Effects of regulatory T cells on macrophage inflammatory responses to *Streptococcus pneumoniae*

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Declaration

I, Gabriella Sophie Szylar, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Streptococcus pneumoniae infection remains a major cause of morbidity and mortality worldwide. An effective inflammatory response is crucial for clearance of the pathogen, but excessive inflammation causes serious complications and host damage. Macrophages are the initiators of inflammation and exhibit plasticity in their responses ranging from highly pro-inflammatory to anti-inflammatory actions. Forkhead box p3 (Foxp3)-expressing Regulatory T (Treg) cells can dampen the inflammatory effects of various cell types.

Co-culture of monocyte-derived macrophages (MDM) with Treg cells prior to or during *S. pneumoniae* infection and measurement of tumour necrosis factor α (TNFα), interleukin (IL)-6 and IL-1β in the supernatant was used to identify anti-inflammatory effects of Treg cells on MDMs. Treg cells potently reduced MDM pro-inflammatory cytokine production when co-cultured prior to infection, in a manner requiring direct Treg-MDM cell contact. This anti-inflammatory effect did not occur upon infection with *Acinetobacter baumannii*, indicating a degree of pathogen-specificity. Treg cells also reduced MDM responses to *S. pneumoniae* when added to the MDMs during infection, but to a lesser extent than pre-infection co-culture. A human intradermal *S. pneumoniae* challenge model was used to examine T cell recruitment, and a potential Treg population was identified. Using normal human lung sections, $Foxp3^+$ and $Foxp3^-$ cells were identified by immunofluorescent (IF) staining, indicating the potential presence of lung-resident Treg cells.

Overall, the data demonstrate that Treg cells can reduce macrophage pro-inflammatory cytokine production to *S. pneumoniae*. Provisional data indicate that Treg cells may recruit in response to *S. pneumoniae* in a human model by 48 hours post-challenge, and $Foxp3^+$ cell are present in normal human lung.

Impact Statement

S. pneumoniae is a major cause of morbidity and mortality, responsible for approximately 1.3 million deaths per year and is the second commonest bacterial cause of death worldwide (Walker et al., 2010; Musher, 1992). Vaccination and antibiotic usage have improved prevention and treatment of pneumococcal disease, but the risks of serotype replacement and antibiotic resistance are a growing problem. Understanding of the immunological response of the human host to *S. pneumoniae* is of great importance in development of treatments. *S. pneumoniae* provokes a large pro-inflammatory response in pneumonia, which can cause complications arising from lung injury and transition of the bacteria from the lungs into the bloodstream and other tissues. Inflammation is required for clearance of *S. pneumoniae* infection but must be controlled, and the mechanisms involved in achieving this balance are poorly understood. As the orchestrators of the immune response to *S. pneumoniae* with the ability to switch between pro-inflammatory and anti-inflammatory activity, macrophages could be targeted for immunomodulative treatment to skew the immune response to clear infection and prevent host damage. The *in vitro* work in this thesis demonstrates that human Treg cells reduce macrophage production of pro-inflammatory cytokines in response to *S. pneumoniae* through Treg-macrophage cell contact. Effector T (Teff) cells were found to either boost or reduce macrophage cytokine responses to infection. Much of the current data on pneumococcal infection comes from mouse studies, and human data is valuable due to differences in immune cell biology. These data add to our understanding of how T cells may modulate the response of innate cells in infection, as the majority of published work focusses on how Treg cells suppress other T cell types. Further work to elucidate the specific mechanism(s) through which Treg and Teff cells affect macrophage inflammatory

responses could pave the way for immunomodulative treatments. Groups with a high risk of pneumococcal infection, i.e. the very young, the elderly, and immunocompromised individuals may benefit particularly from immunomodulative treatment. A novel human *S. pneumoniae* intradermal challenge model identified T cell subsets recruited to the injection site, including a large proportion expressing Treg-associated surface markers. The suppressive ability, cytokine production, macrophage interaction, and a more detailed examination of cell surface and intracellular markers could provide valuable information on the human Treg response to *S. pneumoniae in vivo* and could be achieved through further optimisation of the protocol used in this thesis. These data also provide information on the phenotypes of cells recruiting to infection, such as the memory/effector status of the cells, the proportions of cytotoxic and helper subsets, the expression of a tissue-homing receptor, and the cytokine milieu at the challenge site, which are important considerations in vaccine development and research into treatment. A potential lung-resident Treg population identified in normal human lung, coupled with the *in vitro* finding that Treg cells reduced macrophage responses most potently when these cell types interacted prior to infection, indicate the previously unexplored possibility that local Treg modulation of macrophages in the lung in the absence of disease may influence the response to subsequent infection.

The protocols developed in this work are highly useful for comparing effects of Treg/T cell subsets on macrophage inflammatory responses and recruitment to pneumococcal challenge, and could be used to identify differences in the immune response in at-risk groups. Comparison of Treg recruitment and function in those at risk of pneumococcal disease with healthy controls may reveal important differences in Treg cell number and/or function in response to *S. pneumoniae* challenge. The protocols may also be used to examine the effects of specific treatments on these cellular responses.

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Abbreviations

AAD	allergic airways disease
AP1	activator protein 1
AIM2	absent in melanoma 2
APC	antigen presenting cell
ARDS	acute respiratory distress syndrome
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
BALF	bronchoalveolar lavage fluid
bcl-xl	anti-apoptotic protein B-cell lymphoma extra large
Breg	regulatory B cell
cAMP	cyclic AMP
CARD	caspase activation and recruitment domain
CbpA	choline-binding protein
CCL	CC chemokine ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony-forming unit
ChoP	phosphorylcholine
CLA	cutaneous lymphocyte–associated antigen
COPD	chronic obstructive pulmonary disease
CRP	c-reactive protein
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CTLD	c-type lectin-like domains
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAMP	danger-associated molecular pattern
DC	dendritic cell
DC-SIGN	dendritic cell-specific Intercellular adhesion molecule-3-grabbing non-integrin
Dectin	DC-associated C-type lectin
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
eEF	eukaryotic elongation factor
FcR	Fc receptor
FCS	foetal calf serum
FOXO	Forkhead box O
Foxp3	Forkhead box p3
GARP	glycoprotein A repetitions predominant
GATA3	GATA binding protein 3
G-CSF	granulocyte-colony stimulating factor
GITR	glucocorticoid-induced TNF receptor
GM-CSF	granulocyte-macrophage colony stimulating factor
hBD	human β-defensin
HLA-DR	human leukocyte antigen–DR
HSP	heat shock protein

Ig	immunoglobulin
IF	immunofluorescence
IFNAR	interferon α/β receptor
IFNγ	interferon γ
IL	interleukin
ILC	innate lymphoid cell
IPD	invasive pneumococcal disease
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IκBα	inhibitor of kappa B
IKK	IκB kinase
iNKT	invariant NKT
iNOS	inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF3	interferon regulatory factor
ISPC	immunosuppressive plasmocytes
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
iTreg	induced Treg
JAK	Janus kinase
LAP	latency-associated peptide
LFA-1	leukocyte function-associated antigen-1
LPS	lipopolysaccharide
LRR	leucine-rich repeats
LTBP1	latent TGFβ binding protein 1
MAIT	mucosal associated invariant T
MAPK	mitogen-activated protein kinase
MARCO	macrophage receptor with collagenous structure
MBL	mannose binding lectin
Mcl	myeloid cell leukemia
MCP-1	monocyte chemotactic protein 1
M-CSF	macrophage colony stimulating factor
MDM	monocyte-derived macrophage
MDSC	myeloid-derived suppressor cells
MFI	median fluorescence intensity
MIF	macrophage migration inhibitory factor
MIP-2	macrophage inflammatory protein-2
MMP	matrix metalloproteinase
MR	mannose receptor
MRC-1	mannose receptor C type 1
MULE	Mcl-1 ubiquitin ligase
MyD88	myeloid differentiating factor 88
NADPH	nicotinamide adenine dinucleotide phosphate
NALT	nasal associated lymphoid tissue
NCR1	natural cytotoxicity receptor 1
NET	neutrophil extracellular traps
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	natural killer T cell

NLR	NOD-like receptor
NLRP3	NOD-like receptor family, pyrin domain-containing protein 3
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain
NOS2	nitric oxide synthase 2
nTreg	natural Treg
PAF	platelet activating factor
PAFR	platelet activating factor receptor
PAMP	pathogen-associated molecular pattern
PAR-1	proteinase-activated receptor 1
Pav	pneumococcal adhesion and virulence
PBMC	peripheral blood mononuclear cell
Pce	phosphorylcholine remodelling enzyme
PCV	pneumococcal conjugate vaccine
PD-1	programmed cell death protein 1
PDL-1	programmed death ligand 1
PgdA	enzymes peptidoglycan N-acetylglucosamine deacetylase
PGE2	Prostaglandin E2
PiaA	pneumococcal iron acquisition A
PIgR	polymeric immunoglobulin receptor
Ply	pneumolysin
PPSV	pneumococcal polysaccharide vaccine
PRR	pattern recognition receptor
Psp	pneumococcal surface antigen
pTreg	peripheral Treg
PYD	pyrin domain
RIG1	retinoic acid-inducible gene-1
RIPK1	receptor-interacting serine/threonine kinase
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROR	retinoic acid-related orphan receptors
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
Satb1	special AT-rich sequence-binding protein-1
SD	standard deviation
SIGNR1	specific ICAM-3 grabbing nonintegrin-related 1
SOCS	suppressor of cytokine signalling
SP	surfactant protein
SRA	scavenger receptor A
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
Syk	spleen tyrosine kinase
TAB	TGF-β-activated kinase
TAK	transforming growth factor beta-activated kinase
TANK	TRAF family member-associated NF-kappa-B activator
TBK	TANK-binding kinase
TBX21	T-Box 21
TCR	T cell receptor

Teff	effector T cell
TGFβ	transforming growth factor β
Th	T helper
TI-2	T cell-independent type II
TIGIT	Tim and ITIM domain
TIM3	T cell immunoglobulin 3
TIR	Toll/IL-1 receptor
TIRAP	TIR adaptor protein
TLR	toll-like receptor
TNFα	tumour necrosis factor α
TNFR	tumour necrosis factor receptor
TNFRSF	tumour necrosis factor receptor superfamily
Tr1	type 1 regulatory
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TIR domain-containing adapter molecule
Treg	regulatory T cell
TRIF	TIR domain-containing adaptor-inducing IFNβ
TSDR	Treg-cell-specific demethylation region
tTreg	thymic Treg
ZAP70	zeta-chain-associated protein kinase 70

Chapter 1: Introduction

1.1 *Streptococcus pneumoniae* microbiology

Streptococcus pneumoniae (pneumococcus) is a Gram-positive coccus that is a frequent coloniser of the human respiratory tract and is also one of the commonest causes of serious infections worldwide. *S. pneumoniae* measures approximately 0.5-1.25μm in size and the bacteria are often found in pairs. They are non-motile and do not produce spores. They lack catalase and ferment glucose to lactic acid. *S. pneumoniae* are cultured at 37°C and 5% CO_2 on blood agar to provide a source of catalase to neutralise hydrogen peroxide production. They are α-haemolytic, causing reduction of haemoglobin and resulting in green colonies seen when grown on blood agar. The *S. pneumoniae* intracellular material is contained within a cell membrane surrounded by a cell wall composed of peptidoglycan and decorated with choline and teichoic acid. Surrounding the cell wall is a polysaccharide capsule which varies in chemical composition and therefore determines the *S. pneumoniae* serotype, of which around 100 have been identified so far (Bentley et al., 2006).

1.1.2 Impact of pneumococcal disease

S. pneumoniae is a natural nasopharyngeal commensal of humans (Austrian, 1986). Carriage prevalence in children ranges from 27% to 85%, with higher carriage rates in children in low- and middle- income countries (O'Brien et al., 2003). Colonisation of adults is less common, affecting around 8% of adults in England (Hussain et al., 2005) and with even lower rates in the elderly (Ridda et al., 2010). Colonisation usually occurs without disease and induces B cell immunity (Musher et al., 1997). *S. pneumoniae* can, however, spread from this niche to cause disease including pneumonia, otitis media and sinusitis, and invasive pneumococcal disease (IPD)

including meningitis and septicaemia. *S. pneumoniae* is responsible for approximately 1.3 million deaths per year and is the second commonest bacterial cause of death worldwide (Walker et al., 2010; Musher, 1992). Pneumococcal disease is highest in young children, whose immune system is not fully developed, the elderly, who have a waning immune system, and those who have a compromised immune system. In 2015, 5% (294 000 out of 5.83 million) of deaths among children younger than 5 years old were estimated to be caused by pneumococcal infections (Wahl et al., 2018). *S. pneumoniae* is also the commonest bacterial cause of community acquired pneumonia in adults (Fine et al., 1996). In addition, increasing antibiotic resistance of bacteria makes prevention of *S. pneumoniae* infections increasingly important (Wellcome Trust and Boston Consulting Group Report, 2018)

1.1.3 Vaccination

Due to the impact of pneumococcal disease, effective vaccination is essential and two types of vaccine are in routine use. The pneumococcal polysaccharide vaccine (PPSV23, Pneumovax) vaccine protects against 23 serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F and 33F) of *S. pneumoniae* by inducing an antibody response against the polysaccharide capsule, increasing opsonophagocytosis of the bacteria. This, however, proved to be an ineffective strategy for immunising children, as the PPSV23 vaccine is made entirely of polysaccharide, and therefore has a poor ability to induce a T helper type 1 (Th1) response with immunological memory (Leinonen et al., 1986; de Roux et al., 2008). As children are the main reservoir of pneumococcal carriage and the group most affected by the disease, a more effective vaccine for this age group was required and the 7-valent pneumococcal conjugate vaccine (PCV7, Prevnar) was introduced in 2000. Prevnar contains capsular polysaccharide covalently linked to a carrier protein, and is therefore able to induce a T cell dependent immune response producing immunoglobulin G (IgG) and memory B cells even in infants. PCV7 protects against

seven capsular serotypes (4, 6B, 9V, 14, 18C, 19F and 23F), and is highly effective in preventing IPD in children (Black et al., 2000). Despite this success, the limited number of serotypes protected against by current vaccines allowed the serotypes not included in the vaccine to become more prevalent in the population. Historically, the PCV7 serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were the most commonly associated with IPD in the USA (Shetty, Maldonado, 2013). However, in 2010, the most common serotype causing IPD in children was in the USA was 19A, one third of which were antibiotic resistant (Kaplan et al., 2010). Another study in the same year identified that 1, 5, 6A, 6B, 14, 19F and 23F were the most common causes of IPD in children globally (Johnson et al., 2010). To this end, new PCVs with greater valency have been introduced that protect against 10 or 13 capsular serotypes. Despite routine use of PCV vaccines, replacement disease is still occurring and research into producing a vaccine that offers protection against all pneumococcal strains for both adults and children continues (Ladhani et al., 2018).

