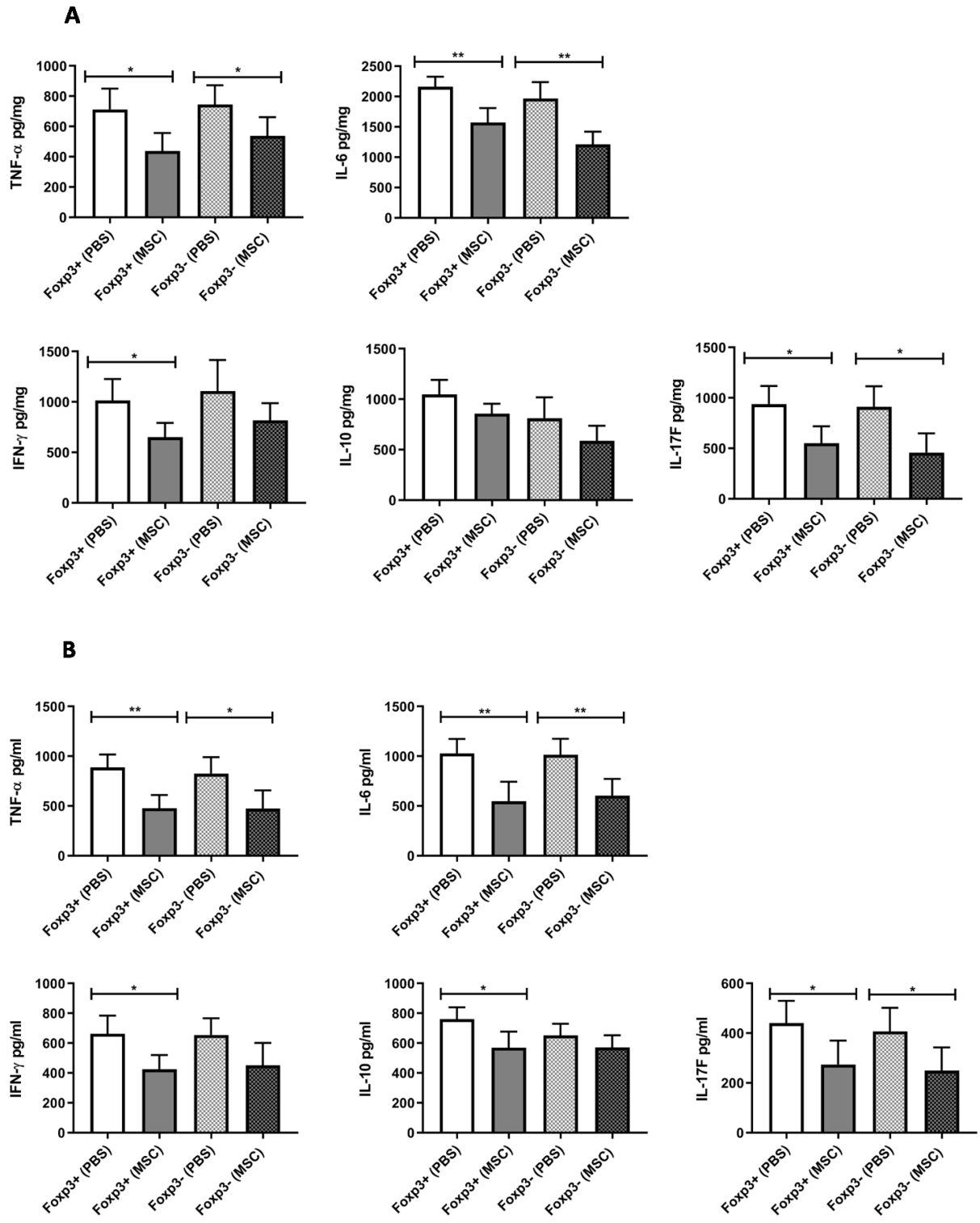


Figure 5.15. Cytokine analysis by ELISA from lung homogenate and BAL 24 hours post *P. aeruginosa* lung infection.

DT-treated Treg deficient IFNγ reporter.Foxp3DTR mice aged 8-12 weeks (n=10) and PBS treated T_{reg} replete mice (n=10) were weighed prior to infection with 10^6 CFU

bioluminescent *P. aeruginosa, Xen 41* i.n. 3 hours post infection 2 groups (n=10) were treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.

(A) Cytokines in lung homogenate. (B) Cytokines in BAL. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01). This experiment was repeated 3 times with consistent results obtained.

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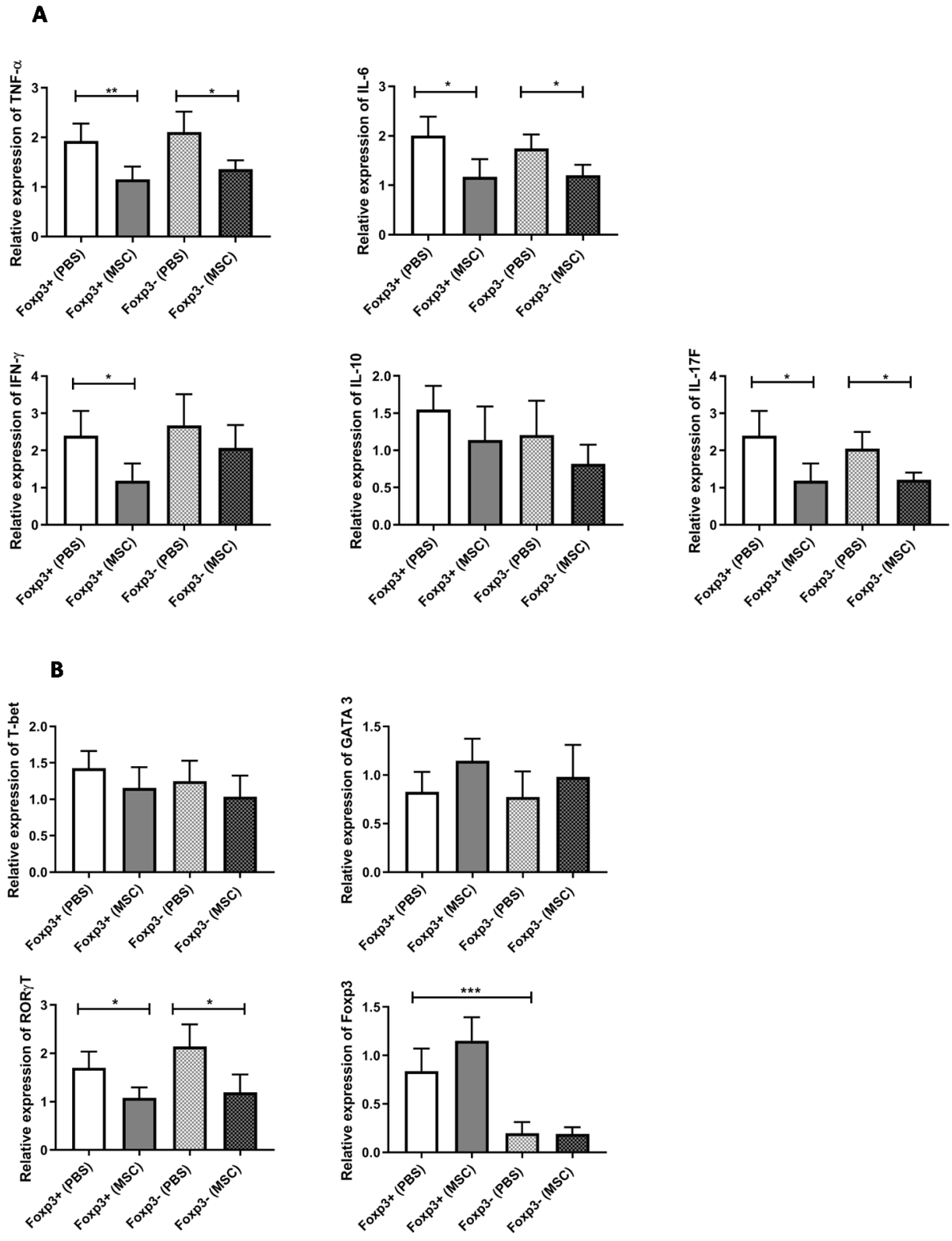
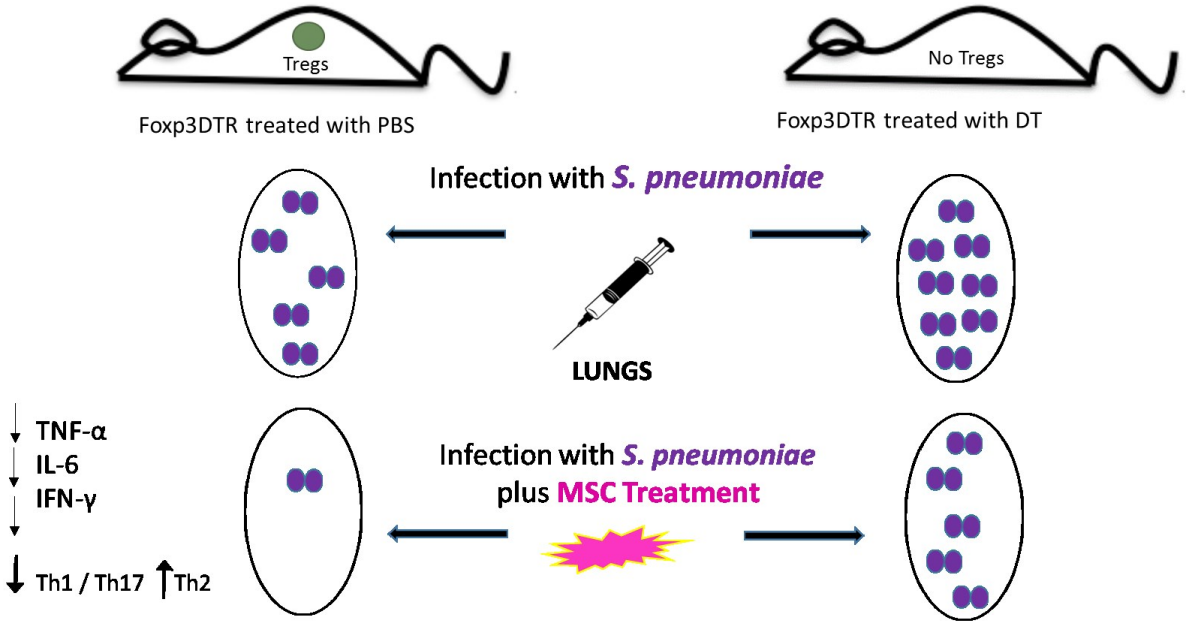