1.1.4 *S. pneumoniae* virulence and interactions with the host

S. pneumoniae have a range of virulence factors to help enable their survival within humans. These aid in colonisation, adherence, translocation, tissue damage and host immune evasion. A selective and non-exhaustive list of virulence factors are summarised in table 1.1.4 (Ferreira and Gordon, 2015). Two of the major virulence factors are the polysaccharide capsule and the pore-forming toxin pneumolysin. A diagram showing the basic structure, some of the major virulence factors and epithelial adhesion components is provided in figure 1.1.4.

Virulence factor	Function
Capsule	Resist opsonophagocytosis by inhibition of complement Reduces trapping by neutrophil extracellular traps (NETs) Prevents mucus binding
Pneumococcal surface antigen A (PspA)	Reduces C3b deposition by blocking formation of C3
Pneumococcal surface antigen C (PspC)	Reduces C3b deposition by binding factor H
Phosphorylcholine (ChoP)	Binds PAFR on epithelial cells Initiates invasion through binding human polymeric immunoglobulin receptor (pIgR)
IgA protease	Cleaves Immunoglobulin A (IgA), reducing opsonophagocytosis and exposes ChoP
Neuraminidase (NanA)	Promotes adherence and resistance to complement deposition
Hyaluronate lyase	Degrades hyaluronan in extracellular matrix, promoting invasion
Pili	Increases adherence and invasion
Pneumococcal adhesion and virulence A (PavA)	Binds fibronectin, facilitating adhesion
Pneumococcal adhesion and virulence B (PavB)	Facilitates colonisation and lung transmigration
Pneumolysin	Cytolysis Complement activation Induction of host inflammatory response Inhibition of ciliary beating on epithelium
Autolysin (LytA)	Induces autolysis, releasing inflammatory bacterial components
Lipoteichoic acid	Induces pro-inflammatory response via TLR2 (and probably TLR4) and PAFR binding
Pneumococcal surface antigen A (PsaA)	Resistance to oxidative stress
Pneumococcal iron acquisition A (PiaA) and iron uptake A (PiuA)	Iron acquisition for bacterial growth
Endonuclease	Degrades neutrophil NETS

Table 1.1.4 Major virulence factors of *S. pneumoniae* and their functions Modified from Ferreira and Gordon, 2015

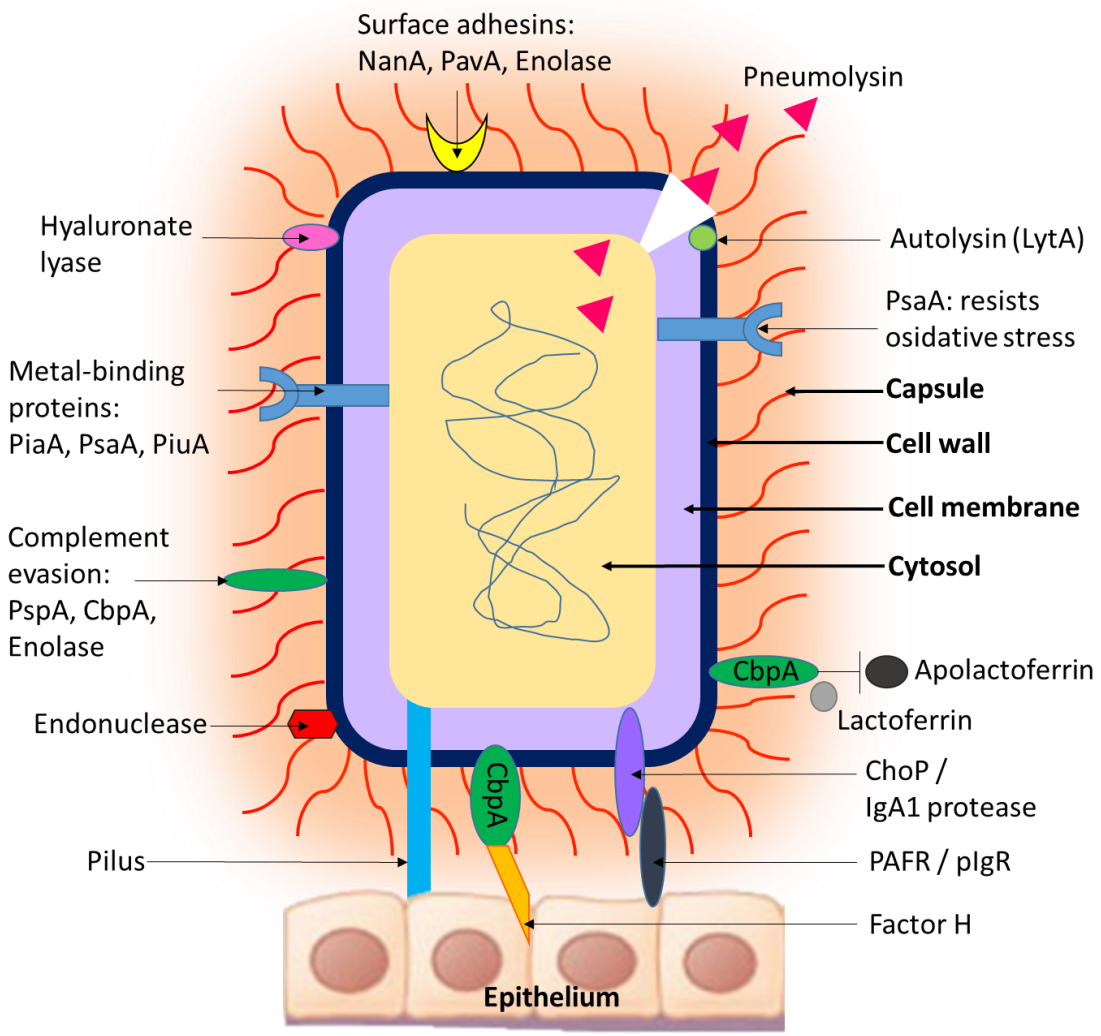

Figure 1.1.4 Structure, virulence factors and epithelial binding components of *Streptococcus pneumoniae*

A diagram representing the basic structure and some of the major virulence factors and epithelial binding components of *S. pneumoniae*. The *S. pneumoniae* intracellular material is contained within a cell membrane surrounded by a cell wall covered by the polysaccharide capsule. Virulence factors help enable bacterial survival within the host and include pneumolysin, the polysaccharide capsule, complement evasion factors (such as PspA, and enolase), metal-binding proteins (such as PiaA, PsaA, and PiuA), surface adhesins (such as NanA, PavA, enolase), hyaluronate lyase, autolysin and endonuclease. Bacterial components such as the pilus, choline-binding protein A (CbpA), and phosphorylcholine (ChoP) facilitate adhesion to the host epithelium.

1.1.5 Capsule

The capsule surrounding *S. pneumoniae* is composed of flexible polysaccharide chains with no stabilisation of the secondary structure which in most strains is covalently bonded to the cell wall (Rutherford et al., 1994). The capsule thickness is variable between strains but is approximately 200nm-400nm (Skov Sorensen et al., 1988; Hyams et al., 2010). The capsule protects the bacteria against entrapment by mucus in colonisation (Nelson et al., 2007). *S. pneumoniae* are known to increase or decrease capsule expression (referred to as opaque and transparent phenotypes, respectively) which aids their survival depending on their location in the host, with transparent variants believed to be better adapted for nasopharyngeal colonisation and opaque variants better adapted for invasive disease (Weiser et al., 1994; Cundell et al., 1995; Kim and Weiser., 1998; Weiser et al., 2001; Briles et al., 2005; Arai et al., 2011). The capsule inhibits IgG, C-reactive protein (CRP), and complement deposition, thereby inhibiting opsonophagocytosis by neutrophils (Hyams et al., 2010). The capsule can also inhibit clearance by alveolar macrophages early in lung infection (Camberlein et al., 2015). However, in some cases the capsule can partly contribute to increased recognition of *S. pneumoniae* by macrophages through dendritic cell-specific Intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), increasing uptake (Koppel et al., 2005). The capsule is either negatively charged or has no net charge, which reduces mucus binding (Nelson et al., 2007). A correlation between negative charge and resistance to neutrophil killing and increased carriage has been reported (Li et al., 2013). Capsular polysaccharide does not appear to be greatly inflammatory. Purified polysaccharide required much higher concentrations than that of heat-killed *S. pneumoniae* in order to release pro-inflammatory cytokines in whole blood (Jagger et al., 2002; Periselneris et al., 2019). In addition, a macrophage cell line (RAW) took longer to release TNFα when exposed to capsular polysaccharide compared to heat-killed *S. pneumoniae* (Simpson et al.,

1994). Unencapsulated strains cause less release of C-X-C motif chemokine ligand (CXCL)-8 from epithelial and monocyte cell lines compared with their isogenic wild-type (Marriott et al., 2012). CXCL8 attracts neutrophils through their chemokine receptors C-X-C motif chemokine receptor (CXCR)-1 and CXCR2.

Pneumococci can undergo capsular switching, whereby an isolate of the bacteria can replace its capsule with that of another serotype through homologous recombination, allowing it to then evade antibodies against its previous capsule type, which further complicates vaccination strategies (Coffey et al., 1991; Brueggemann et al., 2007). Co-colonisation also provides the potential for capsular switching by horizontal gene transfer (Brugger et al., 2010).

1.1.6 Pneumolysin

Pneumolysin (Ply) is a 53 kDa toxin produced by all clinical isolates of *S. pneumoniae* (Kanclerski and Möllby, 1987). It belongs to the cholesterol-dependent cytolysin family of thiol-activated cytolysins produced by some Gram-positive bacteria. Binding to cholesterol is followed by oligomerization and formation of membrane pores. Ply is required for *S. pneumoniae* transmission (Zafar et al., 2017). Ply is highly conserved amongst *S. pneumoniae* serotypes, which has made it an attractive antigen target for vaccination, and mouse studies have found that immunising with the toxin resulted in protection against intranasal challenge with virulent *S. pneumoniae* (Paton et al., 1983). Early studies reported that Ply was located in the cytoplasm of the pneumococcus and is released during bacterial lysis; however, Ply can also localise to the cell wall (Johnson, 1977; Price et al., 2012). The protection elicited by Ply immunisation is presumed to be antibody neutralisation of the free toxin and there is no evidence for a role in opsonophagocytosis. Therefore, Ply immunisation disrupts the kinetics of infection rather than promoting clearance of the bacteria (reviewed by Ogunniyi and Paton, 2015). Ply is released in large amounts upon exposure to antibiotics.

Ply has multiple functional effects, including pore formation in host cell membranes, resulting in lysis. Ply assembles into rings on cholesterol-containing membranes of host cells, a barrel-shaped transmembrane β-sheet forms from β-hairpins which perforates the membrane, causing cytolysis (Tilley et al., 2005). Alternatively, Ply can cause apoptosis through intracellular calcium increase (Braun et al., 2002). The concentration of Ply appears to influence its action on the host cells, with high concentrations resulting in cell lysis.

Sublytic doses of Ply have been found to reduce epithelial barrier integrity (Los et al., 2013). Exposure of Ply to epithelial cells in culture results in activation of p38 mitogen-activated protein kinase (MAPK), increasing the chemokines that attract neutrophils (Ratner et al., 2006). Exposure of A549 cells with *S. pneumoniae*, but not Ply-deficient mutant *S. pneumoniae*, induces upregulation of human β-defensin (hBD)-2 (Kim et al., 2013). It also inhibits the respiratory burst, bactericidal activity and migration of neutrophils, and induces neutrophil prostaglandin and leukotriene production (Paton and Ferrante, 1983; Cockeran et al., 2001). Sublytic concentrations of Ply activates the classical complement pathway (Paton et al., 1984; Rubins et al., 1995; Yuste et al., 2005; Mitchell et al., 1991).

Ply can both promote and inhibit the inflammatory response (Kadioglu et al., 2008). Purified Ply stimulates TNFα and IL-1β production by monocytes (Houldsworth et al., 1994; Periselneris et al., 2019). Ply also decreases the bactericidal activity of monocytes and inhibits respiratory burst, degranulation and phospholipid methylation (Nandoskar et al., 1986). Ply induces nitric oxide (NO), TNFα and IL-6 production by macrophages (Braun et al., 1999; Malley et al., 2003) and contributes to macrophage programmed cell death, a process which is important in bacterial clearance and resolution (Dockrell et al., 2001; Dockrell et al., 2003; Marriott et al., 2004). Ply can also activate dendritic cell (DC) and macrophage inflammasomes such as the nucleotide-binding oligomerisation domain (NOD)-like receptor family, pyrin domain-

containing protein 3 (NLRP3), and absent in melanoma 2 (AIM2), to produce IL-1β (McNeela et al., 2010). Ply can promote also IL-10 secretion, although the overall cytokine profile suggests that it is highly pro-inflammatory (McNeela et al., 2010). Ply can also promote active transforming growth factor β (TGFβ) secretion by human nasopharyngeal epithelial cells and fibroblasts (Neill et al., 2014).