Figure 5.16. RT-PCR analysis of cytokine and transcription factor gene expression in lung 24 hours post *P. aeruginosa* lung infection.

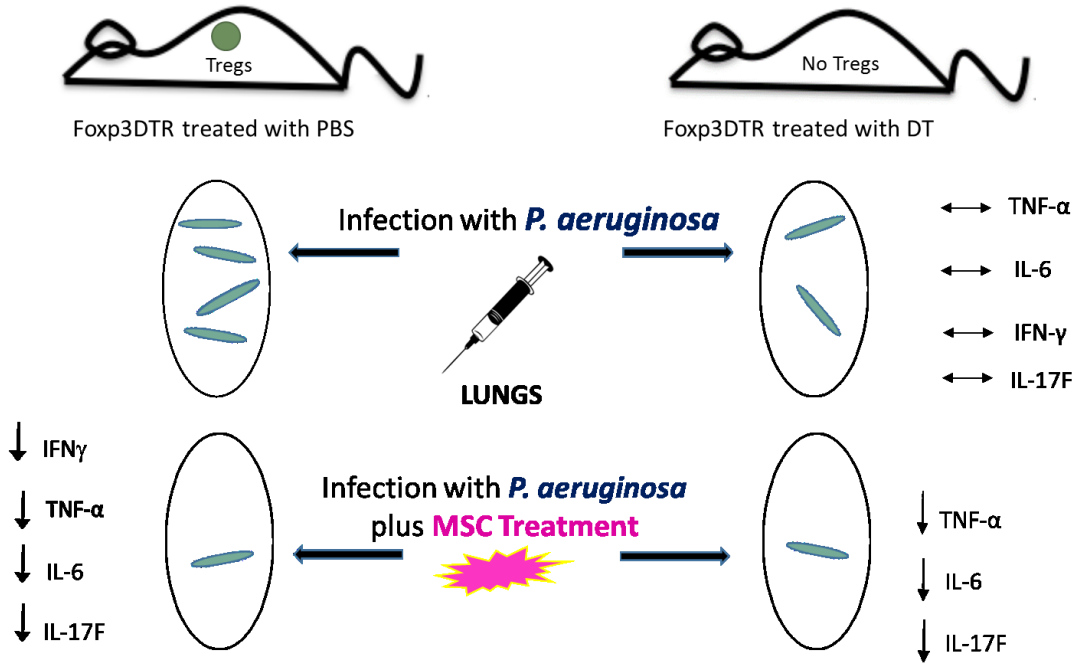
DT-treated T_{reg} deficient IFNg-reporter.Foxp3DTR mice aged 8-12 weeks (n=10) and PBS treated T_{reg} replete mice (n=12) were weighed prior to infection with 10^6 CFU bioluminescent *P. aeruginosa, Xen 41* i.n. 3 hours post infection. 2 groups (n=10) were treated with $5x10^5$ mMSC (DQ8.Abo-Foxp3DTR background, P5) i.v.

(A) Cytokine gene expression. (B) Transcription factor gene expression. Data are presented as mean +/- SEM. Statistical significance was determined using the unpaired student t-test (*p<0.05 **p<0.01 ***p<0.001). This experiment was repeated 3 times with consistent results obtained.

Assisted by C. R.



Infection in the absence of Tregs results in a substantially increased bacterial load and inflammation in lung and BAL. MSC treatment reduces bacterial load and inflammation. Immunity switches away from a Th1 / Th17 towards a Th2 response. This observation is partially Treg dependent.



Infection in the absence of Tregs results in reduced bacterial load in lung and BAL. MSC treatment reduces bacterial burden and inflammation in lung and BAL in presence and absence of Tregs. This observation appears to be independent of Tregs.

Figure 5.17. The impact of MSC treatment post infection with *S. pneumoniae* and *P. aeruginosa* in the presence and absence of Tregs.

Chapter 6

6 Conclusions and Discussion

The work presented in this thesis has produced three main findings: (1) MSCs increase bacterial clearance in lung and BAL in murine models of bacterial pneumonia caused by *Streptococcal pneumoniae and Pseudomonas aeruginosa*; (2) T_{regs} are recruited to the lung following *S. pneumoniae* infection and their presence is beneficial; (3) T_{reg} depletion prior to infection with *P. aeruginosa* does not influence bacterial clearance or inflammatory response. In the following section I will discuss my results and the implications of these findings in the context of existing data on MSC protective effects and the role of T_{regs} in host response to bacterial infection.

6.1 BM-MSCs as novel antimicrobial agents.

Over the last 20 years an array of preclinical studies have demonstrated a role for MSCs in regulating the inflammatory response to tissue injury and infection, supporting the process of tissue repair post inflammation resolution (Ortiz *et al*., 2007, Islam *et al*., 2012; Ahmad *et al*., 2014; Jiang *et al*., 2016). MSCs have multiple capabilities: homing to sites of injury (Ponte *et al*., 2007; Johnson *et al*., 2017; Van Hoeven *et al*., 2018), downregulation of the potentially damaging inflammatory cascade (Nemeth *et al*., 2009; Luo *et al*., 2014; Pedrazza *et al*., 2014), inhibition of apoptosis in vulnerable tissues (Bruno *et al*., 2009; Pedrazza *et al*., 2014; Shabbir *et al*., 2015), activation of resident stem cells with reparative properties and modulation of the activity of multiple immune effector cell types (Crisostomo *et al*., 2008; Weil *et al*., 2010; Gupta *et al*., 2012; Sutton *et al*., 2016; Alcayaga *et al*., 2015). Important for their impact on immune response to infection is their ability to sense their microenvironment and adapt their role to acting as pro- or anti-inflammatory mediators to best suit host requirements (Cho *et al*., 2006; Pevsner-Fischer *et al*., 2007; Wang *et al*., 2009; Li *et al*., 2012; Le Burel *et al*., 2017).

MSC have been demonstrated to be capable of direct antimicrobial activity via the secretion of a range of antimicrobial peptides and proteins (Gupta *et al*., 2012; Alcayaga-Miranda *et al*., 2015; Sutton *et al.,* 2016; Sung *et al*., 2016), putting them in a strong position for

exploitation as a novel cellular antimicrobial therapy to reduce infection-related morbidity and mortality. The need for such a therapy has escalated in recent years with the increasingly widespread problem of antibiotic resistance, and the ageing population, for whom targeted antibiotic treatment alone may not be sufficient (Moellering, 2010; Lin *et al*., 2015). Another recognised mechanism by which MSCs promote bacterial clearance is through enhancing monocyte/macrophage phagocytosis (Hematti *et al*., 2009; Mei *et al*., 2010; Krasnodembskaya *et al*., 2012; Jackson *et al*., 2016, Morrison *et al*., 2017). This has been shown to involve mitochondrial transfer from MSC to host macrophage (Jackson *et al*., 2016; Morrison *et al*., 2017) and the MSC release of EVs (Zhu *et al*., 2014; Park *et al*, 2019). Macrophage transformation from an M1 to an M2 phenotype appears to underpin the enhancement in phagocytic activity (Monsel *et al*., 2015; Johnson *et al*., 2017; Song *et al*., 2017). MSC pre-activation has been reported to enhance MSC antimicrobial effects (Waterman *et al*., 2010; Monsel *et al*., 2015). Co-administration with conventional antibiotic treatment has been shown to reduce mortality where either treatment alone has failed (Mei *et al*., 2010; Alcayaga-Miranda *et al*., 2015).

If MSC therapy is to be safely applied to the treatment of infection, it is important to carefully evaluate MSC effects in a variety of infection models, with the aims of elucidating MSC mechanisms of action and exploring the crosstalk between MSCs and immune effector cells following infectious challenge. The work presented in this thesis demonstrates beneficial anti-microbial and anti-inflammatory effects of murine BM-MSC in mouse models of Gram positive and Gram negative bacterial lung infection. The data suggests that the interaction between MSC and other immune effector cells, specifically T_{regs}, is important for achieving the observed protective effects.

My work began with the exploration of a variety of methods to isolate mMSCs from different anatomic niches (BM, compact bone and adipose tissue) for subsequent use in mouse models of bacterial lung infection. The chosen methodology used a combination of BM and compact bone, demonstrated to be the most successful approach to culturing a homogeneous population of MCS. MSCs are inherently more difficult to isolate from the bone marrow of mice than humans (Phinney *et al*., 1999; Sun *et al*., 2003; Peister *et al*., 2004). Reasons for this include the low frequencies of MSCs in mouse bone marrow (2-5/10^6 BM nucleated cells) (Koc *et al*., 1999), their location near the surface of the bone and within

it (Zhu *et al*., 2010) and the level of contamination of the BM cells with HSCs and fibroblastic cells (Zhao *et al*., 2004). MSCs isolated from different species (human versus murine) and different tissue sites (bone marrow versus adipose tissue) have been demonstrated to display substantial variation in function (Meisel *et al*., 2004; Nauta *et al*., 2007; Dazzi *et al*., 2012). This complicates the interpretation of MSC studies and has been a source of controversy in the field. Even when derived from the same species and tissue of origin, MSCs demonstrate significant donor-to-donor variability, influenced by a range of factors including donor age and health status (Kuznetsov *et al*., 2009; Wang *et al*., 2013). MSC self-renewal capacity and differentiation potential have been reported to decline with increasing age (Stenderup *et al*., 2003; Katsara *et al*., 2011). Taking this into consideration, mice chosen for MSC donation were young (8-12 weeks), healthy and had the same genetic background as the murine infection model to be used.

The technique of MSC isolation, culturing protocols and maturity or passage number have also been shown to influence MSC function, highlighting the importance of standardising preparation techniques (Dazzi *et al*., 2012; Ankrum *et al*., 2014; McLeod and Mauck, 2017). Much of my early work involved going back to first principles to establish a reliable and reproducible method for isolation and expansion of cells with an MSC morphology. On reaching the appropriate passage intended for use, aliquots of these cells were characterised using a panel of cell surface markers established as representative of mMSC (Morikawa *et al*., 2009); mMSC uniformly expressed MHC class-1, Sca-1 and PDGFRα, while being negative for the expression of CD45 (Figure 3.2). These characterised MSC were then validated by exploration of their differentiation potential. *In vitro* differentiation assays demonstrated differentiation capacities of these cells toward adipogenic and osteogenic lineages (Figure 3.3). This rigorous process aimed to achieve standardised preparations for use in the infection models to facilitate comparison of MSC treatment results between studies. Further complexities in MSC heterogeneity have been highlighted by recent studies demonstrating quite extensive variability amongst and within clonal MSC populations: variation in differentiation capacity, transcriptomics and biophysical properties. While the origins of these variations are not yet clear, potential mechanisms such as transcriptional fluctuations an epigenetic instability have been suggested (McLeod and Mauck, 2017).

A remarkable feature of MSCs is their ability to migrate to sites of inflammation and tissue damage (Fiedler *et al*., 2002 and 2006; Ponte *et al*., 2007; Johnson *et al*., 2017; Van Hoeven *et al*., 2018). Following infectious challenge, MSC TLR-ligand interactions allows MSCs to recognise and respond to pathogen-related proteins and traffic towards the bacteria or bacteria-infected cells (Tomchuck *et al*., 2008; Li *et al*., 2013). The "lung barrier" or "pulmonary first-pass effect" has been regarded as a potential impediment to MSC migration to sites of tissue damage because of cell trapping in the lung (Gao *et al*., 2001; Schrepfer *et al*., 2007; Xiang *et al*., 2009). It was necessary to investigate this concept to see if 'lung trapping' was a persistent finding in the mouse models used, even in the absence of lung infection/inflammation directing MSC migration.

In order to explore MSC migration a method was developed for labelling the MSCs *in vitro* and visualizing them (post intravenous administration) *in vivo* and *ex vivo* post organ harvest. MSCs were labelled with a fluorescent dye, DiI, which was demonstrated to have optimal characteristics for *in vivo* detection compared with the other dyes tested: superior brightness, longevity of staining and consequent tissue penetrating capabilities (Figure 3.4). A point of concern was the potential for transfer of fluorescent dye from grafted cells (MSC) to host cells which could be followed by incorrect interpretation of MSC location. To avoid this issue, clear-cut results may be better obtained by a combination of DiI labeling and *alu*-in situ hybridization. However, it has been demonstrated that the degree of transfer of DiI from transplanted cells to the host's tissues depends on the cell type and route of administration (Brulport *et al., 2007*; Schormann *et al.,* 2008). Conditions under which a relatively large fraction of transplanted cells deteriorate, such as direct injection of human cord blood cells into liver tissue resulted in relatively high levels of dye transfer (Brulport *et al.,* 2007). In contrast, administration of MSC via tail vein as was carried out here, followed by active extravasation into lung tissue, resulted in lower levels of dye transfer (Schormann *et al.,* 2008).