Studies have suggested that Ply recognition is by toll-like receptor 4 (TLR4). In a study comparing wild-type mice to TLR4-deficient mice, the macrophages of the wild-type mice were more prone to Ply-induced apoptosis (Srivastava et al., 2005). The same study also found that epithelial cells expressing TLR4 were more likely to undergo apoptosis than cells expressing TLR2 when exposed to pneumolysin. Adding TLR4 antagonists reduced apoptosis. TLR4 but not TLR2 bound Ply-coated ELISA plates. Additionally, Srivastava and colleagues also showed that *S. pneumoniae* elicited more upper respiratory tract cell apoptosis in wild-type mice than in TLR4-defective mice during pneumococcal infection. It has also been suggested that the ability of macrophages to produce cytokines in response to Ply is myeloid differentiating factor 88 (MyD88)- and TLR4-dependent (Malley et al., 2003). However, McNeela and colleagues found that pro-inflammatory cytokine production by DCs and macrophages in response to Ply is TLR4-independent (McNeela et al., 2010). Instead, activation of NLRP3 by Ply and resulting IL-1β secretion promoted IL-17A and interferon-γ (IFNγ) production which induce protective immunity against *S. pneumoniae*. Caspase-1 inhibition and non-haemolytic Ply both reduced IL-1β secretion. Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), an adaptor protein for inflammasome receptors such NLRP3 and AIM2, is required for the induction of caspase-1 activation in macrophages infected with *S. pneumoniae* (Fang et al., 2011). Epithelial cell activation of p38 MAPK and T cell activation of nuclear factor of activated T cells (NFAT) by Ply have been reported as TLR4-independent (Ratner et al., 2006; Koga et al., 2008). Another study found

that multiple TLRs were involved in Ply-induced responses (Dessing et al., 2009). Therefore, the complete role of TLR4 in Ply recognition remains contentious. The pore-forming effect of pneumolysin also allows entry of peptidoglycan in the phagosome into the phagocyte cytosol where it can be recognised by NOD2 (Davis et al., 2011), leading to recruitment of further monocytes/macrophages. Overall, Ply has roles in TLR and NOD2 activation which induces nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) to produce cytokines, and activation of the NLRP3 and AIM2 inflammasomes resulting in IL-1β activation.

In mouse studies demonstrating the role of Ply in *S. pneumoniae* pathogenicity, infection with Ply deficient (Δ*ply*) strains resulted in longer survival, decreased bacteraemia, and decreased lung damage (Berry et al., 1989; Canvin et al., 1995; Rubins et al., 1995). Absence of Ply during *S. pneumoniae* infection in mice resulted in lower numbers of pneumococci in the nasopharynx, trachea and lungs (Kadioglu et al., 2002). Intranasal inoculation of Ply caused raised pro-inflammatory cytokines in bronchoalveolar lavage fluid (BALF) and CXCL2-mediated leukocyte trafficking (Rijneveld, van den Dobbelsteen et al., 2002). Intravenous Δ*ply S. pneumoniae* resulted in decreased systemic IL-6 than wild-type *S. pneumoniae*, and Δ*ply* caused chronic bacteraemia as opposed to acute sepsis (Benton et al., 1998). More recently, a study by Subramanian and colleagues showed that Ply inhibits human DC and mouse alveolar macrophage pro-inflammatory responses through binding to the mannose receptor C type 1 (MRC-1) and activation of suppressor of cytokine signalling (SOCS)-1 (Subramanian et al., 2019). In addition, through interaction with MRC-1, Ply polarised naïve T cells into IFNγ^{low}, IL-4^{high} and Foxp3^{+} cells. Human DCs co-cultured with naïve cluster of differentiation (CD)-4 positive T cells and infected with Ply-expressing (but not Ply-deficient) *S. pneumoniae* induced higher Foxp3 expression, which was abolished upon MRC-1 knockout. Similarly, mouse bone marrow-derived macrophages also increased CD4^{+} T cell Foxp3 and IL-10

expression upon Ply-expressing *S. pneumoniae* infection. Reduced pro-inflammatory cytokine production and neutrophil recruitment were observed upon infection of mice with Ply-expressing *S. pneumoniae* compared to the Ply-deficient mutant, and MRC-1 deficient mice had reduced bacterial loads compared to wild-type mice. Overall, this study demonstrates that Ply-MRC-1 interactions downregulate inflammation and enhance bacterial survival in the airways.

T cells recruit rapidly to the site of pneumococcal invasion early in infection, and the complement-activating activity of pneumolysin, and to a lesser extent its pore-forming activity, contributes to the recruitment of T cells (Kadioglu et al., 2000; Jounblat et al., 2003). Ply also directly activates T cells (Kadioglu et al., 2004). Ply also inhibits lymphocyte proliferation and IgA, IgG and IgM synthesis when pneumococci were added to lymphocytes at the same time as activation (Ferrante et al., 1984).

Some *S. pneumoniae* strains produce non-haemolytic Ply, e.g. 50% of serotype 1 clinical isolates in Scotland have a mutation in the pore-forming region of the pneumolysin gene, preventing haemolytic ability (Kirkham et al., 2006). Non-cytotoxic *S. pneumoniae* cause decreased lung oedema, leukocyte recruitment and mortality in mice, but these bacteria survived better in the blood compared to wild-type or Δ*ply* strains (Harvey et al., 2014; Harvey et al., 2011; Maus et al., 2004, Rijneveld and van den Dobbelsteen, 2002).

1.1.7 *S. pneumoniae* interactions with respiratory tract mucus layer

The ability of *S. pneumoniae* to colonise such a large proportion of the population demonstrates its success to be able to spread and persist in its human hosts. Despite its ability to cause disease, the majority of colonisation occurs asymptomatically, suggesting that this is the evolutionarily advantageous state for transmission. The nasopharynx is the location of pneumococcal colonisation, and adhesion to the nasopharyngeal epithelium is therefore necessary for establishing colonisation. The

nasopharynx is covered with ciliated pseudostratified columnar epithelial cells, coated in a thick mucus layer produced by goblet cells. The mucus presents an initial obstacle to pneumococcal colonisation. Mucus contains mucins coated in negatively-charged sialic acid which bind positively charged particles such as many bacteria, facilitating their removal by mucociliary clearance. However, one common characteristic of all pneumococcal capsule serotypes is that none are positively charged, inhibiting mucus binding (Nelson et al., 2007). *S. pneumoniae* also have the ability to cleave sialic acid residues through expression of exoglycosidases such as neuraminidase (NanA), further preventing binding to the epithelium (King et al., 2005). The mucus layer also contains anti-microbial compounds including lysozyme which can degrade the pneumococcal cell wall. The pneumococcus can defend against lysozyme through alteration of peptidoglycan structure by the enzymes peptidoglycan N-acetylglucosamine deacetylase (PgdA) and O-acetyltransferase (Vollmer and Tomasz, 2000; Crisóstomo et al., 2006). The mucus layer also contains lactoferrin, another antimicrobial glycoprotein which can take up iron that is required for bacterial growth. Pneumococcus can bind lactoferrin using pneumococcal surface protein A (PspA), reducing its antimicrobial ability (Shaper et al, 2004). IgA is induced in response to pneumococcal colonisation, however pneumococci can produce IgA protease which cleaves IgA (Kilian et al., 1979). In a murine study, immunisation with human IgA1 prevented colonisation with an IgA protease-deficient mutant *S. pneumoniae*, and protease-resistant human IgA1 could block colonisation of this mutant and also the wild-type (Roche et al., 2015). In the upper airways, *S. pneumoniae* phosphorylcholine (ChoP) remodelling enzyme (Pce) has been demonstrated to impair platelet activating factor (PAF) in the host and thereby deprive neutrophils of signals necessary for activation (Hergott et al., 2015). Other nasopharyngeal bacterial commensals that are part of the microbiome of the nasopharynx could play a role in the success of pneumococcal colonisation; for

example co-administration of *S. pneumoniae* and *Haemophilus influenzae* to mice resulted in reduced colonisation by *S. pneumoniae* (Lysenko et al., 2005).

1.1.8 *S. pneumoniae* epithelial adhesion

If *S. pneumoniae* can overcome the mucous barrier it can bind to the epithelium through a variety of adhesins. The capsule is down-regulated in order to expose adhesins, such as choline-binding protein (CbpA) which binds the secretory component of the polymeric immunoglobulin receptor (pIgR), ChoP which binds to platelet-activating factor receptor (PAFR), and pneumococcal adherence and virulence protein A and B (PavA and B) and enolase which bind to fibronectin and plasminogen in the extracellular matrix (Rosenow et al., 1997; Zhang et al., 2000; Cundell et al., 1995; Holmes et al., 2001; Bergmann et al., 2001; Jensch et al., 2010). Once attached to the epithelium *S. pneumoniae* replicate locally to form biofilms or can invade through the epithelial barrier by transcytosis or by passing through the tight junctions between cells (Asmat et al., 2014; Clarke et al., 2011; Weight et al., 2019). For example, pneumococci can be endocytosed by epithelial cells through PspC-pIgR interaction then are either killed in the phagolysosome or undergo transcytosis (Asmat et al., 2014). Tight junction components claudin 7 and claudin 10 are downregulated by TLR stimulation resulting in upregulation of Zinc finger protein SNAI1 (SNAIL1), a repressor of the transcription of tight junction proteins, resulting in loss of barrier integrity and allowing pneumococci between epithelial cells (Clarke et al., 2011).

1.2 Overview of the roles of different components of the immune system during *S. pneumoniae* infection

S. pneumoniae has complex interactions with multiple overlapping components of the innate and adaptive immune system. The generally accepted overview of these interactions during the development of *S. pneumoniae* pneumonia is that resident alveolar macrophages respond to the bacteria by production of cytokines and chemokines. These cause vasodilation resulting in increased blood volume and reduced blood velocity at the affected site. Cytokines activate the epithelium and augment the production of chemokines to attract immune cells to the site of infection. These effects improve access of inflammatory cells to the activated endothelium expressing cell adhesion molecules and migration into the tissue aided by increased vascular permeability. Recruited white cells are initially largely neutrophils, followed by monocytes differentiating into macrophages or DCs, and later B and T lymphocytes. Innate lymphoid cells and unconventional T cells assist this early inflammatory response to infection. Systemically, the acute phase response increases the availability of opsonins, chemoattractant molecules, and bactericidal pore-forming complexes as well as contributing to nutritional immunity by reducing availability of nutrients such as iron that are required for bacterial replication. Due to repeated nasopharyngeal colonisation events, almost all humans have an adaptive immune response to *S. pneumoniae* that can alter the progression of infection. The dominant components of this naturally acquired immunity to *S. pneumoniae* seems to vary between anatomical sites, with the evidence suggesting that protection against systemic infection requires antibody responses, clearance of against mucosal infection depends on a T cell response, and protection against pneumonia requires a combination of both humoral and cellular immunity (Ramos-Sevillano et al., 2019). A summary of the immune response to *S. pneumoniae* infection is provided in figure 1.2 and is explained in more detail in this chapter.

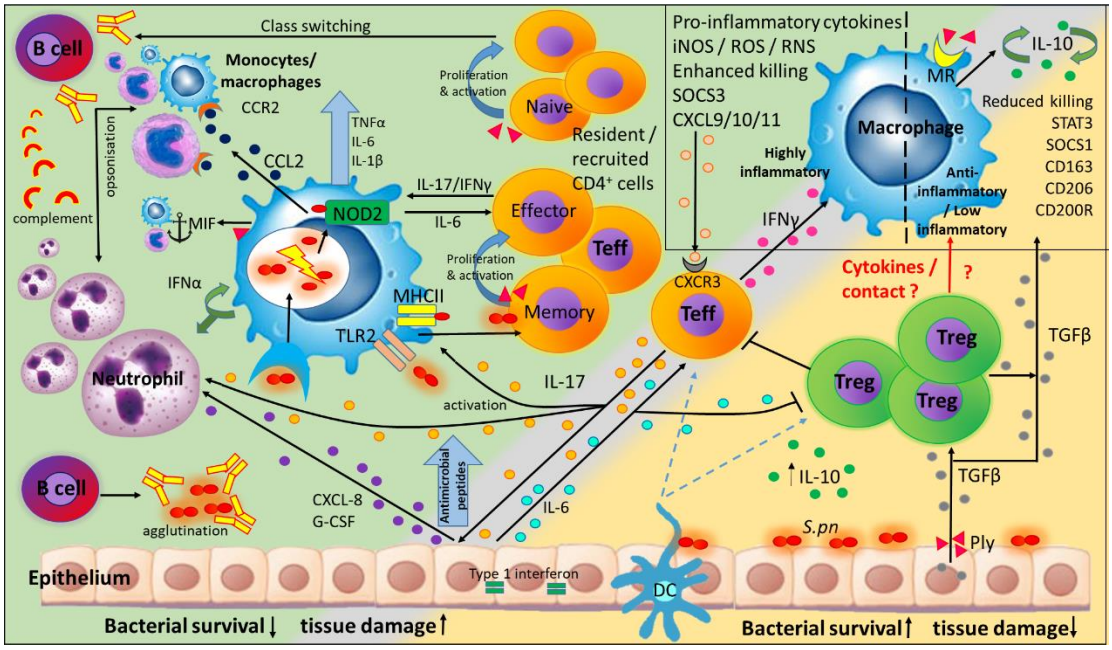

Figure 1.2 The immune response to *S. pneumoniae* infection

Resident macrophages detect *S. pneumoniae* through surface pattern recognition receptors (PRRs) and intracellular PRRs such as NOD2. Macrophage produce pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β, aiding recruitment, differentiation and activation of other cell types. DCs promote T cell responses. Macrophages present antigen on MHCII molecules to $CD4^+$ T cells. $CD4^+$ T cells may be resident memory cells and thus capable of expanding and initiating cytokine production early in response to Ply or other pneumococcal components. IL-17 stimulates epithelial cells to produce neutrophil-attractant chemokines and antimicrobial peptides. IL-17 and IFNα also aid neutrophil and macrophage function. $CD4^+$ T cell numbers increase through expansion or recruitment, for example macrophages produce the chemokines CXCL9, CXCL10 and CXCL11 which attract T cells expressing CXCR3. T cells producing IFNγ skew macrophages into adopting highly inflammatory phenotypes. Macrophages produce chemokines such as chemokine ligand (CCL)-2 which attracts further monocytes/macrophages expressing C-C chemokine receptor (CCR)-2, and macrophage migration inhibitory factor (MIF) which retains the recruited monocytes/macrophages at the site of inflammation. Bacterial uptake is enhanced by antibody and complement opsonisation. Epithelial cells produce TGFβ in response to Ply, promoting Treg development and less inflammatory macrophage phenotypes. Type 1 interferons help preserve the epithelial barrier. While a highly inflammatory environment decreases bacterial survival, in increases tissue damage.