DiD-labelled MSCs, injected via tail vein into mice infected with *P. aeruginosa*, were visualised *in vivo* using IVIS imaging, migrating from the injection site to the thoracic region over a period of 20 minutes. The signal was maintained at 40 minutes, though reduced in intensity. In a separate experiment utilising *S. pneumoniae* as the lung pathogen, comparison was made in the dynamics of MSC distribution between infected and uninfected

mice. Mice treated with MSC post infection exhibited increased fluorescence in the lung compared with liver and spleen at the 40-minute time point, as determined by flow cytometric analysis of fluorescent cells in *ex vivo* organs. This was in contrast to a healthy, uninfected mouse injected with MSCs, which exhibited minimal fluorescence in cells isolated from lung, liver and spleen after 40 minutes (Figure 3.5). Though requiring validation with larger samples, these observations suggest that MSCs may not be routinely trapped in the lung and may preferentially migrate to infected, inflamed lung. Reduction in signal detection from fluorescent MSCs homing to infected lungs, 40 minutes post infusion, suggests a limited life span for these cells. It is widely acknowledged that MSCs can exert potent immunosuppressive and anti-inflammatory effects, despite their lack of engraftment (Eliopoulos *et al*., 2005; Nauta *et al*., 2006; von Bahr *et al*., 2012) and their apparent disappearance within hours of administration (Lee *et al*., 2009; Ankrum *et al*., 2014). A number of studies have demonstrated MSC lysis *in vitro* (Spaggiari *et al*., 2006) and MSC clearance *in vivo* (Eliopoulos *et al.,* 2005*;* Nauta *et al.,* 2006*;* Moll *et al.,* 2012; Li *et al*., 2012*)* Of interest, a recent study showed that MSC apoptosis and subsequent efferocytosis by host macrophages was in fact fundamental to MSC immunomodulatory functions (Galleau *et al*., 2017).

6.2 Paracrine mediators important for MSC function.

Whether resting, or active post migration, BM-MSCs have been shown to express a diverse array of regulatory proteins, growth factors and cytokines which contribute to their immunomodulatory effects. A number or studies using Transwell culture systems to separate MSCs from target cells have demonstrated that many of the observed MSC immunomodulatory effects are mediated through paracrine mechanisms involving one or more of these secreted molecules (Di Nicola *et al*., 2002; Selmani *et al*., 2008; Lee *et al*., 2009; Tatara *et al*., 2011). The molecules expressed by MSC, the MSC 'secretome', can be influenced by exposure of MSC to specific cytokines, MSC 'priming' (Polchert *et al*., 2008; Gupta *et al*., 2012; Le Burel *et al*., 2017). An example is TNF-α primed MSC having enhanced anti-inflammatory effects compared to MSCs which were not exposed to this pro-inflammatory cytokine (Ren *et al*., 2008; Blasi *et al*., 2011). This priming or 'licensing' of MSCs appears to be key to their acquisition of immunosuppressive functions. Blockage of

IFN-γ stimulation with anti-IFN-γ receptor antibodies abrogated the suppressive effect of hMSCs (Krampera et al., 2006). In contrast, MSC pre-treatment with IFN-γ stimulated a 5-fold increase in immunosuppressive activity compared with control MSCs in a murine model of GvHD (Polchert *et al*., 2008). Chapter 3 of this thesis concludes with a small-scale exploration of the secretome of a cohort of the mMSCs used in the *in vivo* infection experiments. The aim was to gain some insight into which paracrine mediators might be important in MSC protective effect in the experimental systems used. The three mediators chosen for investigation (TGF-β, IL-6 and IL-10) have been described by others as contributory to MSC impact on immune response to bacterial infection (Rafaghello *et al*., 2008; Nemeth *et al*., 2009; Cassatella *et al*., 2011).

TGF-β is an important component of the MSC secretome, in part due to its ability to induce T_{regs} (Gollnick *et al*., 1995; Neill *et al*., 2012). Kadioglu and colleagues demonstrated that CBA/Ca mice deficient in TGF-β succumbed to invasive pneumonia more readily than BALB/c mice with intact TGF-β signaling. This observation was attributed to the protective effects of TGF-β-mediated T_{reg} expansion seen in the resistant BALB/c mice, which was lacking in the susceptible CBA/Ca mice (Neill *et al*., 2012). Data presented in Chapter 5 of this thesis supports the notion that T_{regs} are an important component of the immune response to *S. pneumoniae* lung infection. Foxp3DTR mice, depleted of T_{regs} by injection with DT, exhibited increased weight loss and a higher bacterial load in lungs and BAL than T_{reg} replete controls. It seems plausible that TGF-β-mediated induction of T_{regs} could contribute to MSC protective effect, thus it was of interest to explore if TGF-β was detectable in the secretome of the MSCs to be used, and, if exposure to a pro-inflammatory cytokine would enhance its secretion. Unstimulated 'resting' MSCs constitutively expressed low levels of TGF-β and levels were indeed significantly increased by *in vitro* priming with IFN-γ. A 4-fold increase in TGF-β expression was observed at 6 hours post stimulation (Figure 3.6).

MSC secretion of IL-6 has been demonstrated as protective against neutrophil apoptosis and inhibitory to excessive oxidative metabolism (Asensi *et al.,* 2004; Rafaghello *et al*., 2008) which may contribute to MSC anti-microbial and anti-inflammatory effects post infectious challenge. MSC-derived IL-6 has also been shown to prevent the differentiation of monocytes towards antigen-presenting immunogenic cells and skew differentiation towards an anti-inflammatory IL-10 producing cell type (Melief *et al*., 2015). Data presented here

shows that IL-6 is constitutively expressed by unstimulated MSCs and increased significantly post stimulation with IFN-γ, but over a longer time period than TGF-β; expression levels peaked at 48 hours (Figure 3.6). This may imply a lesser contribution of IL-6 to MSC impact on the infection models given that the duration of infection was only 24 hours, at which time IL-6 expression levels may not have been high enough to influence neutrophil activity and survival.

IL-10, like TGF-β, can induce T_{regs} and is known to be immunosuppressive by inhibiting APC secretion of pro-inflammatory cytokines (Asseman *et al*., 1999). IL-10 can be produced by many different cell types: B cells, mast cells, eosinophils, macrophages, DCs, and a T cell subtypes that regulate the synthesis of pro-inflammatory chemokines and cytokines, such as IL-1, IL-6, TNF-α (Zhang *et al*., 2014; Perobelli *et al*., 2015). Its role within the MSC secretome is not well defined, however there is evidence that MSC-derived IL-10 can have beneficial anti- inflammatory effects in a variety of conditions (Qua *et al*., 2012; Wang *et al*., 2018). A recent example of this is IL-10 induced inhibition of hepatocyte pyroptosis (a novel form of programmed cell death associated with an intense inflammatory response) in a murine model of acute liver failure. Systemic treatment with mMSC (shown to secrete IL-10) ameliorated D-Gal-induced hepatocyte damage; IL-10 knock down in MSCs abrogated this protective effect, confirming the therapeutic significance of this cytokine (Wang *et al*., 2018). Murine BM-MSCs have also been shown to inhibit Th17 cell differentiation via IL-10-mediated suppression of Rorγt, the key transcription factor for Th17 cells (Qua *et al*., 2012). Data presented here shows that MSC IL-10 expression levels trended up post stimulation with IFN-γ, rising 2-fold by the 48 hour time point, though statistical significance was not achieved when comparing IL-10 expression levels in unstimulated and stimulated MSCs (Fig 3.6).