1.2.1 Alveolar macrophages

Macrophages can be resident and specialised in tissue, or mature from monocytes in the blood during migration to tissue. Tissue-resident macrophages are usually the first cells to encounter pathogen, subsequently being aided by neutrophils recruiting to the site of infection soon after. Alveolar macrophages are the tissue resident macrophages of the lung characterised by auto-fluorescence, low major histocompatibility complex (MHC)-II expression, CD11c positive expression and lack of CD11b ($C11c^{+}CD11b^{-}$) expression. In response to infection they generate inflammatory responses, phagocytose and kill invading microbes, and present antigen to stimulate adaptive immunity. They are derived from foetal monocytes, populate the airways in the first few days after birth, and self-renew throughout life to maintain their population (Guilliams et al., 2013; Hashimoto et al., 2013; Yona et al., 2013). They are the first cell type to encounter antigen in the airways and are efficient phagocytes that can rapidly clear bacteria (Green and Kass, 1964). Alveolar macrophages have pattern recognition receptors (PRR) including TLRs and NOD-like receptors (NLRs). Macrophages can phagocytose without the help of opsonins via molecules such as mannose receptor (MR, CD206), specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1), macrophage receptor with collagenous structure (MARCO) and scavenger receptor A (SRA) (Macedo-Ramos et al., 2011; Koppel et al., 2005; Arredouani et al., 2006; Dorrington et al., 2013). Recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) triggers the alveolar macrophage inflammatory response. Alveolar macrophages have secondary lysosomes containing the enzymes used to degrade ingested material, which fuse with endocytic vacuoles (Kradin et al., 1986). Unlike neutrophils, alveolar macrophages cannot produce reactive oxygen species (ROS) via myeloperoxidase; however, they can produce ROS via their mitochondria, which recruit to phagosomes in response to TLR1, TLR2 and TLR4 engagement (West et al., 2011). However, ROS is not thought to be required for *S. pneumoniae* killing due

to bacterial adaptations to withstand oxidative stress (Marriott et al. 2007; Biggar et al., 1976). Alveolar macrophages also produce hydrogen peroxide through nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase; however, *S. pneumoniae* are also resistant to this method of killing (Cohen and Cline, 1971; Hampton et al., 1998; Pericone et al., 2003). Despite resistance to ROS killing, ROS also contributes to the production of reactive nitrogen species (RNS). Ply and the *S. pneumoniae* cell wall stimulate production of NO via nitric oxide synthase 2 (-12), and mice lacking NOS2 are less able to clear *S. pneumoniae* (Marriott et al., 2007; Kerr et al., 2004). The effectiveness of NO or RNS at killing *S. pneumoniae* are limited, as pneumococcal components such as PspC reduce NO production (Peppoloni et al., 2006). The protease lysozyme can also be activated in lysosomes to degrade ingested material, including bacterial cell wall peptidoglycan (Ip et al, 2010).

In a non-infected state, alveolar macrophages ingest pathogen-associated material, maintain non-inflammatory steady state and produce TGFβ (Yu et al., 2017). This is partly due to CD200 on the epithelium binding its receptor, CD200R, on macrophages to suppress pro-inflammatory signalling responses. This is enabled by epithelium-derived TGFβ and IL-10 inducing high levels of CD200R expression (Goulding et al., 2011). Alveolar macrophages can suppress functions of T cells and DCs (Holt et al., 1993; Blumenthal et al., 2001). Alveolar macrophage-derived TGFβ and antigen presentation also induces the generation of $CD4^+$ Treg cells from naïve $CD4^+$ cells, which occurs in the lung and not in the draining lymph node (Soroosh et al., 2013). The importance of alveolar macrophages in the lung innate immune response to *S. pneumoniae* has been demonstrated in multiple studies (Sun and Metzger, 2011; Sun et al., 2011; Clement et al., 2008; Dockrell et al., 2003; Phipps et al., 2010; Camberlein et al., 2015). Mice depleted of alveolar macrophages or have alveolar macrophages with reduced intracellular killing capacity are more susceptible to pneumococcal pneumonia (Dockrell et al., 2003; Marriott et al., 2005; Marriott et al.,

2007; Bewley et al., 2011; Camberlein et al., 2015). Alveolar macrophages can ingest a small number of *S. pneumoniae* without producing an inflammatory response (Dockrell et al., 2003). Alveolar macrophage phagocytosis of *S. pneumoniae* is enhanced by antibody and complement opsonisation facilitating binding to the Fc receptor (FcR) and complement receptors, respectively (Gordon et al., 2000). Antibody opsonisation is mainly by capsular polysaccharide-specific IgG. When the inflammatory response is initiated, phagocytosis decreases and production of inflammatory cytokines begins, which provokes neutrophil recruitment (Sharif et al., 2013). Pro-inflammatory cytokines, in particular TNFα, IL-1β and IL-6, as well as chemokines such as the neutrophil-attractant CXCL8, are produced (Tomlinson et al., 2014). If the alveolar macrophage response is overwhelmed, neutrophils become critical for the clearance of invading *S. pneumoniae* in the lung.

Alveolar macrophages have been demonstrated to clear *S. pneumoniae* from the lungs early in infection by phagocytosis and then by apoptosis later in infection (Arredouani et al., 2004; Sun and Metzger, 2008; Phipps et al., 2010; Sun et al., 2011). Alveolar macrophages are also important in resolution of *S. pneumoniae* pneumonia (Knapp et al., 2003; Cox et al., 1995). In the study by Knapp et al., alveolar macrophage-depleted mice had an increased pro-inflammatory response to *S. pneumoniae* in the lung, an elevated and prolonged neutrophil infiltration, and increased mortality, suggesting an important role for alveolar macrophages in modulating the immune response in pneumonia. Efferocytosis of apoptotic cells helps reduce the pro-inflammatory response (Dockrell et al., 2001; Marriott et al., 2006). A detailed discussion of the receptors and molecular pathways that are important for macrophage function is presented in section 1.3.

1.2.2 Epithelium

The epithelium provides a mechanical barrier against infection and contributes to the immune response to microorganisms. Epithelial cells have PRRs that can detect *S. pneumoniae* and contribute to the inflammatory response (Armstrong et al., 2004; Platz et al., 2004; Greene et al., 2005). The presence of *S. pneumoniae* can activate the epithelium to produce cytokines, chemokines and antimicrobial factors. For example, TLR2 expression by primary human airway epithelial cells is increased upon exposure to TNFα and IFNγ (Winder et al., 2009). Human airway epithelial cells in culture release the neutrophil-attractant CXCL8 in response to *S. pneumoniae* (Sorrentino et al., 2008). Ply has been found to be the major factor resulting in CXCL8 production in the human nasopharyngeal cell line Detroit 562 (Baumgartner et al., 2016). Although epithelial cells can produce modest amounts of CXCL8 in response to *S. pneumoniae*, CXCL8 production is increased *in vitro* when alveolar epithelial A549 cells are co-cultured with macrophages or macrophage supernatants, and in particular macrophage IL-1β was required for maximal CXCL8 production by the epithelial cells (Marriott et al., 2012). Epithelial cells can produce cytokines such as IL-6, IL-1β, granulocyte-macrophage colony stimulating factor (GM-CSF) and TGFβ (Gomez and Prince, 2008). Alveolar macrophage-derived TNFα and IL-1β have been demonstrated to induce CC chemokine ligand (CCL)-2 in A549 cells, which recruits monocytes expressing C-C chemokine receptor (CCR)-2 (Standiford et al.,1991). Upon detection of *S. pneumoniae* endopeptidase O, bronchial epithelial cells produce CXCL10, a chemoattractant for $CD4^+$ T cell and natural killer (NK) cells (Zou et al., 2017). CXCL10 also has direct antimicrobial activity against *S. pneumoniae* (Bruce et al., 2017).

Respiratory epithelial cells secrete antimicrobial factors such as hBD-1 constitutively, and express hBD2 and hBD3 upon *S. pneumoniae* infection induced by TLR2 recognition by the epithelial cell (Lee et al., 2004; Scharf et al., 2012). hBDs restrict

bacterial growth and induce lysis and can also modulate cytokine and chemokine responses (Wetering et al., 1997; Hiemstra, 2007). Epithelial cells also produce surfactant protein (SP)-A, which binds to *S. pneumoniae* and functions as an opsonin to increase phagocytosis (Sano et al., 2006).

1.2.3 Neutrophils

Neutrophils are short-lived cells that are present in the blood and bone marrow. They are the first recruited cell type to the site of pneumonia or other infections by chemoattractants and are phagocytic, produce ROS, and can degranulate and form extracellular traps (Kolaczkowska and Kubes, 2013). The main role of neutrophils in pneumococcal pneumonia is phagocytosis and subsequent intracellular killing. Neutrophil phagocytosis is assisted by complement or antibody opsonisation, or through opsonin-independent mechanisms (Hyams et al., 2010). Granules produced by neutrophils contain microbicidal molecules such as serine proteases, defensins and lactoferrin. Supernatants from degranulated human neutrophils can kill *S. pneumoniae* and intracellular killing following complement mediated opsonophagocytosis is mediated by serine proteases (Standish and Weiser, 2009). In the study by Standish and Weiser, *S. pneumoniae* killing was not affected by blocking the neutrophil respiratory burst, suggesting that this ROS is not an effective method of clearance used by the neutrophil. This is further highlighted by the finding that patients with chronic granulomatous disease, in which production of ROS is inhibited, do not have increased incidence of *S. pneumoniae* infection, and neutrophils from these patients can efficiently kill *S. pneumoniae* (Kaplan et al., 1968). In contrast, patients with Chedaki-Higashi syndrome, in which reduced levels of microbicidals are produced, have neutrophils with reduced capability to kill *S. pneumoniae* (Ganz et al., 1988; Root et al., 1972). Activated neutrophils can produce neutrophil extracellular traps (NETs), which contain antimicrobial components (Brinkmann et al., 2004). However, it has been demonstrated that NETs can trap but

not kill *S. pneumoniae,* as the bacteria can escape by degrading the NET with endonuclease (Beiter et al., 2006).

There are also spleen-resident neutrophils that help promote antibody production by marginal zone B cells, which have been termed B helper neutrophils (Puga et al., 2012; Magri et al., 2014; Chorny et al., 2016). Mature neutrophils in the spleen can remove *S. pneumoniae* from red pulp macrophages that take up the bacteria mediated by complement, which is enhanced upon antibody production by marginal zone B cells responding in a T cell-independent manner, while immature neutrophils in the spleen proliferate to increase the effector mature neutrophil population. (Deniset et al., 2017).

Although neutrophils are essential in the clearance of *S. pneumoniae*, they can also contribute to tissue damage and epithelial barrier disruption (Jose et al., 2015). In the study by Jose et al., lung proteinase-activated receptor 1 (PAR-1), was shown to be upregulated during *S. pneumoniae* infection, and promotes neutrophil recruitment resulting in alveolar leak. Antagonising PAR-1 resulted in decreased IL-1β and chemokines CXCL1, CCL2 and CCL7, decreased neutrophil recruitment, and attenuated alveolar leak, but did not impair killing of *S. pneumoniae*, although near-total neutrophil depletion impaired bacterial killing. These data suggest that tightly controlled neutrophil recruitment to the lung is beneficial to the host in terms of both bacterial killing and prevention of tissue damage.

1.2.4 Monocytes and macrophages

Monocytes constitute 1-6% of leukocytes. Monocytes can recognise pathogens through PRRs and similar to macrophages can phagocytose, present antigen, produce cytokines and proliferate in response to infection. Monocytes can be divided into classical inflammatory monocytes expressing $CD14^{+}CD16^{-}CCR2^{+}$ and non-classical patrolling monocytes expressing $CD14^{low}CD16^{+}CX3CR1^{high}$ in humans.

Classical monocytes proliferate in the bone marrow and migrate to the site of infection in response to chemokine concentration gradients through CCR2 (Serbina and Palmer, 2006). Monocytes are capable of differentiating into DCs and macrophages in the lung (Jakubzick et al., 2008; Landsman and Jung, 2007). Monocytes/macrophages can phagocytose *S. pneumoniae* opsonised by complement, antibody or CRP, or may proceed by direct recognition through surface components such as SIGNR1 or MARCO (McCool and Weiser, 2004; Malley et al., 2005; van Lookeren Campagne et al., 2007; Rehm and Coonrod, 1982; Gould and Weiser, 2001; Szalai et al., 1995; Gould and Weiser, 2002).

Monocytes are recruited to the lungs during *S. pneumoniae* pneumonia (Goto et al., 2004). Monocyte recruitment into the pneumonic airspaces occurs predominantly 24-48 hours after the initiation of inflammation, although they sequester in the lung microvasculature much sooner (van Furth et al., 1973; Lundahl and Hed, 1994; Ohgami et al., 1991; Goto et al., 2004). Monocytes taken from *S. pneumoniae* infected mice, labelled and transfused into recipient mice with pneumonia recruit to the lung in 1 hour, where they sequester in the lung microvasculature, therefore there is a delayed migration from the lung blood vessels to the airspaces (Goto et al., 2004). Monocytes recruit to inflamed tissue through CCR2 in response to CCL2, which is produced by alveolar epithelial cells (Serbina and Pamer, 2006; Winter et al., 2007). Mice that over-express CCL2 have increased monocyte recruitment and cleared *S. pneumoniae* more efficiently than wild-type; however, these mice also had greater lung damage, suggesting that similar to neutrophils the monocyte response to *S. pneumoniae* needs tight regulation to ensure recruitment to the site of infection is beneficial rather than harmful to the host (Winter et al., 2007).

Once monocytes are recruited to the lung tissue, they may differentiate into a phenotype resembling alveolar macrophages (Maus et al., 2006). The relative contribution to control of established *S. pneumoniae* pneumonia of alveolar versus

recruited macrophages is a matter of debate. Many of the original alveolar macrophages appear to be lost during inflammation, and may be repopulated by recruited macrophages or through division of the remaining resident populations (reviewed by Hussell and Bell, 2014). Monocytes and macrophages are also likely to regulate T cell activation and thus limit the inflammatory response through Fas ligand mediated apoptosis (Daigneault et al., 2012; Bewley et al., 2011). Fas ligand can induce apoptosis in activated cells during *S. pneumoniae* pneumonia and thus preventing *S. pneumoniae*-induced necrotic cell death which is pro-inflammatory.

1.2.5 Innate lymphoid cells and unconventional lymphocytes

Innate lymphoid cells (ILCs) and unconventional T cells are innate-like cells that can sense infected host cells and respond through a variety of receptors by several functions. ILCs are divided into five subclasses, which are NK cells, ILC1, ILC2, ILC3, and lymphoid tissue-inducer cells. Unconventional T cells include natural killer T (NKT) cells, γδ T cells, and mucosal associated invariant T (MAIT) cells. ILCs do not have antigen-specific receptors and instead respond to danger/stress signals from host cells, whereas unconventional T cells have a limited capacity for antigen recognition along with danger/stress sensors. In terms of function, NK cells, ILC1, ILC2 and ILC3 can be considered as parallels to $CD8^+$ cytotoxic T cells, Th1, Th2 and Th17 cells, respectively, except in their mode of recognition of their targets (reviewed by Borger et al., 2019).

NK cells can kill infected host cells by releasing cytotoxic granules onto the surface of the infected cell and effector molecules that induce programmed cell death are delivered into the cell. NK cells can produce both pro-inflammatory and anti-inflammatory cytokines, such as TNFα, IFNγ, TGFβ and IL-10, and in doing so influence the subtypes of T cells generated through T cell priming. NK cells are increased in the mouse lung upon *S. pneumoniae* infection as early as 6 hours post-infection (Kawakami et al., 2003). There are conflicting reports as to whether NK cells

are beneficial or detrimental to *S. pneumoniae* infection, perhaps arising from their roles in skewing the balance between pro- and anti-inflammatory conditions. NK cells can be important contributors to early immune responses to *S. pneumoniae* through interaction of the NK receptor natural cytotoxicity receptor 1 (NCR1) and its ligands on infected DCs and/or macrophages (Elhaik-Goldman et al., 2011). NK cells are a major source of IFNγ during pneumococcal infection, and disruption of NK cell IFNγ production increases the bacterial burden in the lung during murine pneumonia (Baranek et al., 2017). Conversely, IFNγ has been found to be detrimental in a mouse model of *S. pneumoniae* meningitis in which NK cells were the major contributor of the cytokine (Mitchell et al., 2012), and in mouse models of *S. pneumoniae* co-infection with influenza A (Sun et al., 2007). NK cells can reduce antibody responses to pneumococcal polysaccharides in mice (Khater et al., 1986), but were detrimental in *S. pneumoniae* infections in B cell- and T cell- deficient (*scid*) mice due to elevated inflammatory responses that could be reversed by depleting NK cells or by infection with a Ply-deficient *S. pneumoniae* mutant (Kerr et al., 2005).

ILC3 cells are another innate lymphoid cell type thought to aid the host against *S. pneumoniae*. ILC3 cells are present in the lung in the resting state and are activated by DCs to produce IL-17 and IL-22 in response to *S. pneumoniae* (Van Meale et al., 2014). Therefore, ILC3 cells provide an early source of IL-17 and IL-22 upon *S. pneumoniae* pneumonic infection.

NKT cells have invariant T cell receptors (TCRs) and NK markers that recognise glycoplipid antigens presented by antigen presenting cells (APCs) (Park and Bendelac, 2000; Mattner et al., 2005). They reside in the lung microvasculature and extravasate to the parenchyma when activated (Scanlon et al., 2011). NKT cells are increased in the mouse lung early during *S. pneumoniae* infection (Kawakami et al., 2003). Invariant NKT (iNKT) cells are a subset of NKT cells that have one restricted β chain TCR and an invariant chain that have been shown to produce IL-17A and

IFNγ in response to pneumococcal glycolipids dependent on antigen presentation by CD1d after *S. pneumoniae* infection (Kinjo et al., 2011). Prophylactic administration of the NKT cell activator α-galactosylceramide, which is structurally similar to pneumococcal α-glucosyldiacylglycerol, protects mice against lethal *S. pneumoniae* infections in a manner requiring DCs, IFNγ and IL-17 (Ivanov et al., 2012). Another study reported that NKT cell deficient mice were more susceptible to *S. pneumoniae* infection, and that the Valpha^{+} subset were recruited to the lung by monocyte chemotactic protein (MCP)-1 and promoted neutrophil recruitment (Kawakami et al., 2003). This was later found to be due to NKT cell IFNγ production (Nakamatsu et al., 2007). NKT cells also have an important role in production of anti-pneumococcal antibodies to polysaccharide vaccines in mice, and there is a positive correlation between NKT numbers and IgG after Pneumovax vaccination in humans (Kabrynski et al., 2005; Miyasaka et al., 2012; Miyasaka et al., 2013). In a study comparing both NK and NKT cells, NK cells were found to contribute to mortality in a mouse model of *S. pneumoniae* challenge, as NK cell depletion resulted in decreased bacterial outgrowth and prolonged survival, whereas blocking NKT cell activation did the opposite (Christaki et al., 2015). Inhibition of NKT cell activation resulted in predominance of NK cells and an exaggerated production of IFNγ. NK and NKT cells have been found to be more strongly activated than CD4^{+} T cells, CD4^{-} T cells and monocytes in response to cell wall and capsular polysaccharide (Sundberg-Kövamees et al., 2016).

MAIT cells constitute 1-10% of peripheral blood T cells and 10-20% of lung T cells in humans (Salou et al., 2017). They are found in the lung mucosa and are able to recognise *S. pneumoniae*-infected airway epithelial cells and DCs (Hartmann et al., 2018). Hartmann et al. also demonstrated that MAIT cells responded with varying magnitude of IFNγ production towards different clinical isolates of the 19A serotype, and suggest this was likely to be due to differences in metabolism of riboflavin. An

experimental human colonisation study reported that $CD8^+$ MAIT cells are associated with resistance to carriage acquisition (Jochems et al., 2019). Rapid production of cytokines such as TNFα and IFNγ by MAIT cells upon *S. pneumoniae* detection could enhance recruitment and activation of neutrophils and monocytes.

Another type of innate-like, non-conventional T cells are γδ T cells, which can detect danger signals by either TCR-dependent or TCR-independent mechanisms, through PRRs, TLRs, or NK cell receptors. They are activated by lipids presented by CD1 molecules, C-type lectins, and through APC presentation of microbial products in conjunction with pro-inflammatory cytokines (reviewed by Ivanov et al., 2014). Pulmonary γδ T cells represent a small proportion (approx. 5-10%) of lung T cells and are present in all regions of the lung except the airway mucosa (Wands et al., 2005). Here they make contact with macrophages and DCs, and are capable of altering their development and functional capabilities (Wands et al., 2005; Ismaili et al., 2002; Dalton et al., 2003). They accumulate in the lungs upon pneumococcal infection in mice, and their depletion results in increased bacterial load, defective IL-17, TNFα and macrophage inflammatory protein-2 (MIP-2) production, impaired neutrophil recruitment, and lower survival rate (Ma et al., 2003; Nakasone et al., 2007; Cao et al., 2014). Resolution of lung inflammation is also augmented by γδ T cells following pneumococcal infection by modulating DC and alveolar macrophage numbers to homeostatic levels (Kirby et al., 2007). Previous influenza infection inhibits type I IFN/IL-27-mediated γδ T cell IL-17 production upon subsequent *S. pneumoniae* exposure, increasing susceptibility to infection in mice (Li et al., 2012; Cao et al., 2014).

1.2.6 Dendritic cells

DCs are an important cell type that bridges between innate and adaptive immunity. DCs are resident in most tissues where they survey the tissue for invading pathogens (Steinman and Cohn, 1973; Wright-Browne et al., 1997). They are broadly divided into myeloid (conventional) DCs and plasmacytoid DCs. Myeloid DCs form a network throughout the epithelium of the lung, where they project their dendrites between the epithelial cells to sample the airway lumen (Upham and Xi, 2017). Lung myeloid DCs are reported to activate T cell subsets, whereas subpopulations of lung plasmacytoid DCs are associated with suppression of Teff cell generation and induction of Treg cells through retinoic acid and TGFβ (de Heer, 2004; Lombardi et al., 2012).

DCs migrate through the blood from their source in the bone marrow and arrive at their tissue locations as still-immature cells. DCs can also differentiate from monocytes recruited to the lungs. Upon detection of pathogens via PRRs such as TLRs, DCs endocytose the pathogen-associated material, degrade it, upregulate MHC-II and co-stimulatory markers such as CD40 and CD86, and present pathogen components on their cell surface (Nussenzweig et al., 1980; Schuler and Steinman, 1985; Schuler et al., 1985; Pierre et al., 1997; Young et al., 1992; McLellan et al., 1995). The activated DC also upregulates chemokine receptors such as CCR7 to enable the DC to migrate to the draining lymph nodes for presentation of pathogen components to naïve T cells (Sallusto et al., 1999).

Ply can enable evasion of *S. pneumoniae* from DC surveillance by inhibiting DC maturation, inhibiting production of cytokines and activation of the inflammasome, and induction of apoptosis (Littmann et al., 2009). PavA has been reported to protect *S. pneumoniae* against recognition and phagocytosis by DCs in humans (Noske et al., 2009). *S. pneumoniae* has also been demonstrated to exploit DCs aiding bacterial invasion (Rosendahl et al., 2013). In this study, *S. pneumoniae* induced DC expression of matrix metalloproteinase (MMP)-9, a protease involved in tissue barrier

breakdown, and mice depleted of DCs had better resistance to systemic dissemination and decreased bacterial loads.

1.2.7 B Cells

Naïve B cells are activated to differentiate into antibody-secreting plasma cells and memory B cells upon antigen encounter - this usually requires Th cell help. The effector role of B cells is the production of antibodies, and colonisation results in the development of antibody to *S. pneumoniae* capsular and protein antigens in humans (Turner et al., 2013; Goldblatt et al., 2005; Croucher et al., 2017). The importance of antibody for protection against *S. pneumoniae* is demonstrated by the association of antibody deficiency with increased risk of IPD (Martinot et al., 2014), the protective efficacy of passive vaccination of mice with pooled human IgG (Wilson et al 2017), and the effectiveness of vaccines that induce anti-capsular antibody (Smit et al., 1977; Whitney et al., 2003). Antibodies can bind and neutralise Ply (Salha et al., 2012), and opsonise bacteria for increased phagocytic uptake by binding complement and promoting Fcγ recognition (Wilson et al., 2017, Hyams et al., 2010). Antibody also causes bacterial agglutination, which blocks colonisation, perhaps through blocking access to the epithelium (Roche et al., 2014), and increases *S. pneumoniae* susceptibility to complement (Dalia and Weiser, 2011). It is widely thought that B cell responses to proteins require Th cells and cytokines. T cell-derived IFNγ and IL-10 were found to be important in the regulation of antibody production by adenoidal mononuclear cells in response to Ply and, to a lesser extent, CbpA (Zhang, Bernatoniene et al., 2006a).

S. pneumoniae colonisation-induced protection against subsequent lethal invasive pneumonia by prevention of septicaemia in mice has been demonstrated to be antibody-dependent, as $CD4^+$ T cell depletion still protected mice against septicaemia, whereas antibody deficient mice were not protected (Cohen et al., 2011) It has been proposed that long-lived $CD138^+$ antibody-producing cells mediate this

colonisation-induced protection against pneumococcal septicaemia (Bou Ghanem et al., 2018). In humans, naturally-acquired antibodies to protein antigens rather than anti-capsular antibodies have been found to be important in protection against pneumococcal septicaemia and IPD (Wilson et al., 2017).

Mouse studies have demonstrated that both antibody and T cell-mediated immune responses develop upon *S. pneumoniae* colonisation and are important in protection against subsequent pneumonia, and that antibody responses were against protein antigens as opposed to capsular antigens (Wilson et al., 2015). *S. pneumoniae* capsular polysaccharide antigens stimulate IgM antibody production without T cell help (Beuvery et al., 1982). The capsule instead generates T cell-independent type II (TI-2) responses, induced by high molecular weight polysaccharides with repetitive immunogenic epitopes (Alonso De Velasco et al., 1995; Bachmann et al., 1997; Dintzis et al., 1976). This response generates rapid B cell proliferation and antibody production predominantly of IgM through crosslinking B cell receptors.

IgM memory B cells differentiate into plasma cells and produce IgM antibodies when stimulated with pneumococcal polysaccharides through TLR9 (Capolunghi et al., 2008). Capsular antigens are poorly immunogenic in young children (Douglas et al., 1983). IgM memory B cells are absent in very young children which is associated with the inability to be protected by polysaccharide vaccines and increased susceptibility to pneumococcal infections (Kruetzmann et al., 2003; Carsetti et al., 2005). IgM binding to the capsule activates the classical complement pathway and promotes opsonophagocytosis.

Secretory IgA antibodies are an important factor in preventing *S. pneumoniae* colonisation by preventing *S. pneumoniae* adherence to the epithelium (Kong et al., 2013; Fukuyama et al., 2010; Hammerschmidt et al., 1997; Zhang et al., 2000). Passively administered IgG3 antibodies against ChoP, pneumococcal C-polysaccharide and the serotype 3 capsule are more protective than IgM in a lethal

pneumonia mouse model (Briles et al., 1981). Later, anti-ChoP IgG antibodies were demonstrated to be more protective against septicaemia than IgM when passively administered to mice, and the subclasses IgG1, IgG2a, IgGb and IgG3 all promoted a similar level of protection (Briles et al., 1989, 1984, 1986, and 1992).