6.3 Host immune response to Gram-positive and Gram-negative lung pathogens and the impact of MSC treatment.

The host immune response to lung bacterial infection involves initial defense from resident AM which engulf and kill the pathogen and also to recruit neutrophils to phagocytose the invading organisms (Descamps *et al*., 2012). Neutrophils secrete serine proteases which

contribute to pathogen elimination, and can attack host cells in addition to targeting the bacteria themselves, which can result in tissue damage (Hirche *et al*., 2008; Lavoi *et al*., 2011). Data presented in Chapter four of this thesis demonstrates an enhancement in bacterial clearance following MSC treatment that paradoxically is associated with an overall reduction in the inflammatory response (Figures 4.4 – 4.9). This combination of anti-microbial and anti-inflammatory MSC effect has been demonstrated in several pre-clinical studies investigating MSC impact on ALI induced by endotoxin or live bacteria (Xu *et al*., 2007; Gupta *et al*., 2007; Lee *et al*., 2013; Zhu *et al*., 2014; Park *et al*., 2019) and sepsis (Nemeth *et al*., 2010; Mei *et al*., 2010; Krasnodembskaya *et al*., 2012; Chao *et al*., 2014).

Treatment with MSC was initiated 3 hours post infection induction to allow time for inflammation to develop which is believed to stimulate or 'license' MSCs to have their immunomodulatory effects (Meisel *et al*., 2004; Krampera *et al*., 2006; Ren *et al*., 2008; Park *et al*. 2019). This time delay in treatment also makes the study design more relevant to clinical practice, whereby diagnosis of an infection and the decision to treat doesn't happen in conjunction with the infective insult, but takes hours / days to be recognised. The observed MSC protective effects likely involve a variety of mechanisms. The relatively short duration of the *in vivo* infection experiments undertaken (24 h) supports the notion that MSC paracrine effects or direct cellular interactions of MSC with immune effector cells are probable modes of action contributing to the observed protective effects (Gnecchi *et al*., 2008; Lee *et al*., 2009b; Aslam *et al*., 2009; Chao *et al*., 2014). There is very little support in the literature for MSC engraftment with differentiation or cell fusion being important mechanisms of MSC effect (Mei *et al*., 2007 and 2010; Luo *et al*., 2014).

The observed MSC-mediated suppression of the inflammatory response could itself impact on bacterial growth. In septic patients, bacterial growth has been linked to inflammatory cytokine levels (dos Santos *et al*., 2002). The pattern of bacterial proliferation has been represented as a U-shaped curve, with highest rates of growth at the extremes of the curve, when inflammatory cytokine levels are very high or very low. This data suggests that both excessive inflammation and a hypo-inflammatory state may stimulate bacterial growth or impair bacterial clearance (Meduri *et al*., 1999; Kanangat *et al*., 1999; dos Santos *et al*., 2002). In line with this, Pedrazza and colleagues demonstrated that murine AT-MSCs were protective in a CLP model of polymicrobial sepsis, in part by suppressing pro-inflammatory

cytokines, TNF-α and IL-6, which was associated with a reduction in splenocyte apoptosis (Pedrazza *et al*., 2014). Chao et al also demonstrated a reduction in levels of TNF-α and IL-6 in a rat CLP model treated with hMSCs isolated from bone marrow and umbilical cord (Chao *et al*., 2014). It has been suggested that the anti-inflammatory effects of MSC therapy may suppress the hyper-inflammatory response that accompanies the acute phase of a bacterial lung infection to a level that impedes bacterial growth, supports host cell phagocytic response and limits apoptosis-mediated tissue damage (Bernardo and Fibbe, 2013); Pedrazza *et al*., 2014; Chao *et al*., 2014).

MSC-induced AM reprogramming to produce a predominantly anti-inflammatory, regulatory M2 phenotype may also have contributed to the observed MSC anti-inflammatory effects (Kim and Hematti, 2009; Nemeth *et al*., 2009; Maginni *et al*., 2010; Monsel *et al*., 2015; Song *et al*., 2017). Nemeth et al concluded that reprogramming of endogenous macrophages to increase IL-10 production was a key mechanism of MSC protective effect (Nemeth *et al*., 2009). Data presented here shows that lung and BAL levels of IL-10 and IL-10-associated gene expression were not increased by MSC treatment. In fact, a trend in reduction of IL-10 was observed in the MSC treatment group. This observation is in line with work by Mei and colleagues which demonstrated a profound reduction in IL-10 post MSC treatment in septic mice injured by CLP (Mei *et al*., 2010). A possible explanation for the observed differences in IL-10 levels is timing of MSC administration. Nemeth studied the effect of MSC pre-treatment, administering MSCs 24 hours before CLP, at which time IL-10 production was low. Mei administered MSCs 6 hours post CLP, when IL-10 levels were already maximally elevated, removing scope for further increase (Mei *et al*., 2010). Differences are also noted in the dose of MSC administered; 1 x 10^6 cells in the study conducted by Nemeth, 2.5 x 10^5 in the study conducted by Mei. The MSC treatment studies presented in this thesis utilised 0.5 x 10^5 MSC, given 3 hours post infection, which makes this study closer in design to the Mei Study than to the Nemeth study. IL-10 continues to be a subject of debate in mouse models of infection treated with MSC. Gupta et al reported that MSCs reduce the early macrophage-mediated pro-inflammatory response to *E. coli* pneumonia as measured by BAL levels of macrophage inflammatory protein 2 (MIP-2) and TNFα, but MSC treatment had no effect on the level of IL-10 in the BAL fluid compared with control mice (Gupta *et al*., 2012). Luo et al reported an increase in serum IL-10 following MSC injection 3 hours post infection

induction (Luo *et al*., 2014) whereas Alcayaga-Miranda and colleagues showed a reduction in IL-10 post MSC injection 3 hours after the onset of sepsis (Alcayaga-Miranda *et al*., 2015).

MSC induction of an M2 phenotype in host macrophages has been demonstrated to enhance their phagocytic activity (Krasnodembskaya *et al*., 2010, 2012; Gupta *et al*., 2012; Alcayaga-Miranda *et al*., 2015; Sung *et al*., 2016; Sutton *et al.,* 2016). Duncan Stewart's group noted that co-culture of MSCs with macrophages and fluorescently labelled bacteria did not result in increased macrophage (or MSC) phagocytic activity. However, phagocytosis was shown to be enhanced when assessed *ex vivo* in splenic and peritoneal cells isolated from CLP-injured mice given MSC therapy. This suggests that MSC impact on host cell phagocytic activity is not simply the result of one or more secreted paracrine mediators, but rather depends on exposure to the multifaceted host environment *in vivo*. In support of this is the expression microarray analysis which revealed upregulation in pathways associated with monocyte/macrophage phagocytosis (Mei *et al*., 2010). Subsequent work from other groups has provided further evidence of MSC-enhanced monocyte/macrophage phagocytosis (Krasnodembskaya *et al*., 2012; Lee *et al*., 2013; Monsel *et al*., 2015).

An alternative mechanism by which MSCs might influence macrophage activity is by acting as regulators of the inflammasome. Oh et al found that hMSCs negatively regulated NLRP3 inflammasome activation in human and mouse macrophages that were exposed to LPS and ATP. The hMSCs did not suppress NLRP3 or IL-1β expression at the mRNA level, but significantly suppressed the production of mitochondrial reactive oxygen species (ROS) which is critical for inflammasome activation (Kepp *et al.*, 2011; Oh *et al.*, 2013). Impairment of bacterial clearance was previously reported in a rodent model of *P. aeruginosa* acute lung infection in which alveolar macrophages had been depleted, suggesting the importance of their contribution to bacterial clearance (Hashimoto *et al*., 1996). However, in a more recent study, depletion of AMs improved bacterial clearance and survival in a murine model of *P. aeruginosa* pneumonia (Cohen and Prince, 2013). The investigators concluded that inflammasome signaling, induced by *P. aeruginosa* flagella, stimulated AMs to secrete IL-1β and IL-18 which did not contribute to bacterial clearance, but rather caused excessive lung pathology and increased mortality. Futhermore, IL-18 was observed to suppress IL-17-dependent antimicrobial peptide production from γδ T cells, which may serve to perpetuate bacterial survival (Cohen and Prince, 2013). It follows that MSC-mediated modification of

macrophage behaviour may result in upregulation of macrophage phagocytic capacity and a reduction in inflammasome activation, which may support bacterial clearance and limit collateral tissue damage.

6.4 The role of IL-17 in bacterial clearance and impact of MSC treatment.

It is well established that IL-17 production and associated neutrophil recruitment is crucial to the clearance of extracellular bacterial pathogens from the lungs (Zhang *et al*., 2009; Liu *et al*., 2010; Xu *et al*., 2014). Indeed, the design of anti-*P. aeruginosa* vaccines has included gene products that specifically upregulate T_H17 cell signaling (Wu *et al*., 2012). However, there is accumulating evidence that the timing of IL-17 production is important. Upregulation of IL-17 early in the course of *P. aeruginosa* lung infection has been shown to enhance bacterial clearance (Xu *et al*., 2014). Pre-treatment with IL-17 neutralising antibody was associated with low levels of neutrophil recruitment and impaired bacterial clearance (Liu *et al*., 2011).

My lung transcriptional studies investigating gene expression of a number of cytokines in the infection models used revealed detectable levels of IL-17F but not IL-17A, thus IL-17F was chosen as part of the cytokine panel studied pre and post MSC treatment. IL-17F has been shown to be produced by T_H17 cells, lamina propria T cells, memory $CD4^+$ T cells, γδ T cells, iNKT cells and group 3 ILCs *in vivo* (Yang *et al*., 2008c; Romagnoli *et al*., 2016). Depletion of ILC3s rendered mice highly susceptible to oropharyngeal candidiasis, which is also dependent on IL-17 and IL-17RA (Gladiator *et al*., 2013; Conti *et al*., 2016). In models of bacterial pneumonia, the respective contribution of these different IL-17-producing cell types remains to be determined (Iwanaga and Kolls 2019).

IL-17 was originally found in BAL cells from patients with allergic asthma subjected to ragweed allergen stimulation (Kawaguchi *et al*., 2001) and credited with the ability to induce pro-inflammatory cytokines and chemokines which served as neutrophil chemo attractants (Oda *et al*., 2005). IL-17F-deficient mice, challenged with inhaled *Aspergillus* antigen, failed to demonstrate the same levels of neutrophil recruitment as IL-17F replete controls (Yang *et al*., 2008c). However, overexpression of IL-17F in lung epithelium stimulated the influx of lymphocytes and macrophages in association with mucus hyperplasia, but no evidence of

neutrophil infiltration (Yang *et al*., 2008c). IL-17F, working in synergy with IL-22, has also been demonstrated to induce an array of antimicrobial peptides (Liang *et al*., 2006).

Patients with lung disease secondary to cystic fibrosis who are chronically infected with bacteria and fungi have been shown to have robust Th17 responses to both *P. aeruginosa* and *A. fumigatus* (Chan *et al*., 2013). Neutralization of IL-17 in a murine model of CF lung inflammation induced by the mucoid clinical *P. aeruginosa* isolate M5715, was shown to reduce lung inflammation and bacterial burden in bronchial alveolar lavage fluid (Hsu *et al*., 2016). However, using clinical CF isolates (YH5 and NH57388A) in a similar agar-based model of chronic *P. aeruginosa* infection, IL-17RA KO mice had a higher bacterial burden and a reduced rate of survival (*Bayes et al., 2016*), suggesting that IL17RA is critical for mounting an immune response to these clinical isolates, but the precise ligands required for this effect are unclear (Iwanaga and Kolls, 2019). In support of the concept that Th17 cells contribute to CF lung disease is the observation that high levels of circulating Th17 cells are strongly associated with poorer lung function (Mulcahy *et al*., 2015).

The impact of IL17 (and IL-22) on host response to *S. pneumoniae* lung infection has been demonstrated to be strain dependent (Ritchie *et al*., 2013). IL17RAKO mice were found to be more susceptible to infection with serotype 4 (TIGR4) than IL-17 replete controls, but the opposite was observed in IL17RAKO mice infected with serotype 3 (SRL1). The authors concluded that IL-17 is beneficial in infection caused by a highly invasive (serotype 4) pneumococcus but harmful in localised pulmonary infection caused by a serotype 3 pneumococcus (Ritchie *et al*., 2013). The parent strain of Xen 10 used in my experiments, A66.1, is serotype 3. Data presented in Chapter four suggests that MSC-mediated reduction in lung IL-17F protein levels may be one of the factors contributing to improved outcome as measured by percentage weight loss and bacterial burden (Figure 4.5A). This may not be the case if alternative pneumococcal serotypes were employed as infective pathogens. In future studies, I would like to characterize these variations in far greater detail through side by side comparisons of wild-type, IL-17A and IL-17F knockout strains challenged with different clinical isolates.

Ghannam and colleagues investigated the effects of human BM-MSC on differentiation of the T_H17 lineage and the function of fully differentiated T_H17 cells isolated from human tissue (Ghannam *et al*., 2010). They found that fully polarized T_H17 cells physically adhered

to MSCs, via CCR6 and CD11a/CD18 and their ligands, and transformed into a regulatory T-cell phenotype. This involved both cell-cell contact and paracrine mediator PGE_2 and resulted in MSCs reciprocally modulating the expression of RORC and FOXP3 (Ghannam *et al*., 2010). It is plausible that a possible mechanism for the reduction in lung IL-17F gene expression observed in the MSC treated infected animals in my study is MSC-mediated inhibition of the T_H17 lineage, or even induction of a T_{reg} cell phenotype in mature lung infiltrative T_H17 cells.

These studies are now possible as subsequent work in the lab has led to high granularity phenotypic and functional studies of lung T cell subsets. In support of my data is a study by Luo and colleagues which demonstrated a murine BM-MSC protective effect on acute kidney injury (AKI) in a CLP model of polymicrobial sepsis (Luo *et al*., 2014). MSCs, administered 3 hours post septic insult, abrogated kidney damage and reduced neutrophil infiltration, in association with a reduction in the serum levels and kidney tissue expression levels of IL-17 and IL-17-related chemokines at 24 hours. The authors were not able to explain the precise mechanism by which this occurred but concluded that MSC protective effects were mediated by secreted soluble factors as opposed to cell-cell contact as MSCs themselves were not detected in the kidney (Luo *et al*., 2014).

6.5 The role of T_{regs} in bacterial clearance.

Chapter 5 of this thesis explores T_{reg} effects in lung infection, specifically looking at the impact of T_{reg} depletion on lung bacterial clearance and how this lack of T_{regs} impacts on MSC protective effects. Pre-clinical studies have demonstrated that bacterial infection can promote an expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{regs} in peripheral blood, spleen and draining lymph nodes (O. Carrigan *et al*., 2008 Preston *et al.*, 2011, Okeke *et al.*, 2013). Post intranasal infection with *S. pneumoniae* it has been reported that T cells infiltrated murine lungs early in the infection process, and that this was driven by *S. pneumoniae*-derived pneumolysin. The peak of T cell infiltration coincided with a significant reduction in pneumococcal growth in the lungs (Kadioglu *et al*., 2000, 2004). A significant proportion of these migrating cells have since been demonstrated to be $CD4^+$ $CD25^+$ Foxp3 regulatory T cells (Neill *et al*., 2012). T_{regs} have also been shown to activate (as opposed to suppress)

immune effector cells to counter infection in a murine model of fungal infection with *Candia albicans*. In this study T_{regs} upregulated the expression of T_H17-associated cytokine genes *il-17a*, *il-17f* and *il-22* in $CD4^+$ T cells to enhance fungal clearance (Pandiyan *et al*., 2011).