1.2.8 T cells

$CD4^+$ and $CD8^+$ T cells drive the cellular adaptive immune response. $CD4^+$ T cells, known as T helper (Th) cells, recognise antigen presented on MHC-II molecules plus co-stimulatory molecules to activate, proliferate and differentiate into effector cells that produce cytokines, and activate B cells and cytotoxic $CD8^+$ T cells. There are conflicting data as to the importance of $CD4^+$ T cells and whether their roles are protective or detrimental in *S. pneumoniae* colonisation and infection, perhaps due to the fact that these cells have both inflammatory and anti-inflammatory subsets.

Th1 development is controlled by the transcription factors T-Box 21 (TBX21), Eomesodermin (EOMES), and signal transducer and activator of transcription (STAT)-4. One of the main effector cytokines they produce is IFNγ. Th1 cells express the CXCR3 receptor and recruit to the lungs in response to CXCL9, CXCL10 and CXCL11 produced by epithelial cells (Tudhope et al., 2007). Production of these chemokines is stimulated by IFNγ, therefore Th1 cells can augment their own recruitment. IFNγ activates macrophages to produce pro-inflammatory cytokines and increase antimicrobial functions. Th1 cells promote class switching to IgG2a, which is correlated with enhanced phagocytosis of *S. pneumoniae* in mouse models (Lefeber et al., 2003).

Th2 development is controlled by the transcription factors GATA binding protein (GATA)-3 and STAT6. Effector cytokines of Th2 cells include IL-4 and IL-13. IL-4 is important in B cell proliferation and IL-13 increases the expression of mucins (Atherton et al., 2003). Th2 cells promote class switching to IgG1, IgA and IgE.

Macrophage activation in response to Th2 cytokines shifts the macrophage towards a wound-healing phenotype and stimulates arginase activity, which has been shown to worsen lung-protective immunity against *S. pneumoniae* (Knippenberg et al., 2015).

Th17 development is controlled by retinoic acid-related orphan receptors (ROR)-γt, RORα and STAT3 in the presence of TGFβ, IL-6, IL-21, and lineage commitment is stabilised by IL-23. They produce the cytokines IL-17 and IL-22. The IL-17 receptor is expressed on cells including leukocytes and vascular endothelial cells and signalling results in granulocyte-colony stimulating factor (G-CSF), IL-6 and CXCL8 production, neutrophil recruitment and hBD2 production. Th17 cells are important in the immune response to colonisation (Kadioglu et al., 2004; Basset et al., 2007; van Rossum et al., 2005). Th17 derived IL-17A promotes monocyte/ macrophage and neutrophil recruitment and killing (Lu et a., 2008). Prior *S. pneumoniae* colonisation in a mouse pneumonia model resulted in cross-protection upon subsequent infection which was abrogated by IL-17A blockade, suggesting the importance of a memory Th17 cell response in protection against pneumonia (Wang, Jiang et al., 2017).

A subset of $CD4^+$ T cells with characteristics of both Th1 and Th17 have been described (Annunziato et al., 2007). In a study comparing peripheral blood memory $CD4^+$ T cell subset responses to *S. pneumoniae*, reactive cells were predominantly $CCR6^+CXCR3^+IFN\gamma^+$ cells and $CCR6^+CCR4^+$ cells producing IL-17 and IL-22 (Engen et al., 2014). This suggests that memory cells responsive to *S. pneumoniae* are predominantly $CCR6^+$ cells that produce IFNγ, IL-17 and IL-22. $CCR6^+$ Th1 cells are a recently described subset which produce both IFNγ and IL-17 and have Th17 ancestry (Annunziato et al., 2007). In the study by Engen et al., the capsule was not responsible for recognition by $CD4^+$ memory T cells. Pneumococcal carriage in humans increases $CD4^+IL\text{-}17A^+TNF\alpha^+IFN\gamma^+$ Th cell numbers in the blood and lungs (Wright et al., 2013).

Treg cells have anti-inflammatory functions which can limit tissue damage and aid resolution of inflammatory responses. The best available marker for Treg cells is Foxp3 in combination with surface markers $CD4^+CD25^+CD127^{low}$. They may be protective where inflammation is excessive, however they may be detrimental if they prevent protective immune responses. The balance between Treg and Th17 cells is considered particularly important in the clearance of *S. pneumoniae* colonisation (Zhang et al., 2011; Pido-Lopez et al., 2011; Neill et al., 2014; Jiang et al., 2015, Mubarak et al., 2016). Treg cells also have been demonstrated to be protective against IPD (Neill et al., 2012). Part 1.2.11 discusses the role of Treg in *S. pneumoniae* colonisation, part 1.2.12 discusses the role of Treg in *S. pneumoniae* pneumonia, and part 1.4 outlines Treg biology in more detail.

$CD8^+$ T cells recognise antigen presented on MHC-I molecules and are usually associated with defence against intracellular pathogens. As *S. pneumoniae* is an extracellular pathogen, the $CD8^+$ T cell response has not been studied extensively, however recent findings that *S. pneumoniae* can replicate within splenic macrophages and reside in epithelial cells may suggest a previously underappreciated role for $CD8^+$ T cells (Weight et al., 2018; Ercoli et al., 2018). Effector functions of $CD8^+$ T cells include production of cytokines such as TNFα and IFNγ, release of cytotoxic granules such as perforin and granzyme, and induction of apoptosis of infected cells. In addition, $CD8^+$ Treg cells can inhibit immune responses, have effector functions such as production of the anti-inflammatory cytokines IL-10 and TGFβ, and influence DC co-stimulatory molecule CD80 and CD86 expression. $CD8^+$ Treg cells can inhibit $CD4^+$ T cells in a contact-dependent manner through cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and membrane-bound TGFβ. In a study comparing CD4 knockout and CD8 knockout mice in a serotype 3 *S. pneumoniae* pneumonia model, CD8 knockout mice had decreased survival associated with increased inflammation, whereas CD4 knockout mice were better

protected, suggesting that $CD8^+$ T cells had a protective anti-inflammatory effect in serotype 3 pneumonia (Weber et al., 2011). Another study found that vaccination with serotype 3 capsular polysaccharide tetanus toxoid-conjugate protected wild type, CD4 knockout, and secretory IgM knockout mice from both systemic and pulmonary infection with serotype 3, but CD8 knockout mice were only protected from systemic and not pulmonary infection, suggesting that $CD8^+$ T cells are important in antibody immunity to serotype 3 (Tian et al., 2007). *S. pneumoniae*-specific $CD8^+$ T cells have been detected in peripheral blood of Gambian adults (Mureithi et al., 2009). Resident memory $CD8^+$ T cells were increased in subjects protected against *S. pneumoniae* carriage in an experimental human pneumococcal challenge study (Jochems et al., 2019).

1.2.9 Acute phase response

Acute phase response protein production is initiated by TNFα, IL-1β and IL-6 acting on liver hepatocytes. An important component of the acute phase response to *S. pneumoniae* is complement. The complement system is composed of a large number of proteins in the plasma that help fight infection through opsonisation and recognition by phagocytes with complement receptors. Complement components also act as chemoattractants to recruit phagocytes, activate phagocytes, enhance B cell responses, and by cause direct to damage bacteria through creating pores in the bacterial membranes. The complement cascade can be activated via three pathways: the classical, lectin and alternative pathways. The classical pathway is initiated by binding of C1q to either the bacterial surface, to CRP which binds to phosphorylcholine residues in pneumococcal C polysaccharide (teichoic acid) of the cell wall, or binding to antigen-antibody complexes (Prellner, 1981; Mold et al., 2002; Yother et al., 1982). The antibody that most efficiently binds C1q is natural IgM, which has broad specificity and is produced in the absence of infection and is considered part of the innate immune system allowing an immediate response to infection, and

are important for immunity to *S. pneumoniae* (Boes et al., 1998; Mold et al., 2002; Briles et al., 1981). People with deficiency of the classical complement pathway have an increased susceptibility to pneumococcal septicaemia, meningitis and pneumonia (Jönsson et al., 2005; Yuste et al., 2005). The lectin pathway is initiated by binding of proteins such as mannose binding lectin (MBL) binding to pathogen carbohydrates, and the alternative pathway can be initiated by spontaneous complement component C3 binding to the pathogen. All pathways result in the production of C3 convertase covalently bound to the pathogen, where C3 is cleaved to produce C3a and C3b, acting as a mediator of inflammation and an opsonin, respectively. Subsequent events lead to the production of the pore-forming membrane attack complex. The complement system is important for innate immunity to *S. pneumoniae* (Alper et al., 1970; Gross et al., 1978; Winkelstein 1981; Mold et al., 2002). The classical pathway has been demonstrated to be the dominant pathway for activation of the complement system in innate immunity to *S. pneumoniae* in mice, partially mediated by natural IgM binding to bacteria (Brown et al., 2002). Opsonisation of *S. pneumoniae* by complement in the absence of specific IgG is mainly dependent on the classical pathway, whereas complement activation is amplified by the alternative pathway, leading to denser C3 deposition on the bacteria (Brown et al., 2002). Disrupting the classical pathway has also been shown to reduce the numbers of macrophages and lymphocytes at the site of *S. pneumoniae* infection in mice, suggesting effects on the adaptive immune response (Brown et al., 2002). The *S. pneumoniae* capsule can inhibit binding of complement opsonisation and activity and therefore inhibiting neutrophil phagocytosis (Hyams et al., 2010). Complement component C3 has an important role during the early hours of pneumonia in mice, demonstrated by C3 increase within 1 hour of pneumonia onset correlating with decreased bacterial loads in the lung, and C3 depletion resulting in overwhelming inflammation and decreased survival times (Kerr et al., 2005).

In addition to its role in complement activation, CRP also blocks *S. pneumoniae* adherence through PAFR (Gould and Weiser, 2002). Transgenic mice that express the human CRP gene are protected against *S. pneumoniae* infection (Szalai et al., 1995).

1.2.10 Cytokines and chemokines

S. pneumoniae is recognised by lung resident cells expressing PRRs, which generates an inflammatory response by production of cytokines and chemokines. Cytokines are small proteins that can affect cells in an autocrine, paracrine or endocrine manner by binding their specific receptors. Upon intranasal administration of *S. pneumoniae* in mice models, TNFα is rapidly increased in the BALF, followed by IL-1β then IL-6.

1.2.10.1 Interferon-α/β

Interferon-α and interferon-β are referred to as type 1 interferons. They signal through the interferon α/β receptor (IFNAR). Epithelial cells, DCs, monocytes and macrophages are some of the cells that can produce type 1 interferons. They have various functions depending on the infecting organism and are particularly associated with antiviral responses. However, *S. pneumoniae* also activates type 1 interferon signalling (Parker et al., 2011). In mouse *S. pneumoniae* infection, IFNα has been found to aid the host response by increasing neutrophil and macrophage bacterial killing and reducing tissue damage (Damjanovic et al., 2014). Mice without IFNAR had increased bacteraemia upon intranasal *S. pneumoniae* infection (LeMessurier et al., 2013). By contrast, treatment of mice with IFNβ reduced bacteraemia, the effect of which was upregulation of tight junction proteins and downregulation of PAFR, thereby reducing PAFR-mediated transcytosis and migration through disrupted tight junctions (LeMessurier et al., 2013). Type 1 interferon also contributes to the immune

response to *S. pneumoniae* carriage, as mice deficient in IFNAR had increased nasal colonisation compared with that of wild-type mice (Parker et al., 2011).

1.2.10.2 Interferon-γ

IFNγ is produced as part of the innate response by NK cells, NKT cells and monocytes, and also by $CD4^+$ and $CD8^+$ T cells in the adaptive response in antigen-specific immunity. Neutrophils may also be an important source of IFNγ in pneumococcal pneumonia (Gomez et al., 2015). A main role of IFNγ is activation of macrophages into a host-defence phenotype with increased microbicidal activity and production of pro-inflammatory cytokines (Mackaness, 1977; O'Shea et al., 2008). The contribution of IFNγ in the immune response to *S. pneumoniae* is contentious. IFNγ has been demonstrated to enhance chemokine expression and resulting neutrophil recruitment, improving survival rates in *S. pneumoniae* challenge (Sun, Salmon et al., 2007). IFNγ knockout mice have been found to have increased mortality than immunocompetent mice in a *S. pneumoniae* pneumonia model, suggesting a protective role for IFNγ (Rubins and Pomeroy, 1997) However, another study found that IFNγ-deficient mice had reduced bacterial loads in the lungs and decreased mortality in *S. pneumoniae* pneumonia, suggesting a deleterious effect of IFNγ in the host response (Rijneveld, Lauw et al., 2002). In addition, IFNγ has been demonstrated to have a negative effect on the outcome of pneumococcal meningitis, as depletion with an anti-IFNγ antibody increased survival (Mitchell et al., 2012; Pettini et al., 2015). The main source of the IFNγ was NK cells and production was by ASC-dependent inflammasome (Mitchell et al., 2012). IFNγ gene deletion did not affect neutrophil recruitment but did decrease monocyte recruitment and enhanced bacterial clearance. IFNγ raised NOS2 levels at 48 hours in the murine meningitis model, and knock-out of NOS2 delayed mortality and abolished blood-brain barrier dysfunction (Yau et al., 2016). IFNγ produced in response to influenza virus predisposes for subsequent *S. pneumoniae* infection by decreasing alveolar

macrophage-mediated clearance in the early stages of infection (Sun and Metzger, 2008).

1.2.10.3 Interleukin-1β

There are three forms of IL-1: IL-1α, IL-1β and IL-1R-antagonist (IL-1Ra). IL-1α and IL-1β are pro-inflammatory, whereas IL-1Ra acts to regulate the IL-1 pro-inflammatory response by competing with IL-1α and IL-1β for the IL-1 receptor (IL-1R). Maturation of the IL-1β precursor is promoted by caspase 1 which is activated by inflammasomes. The main source of IL-1β are monocytes and macrophages and it can also be produced by NK cells, B cells, DCs and epithelial cells. IL-1β stimulates acute phase protein production, induces fever, promotes histamine release causing vasodilation, and increases cell adhesion molecule expression on endothelial cells and leukocytes (Arango Duque and Descoteaux, 2014). IL-1β also acts as a chemoattractant for neutrophils (Oliveira et al., 2008). $CD4^+$ T cell antigen-driven expansion and differentiation is enhanced by IL-1β (Ben-Sasson et al., 2009). Mouse models deficient in IL-1R have been demonstrated to have increased susceptibility to *S. pneumoniae* pneumonia and meningitis (Zwijnenburg et al., 2003). IL-1R deficient mice also had prolonged pneumococcal carriage with reduced neutrophils early in infection and reduced macrophages later in carriage (Lemon et al., 2015).