My data is in line with these studies, showing an increase in the percentage of T_{regs} within the $CD4^+$ T cell population in both lung and spleen, measured *ex vivo* by flow cytometry, 24 hours post infection with *S. pneumoniae* (Figure 5.2). Infection-induced T_{reg} expansion may reflect local TCR- or cytokine-driven expansion, chemokine-based recruitment of circulating T_{regs}, conversion of local and/or circulating conventional $CD4^+$ T cells, or prolonged survival of existing T_{regs} (Kadioglu *et al*., 2004; Sather *et al*., 2007; Thorburn *et al*., 2010). T_{regs} have been shown to express diverse patterns of homing receptors that facilitate migration (Huehn *et al*., 2004; Siegmund *et al*., 2005). An example is CCR4, which appears to contribute to the accumulation of T_{regs} in the airways and lung tissue. The CCR4 ligand, CCL17, is constitutively expressed by bronchial epithelial cells in the lung and its expression has been demonstrated to increase in the inflamed lung (Sekiya et al., 2000). CCR4KO mice have been reported to behave similarly to anti-CD25-treated mice rendered deficient in $T_{regs,}$ exhibiting more severe inflammation than T_{reg} replete controls in a murine model of allergic lung disease (Faustino *et al*., 2013). Another consideration is the potential contribution of tissue-resident T_{regs}. T_{regs} residing in fat (visceral adipose tissue (VAT) T_{regs}) (Feuerer *et al*., 2009), bowel lamina propria (Kim *et al*., 2016), skeletal muscle (Burzyn *et al*., 2013) and lung (Arpaia *et al*., 2015) have been identified.

Depletion of T_{regs} in an inducible Foxp3.DTR knockout mouse experimental model prior to infection with *S. pneumoniae* resulted in a significant increase in lung bacterial burden, suggesting that T_{regs} are beneficial to the host response to this bacterium.

The precise mechanism of this T_{reg} effect is not clear, however it is interesting that T_{reg} depletion was associated with a general increase in the expression of genes encoding inflammatory cytokines; TNF-α was significantly elevated in the DT-treated group (Figure 5.6). The observed trend in elevation of KC may have contributed to an increase in lung neutrophils in the T_{reg} deplete group, as observed in the 8-hour model. It is somewhat surprising that the relative lung neutrophilia in DT-treated mice is associated with reduced

bacterial clearance given the importance of this cell type in phagocytosis and containment of bacterium in the early stages of infection. However, other studies have shown that a reduction in the number of infiltrating neutrophils can be associated with improved clinical outcome (Marks *et al*., 2007; Kadioglu *et al.,* 2011; Luo *et al*., 2014). Indeed, uncontrolled lung neutrophil influx may contribute to lung tissue damage that could facilitate bacterial dissemination.

It is possible that T_{regs} which are adapted to function in a particular 'niche' within a specific tissue, such as lung, may be important, not only in tissue homeostasis, but in defense against pathogen invasion (Bapat *et al*., 2015; Cipolleta *et al*., 2015). The expansion of lung resident T_{regs}, which may be specifically suited to responding to the inflammatory micro-environment induced by lung infection, is also a possible explanation for the observed T_{reg} expansion and detrimental impact of T_{reg} depletion. Kadioglu's group have previously shown that infection with *S. pneumoniae* induces T_{reg} expansion to a greater extent than infection with other Gram-positive bacteria. They concluded that T_{regs} are critical regulators of responses to *S. pneumoniae* infection, functioning to reign in the initial inflammatory response which can be overly exuberant and potentially damaging to host cells (Kadioglu *et al*., 2014).

In support of this is the data obtained from the IFN-γ reporter-Foxp3.DTR mouse which produces a bioluminescent signal when expressing IFN-γ, detectable by IVIS imaging (Reynolds, C. *et al*., 2019). In addition to this, the IFN-γ reporter-Foxp3.DTR transgenic is an inducible knockout of $Foxp3^{+}GFP^{+}$ cells (T_{regs}) so comparison in the expression levels of IFN-γ can be made between T_{reg} depleted versus Treg replete mice post infection with *S. pneumoniae*. As anticipated from the RT-PCR data from lung which showed a trend of increased expression of IFN-γ in DT-treated infected mice (Figure 5.6), IFN-γ reporter-Foxp3.DTR mice depleted of T_{regs} prior to infection with *S. pneumoniae* expressed higher levels of IFN-γ than infected T_{reg} replete mice as determined by bioluminescent signal strength (Figure 5.8). Collectively, these data demonstrate an important role for T_{regs} in the host response to pneumococcal pneumonia and highlight the significance of balancing lung based pro-inflammatory responses with anti-inflammatory and immunomodulatory activity. Of interest, the association of increased lung inflammation with impairment of bacterial

clearance is complimentary to the observation that MSC-enhancement of pathogen eradication is accompanied by a reduction in pro-inflammatory cytokines in lung and BAL.

T_{reg} depletion during *P. aeruginosa* lung infection did not impact on bacterial burden or inflammatory response, suggesting no role for T_{regs} in the host response to this bacterium.

T_{reg} depletion prior to infection with *P. aeruginosa* did not impact on bacterial burden or inflammatory response, in fact there was a trend of enhanced bacterial clearance in the absence of T_{regs} (Figure 5.14) and no significant difference was seen in the levels of pro-inflammatory cytokines in the DT- versus PBS-treated infected mice (Figure 5.15). These data are in agreement with that of O. Carrigan and colleagues who found no difference in survival outcome between anti-CD25 (T_{reg} depleted) and control Ab-treated (T_{reg} replete) mice infected with *P. aeruginosa*. This was despite observing that lung infection with *P. aeruginosa* induced an increase in nT_{regs} in mouse spleen (O.Carrigan *et al*., 2008). It is reasonable to suggest that distinct types of invading pathogens require differing approaches to elimination, which necessitates mobilisation of discrete components of the microbicidal host response, which may be more or less reliant on a particular immune cell subset. Worthy of further investigation is the possibility that eradication of Gram-positive bacteria may be more reliant on T_{regs} than is the case for Gram-negative organisms.

6.6 BM-MSCs and their cross talk with T_{regs}.

A key advantage that cellular immunotherapy may have over antibiotic treatment or targeted inhibition of a specific pro-inflammatory mediator is the capacity to stimulate multiple pathways in a coordinated way (dos Santos *et al*., 2012). One of the pathways which may be contributory to MSC protective effects is the interaction of MSC with T_{regs}. MSC-mediated T_{reg} recruitment has been shown to impact positively on clinical outcome in pre-clinical and clinical studies of sepsis (Monneret *et al*., 2003; Heuer *et al*., 2005; Wisnoski *et al*., 2007; Okeke *et al*., 2013; Chao *et al*., 2014; Neill *et al*., 2014). Of interest, Chao and colleagues demonstrated that MSC treatment not only increased the percentage of circulating CD3+CD4+CD25+ T_{reg} cells and the ratio of T_{reg} cells/T cells in the peripheral blood

of septic rats, but also restored T_{reg} cell suppressive function. In contrast, the suppressive capacity of CD4+CD25+ T_{reg} cells from rats receiving PBS only after CLP was significantly decreased (Chao *et al*., 2014). The investigators propose that the observed reduction in levels of serum IL-6 and TNF-α occurred because of increased T_{reg} activity. Previous reports have suggested that T_{regs} can control production of pro-inflammatory cytokines during infection (Cambos *et al*., 2008; Gonzalez-Rey *et al*., 2009). Chapter 5 of this thesis explores MSC-T_{reg} interaction in mouse models of bacterial lung infection, specifically focusing on the impact of T_{reg} depletion on MSC protective effect.

Immunity shifted away from a Th1/Th17 towards a Th2 response.

In the T_{reg} replete mice infected with *S. pneumoniae*, the observed impact of MSC treatment on the relative expression of genes encoding transcription factors important for different subtypes of effector T cells raises some interesting questions about mechanism of MSC effect. A significant reduction in *t-bet* and *rorγt* was observed in MSC treated mice compared to PBS treated controls. In conjunction with this, an increase in expression levels of *gata-3* was noted. This might suggest that one mechanism by which MSCs exert their anti-inflammatory effects is by promotion of a Th-2 cell response in preference to the established Th1/Th17 response to lung infection with *S. pneumoniae*. MSCs have been shown to stimulate a shift in T cell response from T_H1 to T_H2 cell predominance as a way of ameliorating an overzealous inflmammatory response (Aggarwal and Pittenger, 2005, Bai *et al*., 2009). This trend in transcription factor expression was evident in the MSC treated Treg deplete mice, but to a lesser extent, suggesting a possible reduction in MSC impact in the absence of T_{regs} (Fig 5.13B).

It has previously been shown that IL-17 neutralisation can enhance bacterial clearance of serotype 3 *S. pneumoniae*, but not the more invasive serotype 4 *S. pneumoniae* (Ritchie *et al*. 2013). In the serotype 3 infection model utilised in this study it appears that suppression of IL-17F was indeed associated with MSC protective effects. Possible mechanisms of MSC-mediated IL-17F suppression include physical adherence to T_H17 cells inducing their transformation into T_{regs} via reciprocal modulation of RORγt and Foxp3 (Ghannam *et al.*,

2010) or inhibition of T_H17 differentiation via IL-10-mediated suppression of RORγt (Qua *et al*., 2012).

In *P. aeruginosa* infection model MSC-treated T_{reg} replete mice exhibited a significant reduction in the expression of transcription factor *rorγt,* in line with the reduction in levels of IL-17F seen post MSC treatment. Reduced serum levels of IL-17 associated with enhanced bacterial clearance and a reduction in lung inflammatory infiltrate post *P. aeruginosa* infection has previously been reported (Hsu *et al*., 2016). The observed reduction in *rorγt* expression was also evident in the T_{reg}-deficient cohort treated with MSC (Fig 5.16). This might suggest that one mechanism by which MSCs exert their anti-inflammatory effects is by decreasing the T_H17 cell response which is known to be active in lung infection with *P. aeruginosa.* An alternative explanation is if an entirely different mechanism of MSC activity was at work, for example inhibition of the inflammasome of host macrophages. This has been demonstrated to be one of the ways in which MSC can influence macrophage behaviour to increase phagocytic capacity and enhance bacterial clearance (Kepp *et al*., 2011; Oh *et al*., 2013, Cohen and Prince, 2013).

6.7 In Summary

Cultured mMSC have been demonstrated to home to mouse lung following infection with *S. pneumoniae* and *P. aeruginosa*, where they act to reduce lung bacterial burden. Demonstration that MSC treatment facilitates enhanced bacterial clearance from the lung is important because MSCs have previously been thought to have predominantly immunosuppressive effects, which could impair host defense to bacterial infection. Efforts to delineate immune correlates of this MSC-mediated protection have indicated paradoxical effects through reducing levels of inflammatory cytokines. It seems likely that a complex set of mechanisms is at play, whereby MSC-mediated anti-inflammatory activities are counterbalanced by direct antimicrobial effects and indirect enhancement of host cell phagocytosis (Mei *et al*., 2010; Krasnodembskaya *et al*., 2012; Alcayaga-Miranda *et al*., 2015; Sung *et al*., 2016; Sutton *et al.,* 2016). In this way, antimicrobial and immunomodulatory MSC effects are coordinated in eliminating invading pathogen and preserving host tissue integrity.

Little is currently known about the role of T_{regs} in MSC protective effect. MSCs enhance T_{reg} recruitment, but it is unknown whether T_{regs} are pivotal for MSC effects. In this study T_{regs} were shown to be recruited to the infected lung and MSC-mediated protection appears to be partially influenced by T_{reg} depletion for *Streptococcal pneumoniae* infection, but not for *Pseudomonas aeruginosa* infection. This pathogen specific difference in MSC interaction with T_{regs} is interesting and warrants further investigation. It illustrates how important it is to fully understand the multitude of ways in which MSCs can interact with the different immune cell subtypes, in any given context, if we are to safely and effectively utilise MSC treatment for bacterial infection caused by a variety of pathogens.

The ability of MSCs to sense microbial invasion, which may be more pathogen specific than has so far been appreciated, and interact with host cells to modify immune effector cell behaviour, may allow MSCs to respond in a biphasic manner to an encounter with pathogen (Pevsner-Fischer *et al*., 2007; Wang *et al*., 2009; Li *et al*., 2012). The first phase of MSC impact is focused on augmentation of antimicrobial response to reduce bacterial burden. The second phase is dominated by anti-inflammatory activities which limit collateral tissue damage, facilitate tissue repair and support dysfunctional immune cells, which may also curb bacterial growth (Meduri, 2002; dos Santos *et al*., 2002; Mei *et al*., 2010; Auletta *et al*., 2012; Le Burel *et al*., 2017). In response to infection with Gram Positive organisms this latter phase may involve the recruitment of T_{regs} which serve to re-establish tissue homeostasis post pathogen elimination (Wisnoski *et al*., 2007; Okeke *et al*., 2013). It is of paramount importance to the field that our understanding of MSC mechanistics continues to grow, if this immunotherapy is to be put to maximal use for treatment of the array of pathologies for which it may be applicable.

6.8 Future Work

This thesis has produced new insights into MSC protective effects, T_{reg} function and MSC-T_{reg} interaction during bacterial lung infection with Gram-positive and Gram-negative pathogens. The work carried out raised a number of interesting questions which should be pursued in the next phase of work. The overall aim would be to fully elucidate the

mechanisms of MSC protective effects critical to understanding MSC biology and would assist the pathway to clinical translation.

Further experiments could aim to characterise changes to the lung infiltrating population in the presence of MSC treatment. This could be achieved using multi-parameter flow cytometry analysis of lung tissue from infected mice, with or without MSC, and could include characterisation of potential anti-bacterial immune populations by utilising intracellular staining to investigate cytokine differences. In parallel with this, Affymetrix array transcriptomic studies could be used to investigate transcriptional changes in lung tissue in the presence or absence of MSC. It would be interesting to explore MSC-mediated changes in neutrophil behaviour in the infection models: degree of apoptosis, phagocytic activity and oxidative metabolism. In parallel with this, investigation of MSC-induced macrophage re-programming and the possibility of enhancement in macrophage phagocytic capabilities could be key to understanding MSC impact on bacterial clearance.

Further investigation of T_{reg} effects in these infection models could be achieved by sorting $CD4^{+}GFP^{+}$ T_{regs} to compare systemic (spleen) with lung T_{regs} in the presence and absence of infection, allowing RT-PCR comparison of transcripts. The aim would be to gain insights into apparent anti-bacterial effector functions of T_{regs}. This would in turn feed into the related studies on their role in MSC-induced protection. These experiments were attempted during my project, but due to the small numbers of T_{regs} acquired it was difficult to produce meaningful data from the RNA isolated. Initial attempts looking at transcriptomic differences in lung versus splenic T_{regs} from healthy animals exploring markers of migration and tissue residency (BIMP-1, HOBIT, S1pr1 and CCR4) were limited by the amount of recoverable RNA. An additional magnification step prior to transcriptomic analysis may be required. Also worthy of further investigation is the mechanism by which MSCs interact with T_{regs}. MSC-mediated T_{reg} induction has been demonstrated to involve both soluble and cell contact-dependent factors. However, little is understood about the contribution of each to MSC-mediated T_{reg} effects in the context of bacterial infection.

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