1.2.10.4 Interleukin-6

IL-6 can be produced by a wide range of cells including monocytes, macrophages, T cells, B cells, endothelial cells, epithelial cells. IL-6 enhances neutrophil functions such as oxygen radical production and prolongs their lifespan by delaying apoptosis (Biffl et al., 1996). IL-6 also induces CRP, levels of which correlate in community-acquired pneumonia (Ortqvist et al., 1995). IL-6 also encourages the differentiation of Th17 cells from naïve $CD4^+$ cells and inhibits Treg cell development (Bettelli et al., 2006). IL-6 signalling promotes recruitment of monocytes (Hurst et al., 2001). IL-6

also promotes B cell differentiation into plasma cells (Yoshizaki et al., 1984). IL-6 deficient mice died earlier and had increased *S. pneumoniae* colonies in the lung than wild type in a pneumonia model (van der Poll et al., 1997). Acute phase protein production in *S. pneumoniae* infection requires IL-6 to activate STAT3 in liver cells (Quinton et al., 2009).

1.2.10.5 Interleukin-10

IL-10 is an anti-inflammatory cytokine produced by DCs, macrophages, neutrophils, B cells and T cells. Macrophage activation and production of pro-inflammatory cytokines can be inhibited by IL-10 (Bogdan et al., 1991; Fiorentino et al., 1991). It can also reduce secretion of pro-inflammatory cytokines by DCs and neutrophils (Mege et al., 2006; Cyktor and Turner, 2011). IL-10 is produced by macrophages in response to *S. pneumoniae* infection through TLR2 or NOD2 binding of cell wall fragments triggering MyD88 and receptor-interacting serine/threonine kinase (RIPK)-2 signalling (Moreira et al., 2008).

MHC-II expression by macrophages is reduced after exposure to IL-10, therefore inhibiting antigen presentation (Chadban et al., 1998). Th1 and NK cell IFNγ production is also inhibited by IL-10, however IL-10 induces differentiation and antibody production by B cells (Fiorentino et al., 1991; Defrance et al., 1992; Rousset et al., 1992). When pre-treated with recombinant IL-10, a murine macrophage cell line was found to produce less NO and NOS upon exposure to lipopolysaccharide (LPS), and this suppressive effect was not seen if IL-10 was added simultaneously with or after LPS stimulation (Cunha et al., 1992). IL-10 promotes increased expansion of induced Treg cells (Hsu et al., 2015). IL-10 in the lungs is increased at 48 hours post-infection in mouse pneumonia model (Peñaloza et al., 2015). After *S. pneumoniae* infection, mice lacking IL-10 have been demonstrated to have increased lung damage, mortality, pro-inflammatory cytokine levels, and neutrophils in the lung, however these mice also had lower bacterial burden in the lungs, blood, spleen and

brain (Peñaloza et al., 2015). This suggests that deficiency of IL-10 allows better bacterial clearance but increased mortality due to uncontrolled inflammation. IL-10 has been found to be lower at both baseline levels and upon infection with *S. pneumoniae* in aged mice compared to in young mice (Williams et al., 2015). Aged mice had increased neutrophil recruitment but did not have statistically significant differences in bacterial burden than young mice. IL-10 has also been reported to be detrimental in mouse pneumonia models in which recombinant IL-10 was intranasally administered along with *S. pneumoniae* resulting in decreased TNFα and IFNγ in the lungs associated with increased bacterial burden in the lungs and blood and early death (van der Poll et al., 1996). Conversely, anti-IL-10 treatment in this mouse model improved survival. Treatment of a pneumonia in a mouse model with ceftriaxone with IL-10 resulted in a 95% survival rate, treatment with ceftriaxone alone resulted in 54% survival, and untreated mice had 100% mortality (Wang et al., 2005).

1.2.10.6 Interleukin-17

IL-17 is produced by Th17 cells, γδT cells, NKT cells, and innate lymphoid cells. IL-17 exists as six homologous molecules – IL-17A through IL-17F. IL-17A and IL-17F are highly homologous, have similar functions and interact with the same receptors. IL-17 activates neutrophils, promotes their expansion by regulation of G-CSF and G-CSF receptor, and regulates their recruitment through regulating chemokine expression (Gaffen 2009). IL-17 also activates IL-6, CXCL8 and G-CSF production in epithelial cells and fibroblasts. IL-17A, along with TLR2 and $CD4^+$ T cells, promote monocytes, macrophages and neutrophils during pneumococcal carriage (Zhang et al., 2009). IL-17A increases alveolar macrophage phagocytosis and killing of *S. pneumoniae in vitro* (Wright et al., 2013). In a mouse pneumonia model, IL-17A has been found to peak at 24 hours post-infection and γδ T cells are a major source (Cao et al., 2014). In the study by Cao et al., IL-17A neutralisation resulted in decreased neutrophil recruitment and decreased survival, however administration of

recombinant IL-17A enhanced lung neutrophil recruitment. In a mouse model, intranasal administration of recombinant IL-17F resulted in decreased bacterial burden and increased leukocyte, neutrophil and macrophage recruitment to the lungs upon *S. pneumoniae* infection (Chen et al., 2015).

1.2.10.7 Interleukin-22

The major role of IL-22 is in epithelial regeneration following injury and is produced by $CD4^+$ T cells, γδ T cells, NKT cells, ILCs and alveolar macrophages (Duhen et al., 2009; Hannsson et al., 2013). Of the human $CD4^+$ cells, 15% are Th17 cells which co-express IL-17, 33% are Th1 cells which co-express IFNγ, and 50% are Th22 cells which produce IL-22 alone (Duhen et al., 2009; Dudakov et al., 2015). IL-22 is induced in the lung as early as 5 hours post-infection with *S. pneumoniae* and IL-22 knock-out mice have been found to be more susceptible to pneumococcal infection (Trevejo-Nunez et al., 2017). In the study by Trevejo-Nunez et al., administration of recombinant IL-22 decreased bacterial burden in the lungs, liver and spleen, and increased complement component C3 in the liver which subsequently improved *S. pneumoniae* phagocytic uptake. The major source of IL-22 was γδ T cells. By contrast, pneumococcal whole cell antigen-stimulated peripheral blood mononuclear cells (PBMCs) from healthy adults produced IL-22 and IL-17A, the vast majority of which was produced by $CD4^+$ effector/memory T cells and was dependent on MHC-II interaction (Lundgren et al., 2012). The majority of these cells expressed either IL-17 or IL-22 with very little co-expression, suggesting that they are produced by different subsets.

1.2.10.8 Transforming growth factor β

TGFβ is produced by cells including platelets, T cells, macrophages, and epithelial cells. TGFβ has three isoforms, TGFβ1, TGFβ2 and TGFβ3 and their functions can be broadly grouped into modulation of inflammation, cell differentiation and control of

extracellular matrix production. TGFβ is secreted as complexes composed of latency associated peptide (LAP) and usually another protein called latent TGFβ binding protein 1 (LTBP1). TGFβ+LAP is known as the small latent complex, and TGFβ+LAP+LTBP1 is known as the large latent complex. TGFβ deactivates macrophages (Tsunawaki et al., 1988). TGFβ1 inhibits TNFα and IL-1β production by mouse peritoneal macrophages upon LPS stimulation (Imai et al., 2000). TGFβ promotes the differentiation of human monocytic cell line THP-1 and tumour-associated macrophages into an anti-inflammatory phenotype characterised by increased IL-10 production and decreased TNFα and IL-12 production (Zhang, Wang et al., 2016). Pneumococcal neuraminidases can activate TGFβ by removing sialic acids from LAP (Gratz et al., 2017). TGFβ also promotes the differentiation of Treg cells (Chen et al., 2003). TGFβ and Treg cells have been demonstrated to protect mice against invasive pneumococcal pneumonia (Neill et al., 2012).

1.2.10.9 Tumour necrosis factor-α

TNFα is typically produced by macrophages, NK cells, and T cells. TNFα exists as a transmembrane or soluble form. Soluble TNFα acts by binding to TNF receptors (TNFR)-1 or TNFR2 and the transmembrane form acts on TNFR2 (Horiuchi et al., 2010). TNFα is one of the first cytokines to be secreted in response to pathogens. It increases CRP production, induces vasodilation and aids expression of cell adhesion molecules (Arango Duque and Descoteaux, 2014; Vieira et al., 2009). TNFα with IL-17 promote neutrophil recruitment by causing CXCL1, CXCL2 and CXCL5 production (Griffin et a., 2012). TNFα is synthesised in the endoplasmic reticulum and migrates to the cell surface in vesicles fused with recycling endosomes (Arango Duque and Descoteaux, 2014). The TNFα cascade is activated in airway epithelial cells and alveolar macrophages upon recognition of *S. pneumoniae*. Alveolar macrophages are the main producers of TNFα in *S. pneumoniae* pneumonia (Kirby et al., 2005). Blocking TNFα in mouse models of *S. pneumoniae* pneumonia results in increased

bacterial load, decreased neutrophil recruitment and accelerated death (Takashima et al., 1997). TNFα has been shown to increase the oxidative burst by neutrophils stimulated with *S. pneumoniae in vitro* (Kragsbjerg and Fredlund 2001). Patients on anti-TNFα therapy have an increased risk of IPD (Colombel et al., 2004; Baghai et al., 2001). However, excessive TNFα resulting in excessive neutrophil activation contributes to lung injury and sepsis (Wright et al., 2004; Hildebrandt et al., 2004; Tracey et al., 1987). TNFα also increases expression of receptors that allow *S. pneumoniae* invasion, for example PAFR on endothelial cells promoting bacteraemia (Cundell et al., 1995). TNFα also regulates endothelial cell receptors for neutrophil adherence (Cohen, 1994). Phagocytosis of apoptotic macrophages reduces TNFα production and neutrophil production, contributing to resolution (Marriott et al., 2006).

Cytokine	Sources	Functions in *S. pneumoniae* response	Reference
IFNα/β	Epithelial cells, DCs, monocytes and macrophages	Downregulates PAFR and upregulates tight junctions increasing neutrophil and macrophage bacterial killing	LeMessurier et al., 2013 Damjanovic et al., 2014
IFNγ	NK cells, NKT cells, monocytes, T cells	Detrimental in pneumococcal meningitis Predisposes for secondary pneumonia after influenza Enhanced neutrophil recruitment Activates highly inflammatory macrophages	Mitchell et al., 2012 Sun and Metzger, 2008 Sun, Salmon et al., 2007
IL-1β	Monocytes, macrophages, NK cells, B cells, DCs, epithelial cells	IL-1R deficiency prolongs carriage and increases susceptibility to pneumonia and meningitis in mice	Zwijnenburg et al., 2003 Lemon et al., 2015
IL-6	Monocytes, macrophages, T cells, B cells, endothelial cells, epithelial cells	Deficiency increases mortality in mouse pneumonia Required for acute phase response Th17 differentiation	van der Poll et al., 1997 Quinton et al., 2009
IL-10	DCs, macrophages, neutrophils, B cells, T cells	Deficiency in mice increases tissue damage and increases mortality but reduced bacterial numbers	Peñaloza et al., 2015
IL-17	Th17 cells, γδT cells, NKT cells, innate lymphoid cells	Promotes monocyte, macrophage and neutrophil recruitment in carriage Increases alveolar macrophage phagocytosis and killing	Zhang et al., 2009 Wright et al., 2013
IL-22	T helper cells, γδ T cells, NKT cells, ILCs and alveolar macrophages	Decreases bacterial burden in lung, spleen and live Increases opsonophagocytosis	Trevejo-Nunez et al., 2017
TGFβ	Platelets, T cells, macrophages, epithelial cells	Promotes Treg cells protective against IPD	Neill et al., 2012
TNFα	Macrophages, NK cells, T cells	Increase the oxidative burst by neutrophils Anti-TNFα therapy increases risk of IPD Increases PAFR expression	Kragsbjerg and Fredlund 2001 Colombel et al., 2004; Baghai et al., 2001 Cundell et al., 1995

Table 1.2.10 A non-exhaustive list of major cytokine functions in *S. pneumoniae* infection

1.2.10.10 Chemokines

Chemokines are chemoattractant cytokines and can be broadly separated into two groups – the CC chemokines, which have two adjacent cysteines near the amino terminus, and the CXC chemokines which have two separated cysteines separated by an amino acid. Chemokines attract cells with their specific receptor along a concentration gradient towards the site of infection, and they can change the conformation of integrins in the endothelium, allowing cell adherence.

CCL2 is a monocyte chemoattractant produced by epithelial cells. Knockout of CCL2 has been demonstrated to decrease survival in mouse models of *S. pneumoniae* pneumonia by inhibition of macrophage and DC recruitment to the lung, prolonging neutrophil recruitment and promoting sepsis (Winter et al., 2009). CCL5 binds CCR1, CCR3, CCR4 and CCR5, is produced by epithelial cells, lymphocytes, and platelets. It recruits monocytes, memory T cells, NK cells and DCs (Palaniappan et al., 2004). Blockade of CCL5 in mouse *S. pneumoniae* infection models has been demonstrated to reduce recruitment of $CD4^+$ T cell, $CD8^+$ T cell and $CD11b^+$ cells, reduce antibody levels, and promote the progression from carriage to lethal pneumonia (Palaniappan et al., 2005). CCL7 inhibition has been demonstrated to inhibit neutrophil recruitment in a mouse pneumococcal pneumonia model in a manner that did not affect bacterial clearance but did reduce lung damage (Jose et al., 2015). CCL20 is a chemokine produced by lung epithelial cells in response to IL-1β and TNFα (Starner et al., 2003). It is the only known ligand of CCR6, expressed on subsets of effector/memory T cells. Almost all mucosa- and skin-homing memory/effector T cells, and a higher percentage of $CD4^+$ T cells than $CD8^+$ T cells, express CCR6 (Schutyser et al., 2003).

CXCL8 is a neutrophil attractant chemokine. *In vitro* exposure of respiratory tract epithelial cells to *S. pneumoniae* resulted in low level of CXCL8 production, however IL-1β produced by addition of THP-1 cells or MDMs increased CXCL8 production (Marriott et al., 2012). CXCR2 is a receptor for several chemokines involved in

neutrophil recruitment, and inhibition of CXCR2 in mouse models decreases the recruitment of both neutrophils and macrophages to the lung, resulting in greatly increased bacterial outgrowth and mortality (Herbold et al., 2010). IFNγ can induce production of the chemokines CXCL9, CXCL10 and CXCL11. Knockout of CXCR3, which is the receptor for CXCL9, CXCL10 and CXCL11, in mouse pneumonia models resulted in reduced virulence of serotype 3 but not serotype 8 *S. pneumoniae*, suggesting that the chemokine-driven inflammatory response to *S. pneumoniae* in the lung depends on the infecting serotype (Seyoum et al., 2011). Where virulence was attenuated, mice had fewer neutrophils, more alveolar macrophages, fewer blood colonies and less tissue damage than the wild type.

Chemokine	Sources	Functions in *S. pneumoniae* response	Reference
CCL2	Epithelial cells	macrophage and DC recruitment to the lung	Winter et al., 2009
CCL5	Epithelial cells, lymphocytes, and platelets	recruitment of $CD4^+$ T cell, $CD8^+$ T cell and $CD11b^+$ cells, promotes antibody	Palaniappan et al., 2005
CXCL1	Epithelial cells, macrophages, neutrophils	Neutrophil recruitment in mouse pneumonia	Jose et al., 2015
CXCL8	Epithelial cells, macrophages	Neutrophil recruitment in pneumonia	Marriott et al., 2012
*CXCR3	CXCL9, CXCL10, CXCL11 expressed by macrophages, neutrophils, epithelial cells, endothelial cells	Neutrophil recruitment in serotype 3 but not serotype 8 *S. pneumoniae* pneumonia	Seyoum et al., 2011

* Receptor for CXCL9, CXCL10 and CXCL11

Table 1.2.10.10 A non-exhaustive list of major chemokine functions in *S. pneumoniae* infection

1.2.11 Immune response in *S. pneumoniae* colonisation

Most of the data on the immune response to *S. pneumoniae* colonisation have been obtained using murine infection models. *S. pneumoniae* colonisation in mice results in an acute inflammatory response with neutrophil recruitment to the paranasal spaces, but the neutrophils are not sufficient to control initial colonisation (van Rossum et al., 2005). Despite being the first cells recruited to the presence of *S. pneumoniae* after colonisation, neutrophil presence in the nasopharynx does not correlate with clearance and does not appear to affect bacterial burden (McCool and Weiser, 2004; Nelson et al., 2007; Matthias et al., 2008). Instead, neutrophils have been demonstrated to enhance the adaptive immune response to colonisation by increased release and delivery of antigen to the nasal associated lymphoid tissue (NALT) and associated increased clearance (Matthias et al., 2008; Hergott et al., 2015).

In contrast, macrophages are vital for carriage clearance. Mice deficient in PRRs had decreased local chemokine production resulting in decreased recruitment and retention of macrophages in the upper airway, which inhibited clearance of *S. pneumoniae* (Zhang et al., 2009; Matthias et al., 2008; Davis et al., 2011). Clearance is dependent on a sustained recruitment of monocytes/macrophages, which phagocytose the bacteria and help generate the adaptive immune response (Zhang et al., 2009). Monocyte-macrophage recruitment is dependent on CCR2, and retention of monocyte-macrophages is dependent on macrophage migration inhibitory factor (MIF), a cytokine produced in response to Ply (Davis et al., 2011; Das et al., 2014).

Although the introduction of capsular-specific vaccines prevents human colonisation with vaccine serotype *S. pneumoniae* strains and anti-capsular antibody can prevent establishment of colonisation in mice by agglutination of *S. pneumoniae* (Roche et al., 2015), naturally acquired antibody at least to capsular antigens seems to be less

important for prevention of colonisation. In children over 2 years old, there is a decrease in carriage rate with age before anti-capsular antibody is detected (Lipsitch et al., 2005; Högberg et al., 2007). In murine models, B cell depleted mice were found to be unaffected in density or duration of colonisation, suggesting that antibody was not required for clearance of colonisation (McCool and Weiser 2004). A study using MHC-II deficient mice showed that $CD4^+$ T cells were important for early protective immunity, and that Ply induces $CD4^+$ T cells migration (Kadioglu et al., 2004). A mixture of the pneumococcal proteins PspC, adhesin A and pneumolysoid with cholera toxoid administered mucosally was also found to protect against colonisation in a $CD4^+$ T cell dependent, antibody-independent manner in mice (Basset et al., 2007). Further studies found that immunity to *S. pneumoniae* colonisation in mice was $CD4^+$ T cell dependent and antibody-independent, $CD8^+$ T cells were not required, and that absence of TLR2 delayed clearance of colonisation (van Rossum et al., 2005; Malley et al., 2005; Trzciński et al., 2005). The cytokine IL-17A was found to be implicated in the T cell-mediated resistance to colonisation after intranasal administration of cell wall polysaccharide in mice (Malley et al., 2006). Later, it was found that Th17-derived IL-17A mediated pneumococcal immunity in mice by increasing neutrophil killing, in a manner independent of antibody or complement (Lu et a., 2008).

IL-17A, TLR2 and $CD4^+$ T cells have been found to promote recruitment of monocytes and macrophages during primary and secondary colonisation events in mice, and neutrophils during secondary colonisation events (Zhang et al., 2009). In the study by Zhang et al., decline in colonisation correlated with increased monocytes/macrophages, and depletion of monocytes/macrophages decreased clearance of colonisation. Monocyte/macrophage recruitment was dependent on TLR2 but not TLR4. Depletion of $CD4^+$ T cells had no effect on early colonisation, but resulted in delayed late clearance and decreased recruitment of

monocytes/macrophages. Depletion of IL-17A completely attenuated monocyte/macrophage recruitment and resulted in loss of clearance. Secondary colonisation resulted in more rapid clearance of *S. pneumoniae*, higher numbers of monocytes/macrophages, and an increased neutrophil response. Mice previously depleted of $CD4^+$ T cells or TLR2 had no increased expression of monocyte chemotactic protein 1 (MCP-1) during the secondary colonisation. Blocking $CD4^+$ T cells or IL-17A abolished the enhanced clearance, and resulted in loss of the monocyte/macrophage and neutrophil recruitment. This study found the neutrophil response in the secondary colonisation to be important in clearance. Overall this study found that primary colonisation results in IL-17A-dependent, TLR2-dependent monocyte/macrophage-mediated clearance and generation of $CD4^+$ memory T cells which clear colonisation more rapidly through recruitment of neutrophils upon secondary colonisation.

In addition to mouse work, several studies on human cells including NALT and PBMCs, have provided further insight into cellular immunity to *S. pneumoniae*. Ply has been found to induce $CD4^+$ T cell proliferation in PBMCs and in adenoidal mononuclear cells in UK children, suggesting natural mucosal $CD4^+$ T cell immunity to pneumococcal protein antigens (Zhang et al., 2007). This study found less Ply-induced $CD4^+$ T cell proliferation in PBMCs from culture positive children than culture negative children, but no such difference in adenoidal cell proliferation, and proposed that antigen-specific memory $CD4^+$ T cells in the peripheral blood migrate to the nasopharynx and became sequestered in the adenoids, resulting in fewer antigen-specific memory T cells remaining in the peripheral blood. They found that CbpA induced only memory $CD4^+$ T cell proliferation, whereas Ply could induce proliferation of both naïve and memory $CD4^+$ T cells in a manner partly dependent on TLR4. In addition, IL-10 along with IFNγ was produced in response to Ply by adenoidal cells but not PBMCs, suggesting local regulation of inflammatory responses. A further

study found that domain 4 of Ply could elicit memory Th17 cell responses in NALT cells (Gray et al., 2014).

A study investigating PBMC T cell responses in Gambian adults found that effector memory $CD4^+$ cells ($CD4^+CD45RO^+CCR7^-$), implicated in pathogen clearance, and resting memory $CD4^+$ cells ($CD4^+CD45RO^+CCR7^+$), implicated in immune surveillance, proliferate in response to pneumococcal antigens (Mureithi et al., 2009). Although both $CD4^+$ T cell and $CD8^+$ T cell proliferative responses were observed, $CD4^+$ T cell responses predominated. The potency of the immune responses of these blood cells from Gambian adults did not correlate with lower carriage rates, which contrasts the findings of the mucosal response of children in the UK (Zhang et al., 2007).

Differences in the responses of PBMCs to pneumococcal antigens were also seen between Swedish and Bangladeshi donors (Lundgren et al., 2012). IL-17A production by PBMCs from Bangladeshi adults and children was higher than that of Swedish donor PBMCs. In the Swedish groups, a stronger Th17 response upon pneumococcal antigen stimulation was seen in adults compared to children. In the Bangladeshi groups, a strong Th17 response was seen in both adults and children. In the same study, IL-17A and IL-22 were raised upon stimulation with whole cell antigen, and that these cytokines came from separate populations of $CD4^+$ effector/memory T cells, suggesting Th22 involvement.

Treg cells have also been detected in NALT in higher proportions than in peripheral blood and with higher proportions largely consisting of the effector/memory $CD45RO^+$ subtype (Zhang et al., 2011). In the study by Zhang et al., Treg cells were found in higher proportions in the NALT of *S. pneumoniae* culture positive children than culture negative children. Treg cells proliferated in response to pneumococcal whole cell vaccine *in vitro*, and this increase was higher in cells from culture positive children. Depletion of Treg cells resulted in increase in $CD4^+$ effector T cell proliferation in

response to whole cell antigen, and this increase was higher in culture positive children than culture negative children. Depletion of Treg also increased TNFα, IFNγ and IL-17. These data suggest that adenoidal effector/memory Treg cells with antigen-specific inhibitory effects on $CD4^+$ T cells are present in the adenoids of children promoted by local pneumococcal colonisation.

A study comparing the numbers of Treg cells and Th17 cells in the adenoids of *S. pneumoniae* carriage positive and carriage negative children found that Treg cells were upregulated and Th17 cells were downregulated in carriage positive children (Jiang et al., 2015). Stimulation of these cells with supernatant from cultured serotype 2 strain D39 found greater increases in Treg numbers in the carriage positive group than in the carriage negative group, whereas increase in Th17 was greater in the culture negative group. In addition, IL-6 and IL-17A levels were higher in the carriage negative group, whereas TGFβ levels were higher in the carriage positive group. The authors of this study suggested that Treg cells could downregulate Th17 cells in the adenoids and promote chronic carriage.

Age has been found to be a determinate in the Treg/Th17 cell balance in colonisation. Treg cell numbers in the NALT have been found to decrease with age in a patient sample from children and young adults, whereas Th17 increases (Mubarak et al., 2016). The Th17:Treg ratio was higher in carriage negative than carriage positive, and tonsil cells from carriage negative children produced a higher Th17:Treg and IL-17A:IL-10 ratio than cells from carriage positive children upon pneumococcal stimulation. A stronger memory Th17 and higher IL-17:IL-10 ratio were elicited by carriage negative compared to carriage positive children. Depletion of $CD4^+$ T cells abrogated IL-17A production, confirming that $CD4^+$ T cells were the primary source.

Depletion of $CD25^+$ Treg cells from cultures of tonsil mononuclear cells taken from adults resulted in increased proliferation of the remaining $CD4^+$ T cells to Ply and to D39 *S. pneumoniae* culture supernatant (Pido-Lopez et al., 2011). Treg cells inhibited

effector $CD4^+$ T cell proliferation in a manner partly dependent on CTLA4 and programmed death ligand 1 (PDL-1). In contrast, no difference in proliferation was seen when cells were taken from donors less than 17 years old, suggesting Treg cells control $CD4^+$ effector T cell proliferation in response to *S. pneumoniae* only in adults.

In a mouse model of allergic airways disease (AAD), pneumococcal serotype 3 capsular polysaccharide and F433 pneumolysoid administered together were found to induce expansion of Treg cells in mediastinal lymph nodes (Preston et al., 2011; Thorburn et al., 2012; Thorburn et al., 2013). These Treg cells were found to decrease IL-6 and increase TGFβ in the lung, and reduced Th17 induction and function in the AAD mouse model (Thorburn et al., 2013).

S. pneumoniae have also been found to be able to induce active TGFβ1 production by human nasopharyngeal epithelial cells and fibroblasts *in vitro*, and elevated TGFβ1 and Treg cell numbers are crucial for prolonged carriage in a mouse model and a human challenge model (Neill et al., 2014). Low density of pneumococcal carriage was associated with prolonged elevation of TGFβ1 and increased Treg cell number, whereas high density carriage resulted in a transient TGFβ1 increase and no statistically significant Treg cell increase. Low density colonisation was also associated with a more rapid decline in neutrophil numbers than with high density, and macrophages showed increased mannose receptor, a marker of alternative activation. Ply was found to be required for the induction of active TGFβ1. Blockade of TGFβ1 with P17, a peptide inhibitor of TGFβ1 (Gil-Guerrero et al., 2008), in mice prior to low dose intranasal administration of bacteria resulted in reduced carriage, a transient bacterial translocation to the lungs, failure to induce Treg cell responses and exacerbated neutrophil recruitment. IL-10 was detected but was not statistically significantly different between control and TGFβ-blockade mice, suggesting that Treg cells were not the exclusive source of IL-10, however the human carriage model showed higher IL-10 in carriage positive compared to carriage negative individuals,

suggesting a role for IL-10 in carriage. Overall, this work suggests that *S. pneumoniae* induce epithelial TGFβ1 production, generating a Treg response which limits pro-inflammatory responses, increasing carriage duration but preventing tissue damage and bacterial spread. This prolonged, contained carriage mediated by Treg cells allows time for protective memory immune responses to develop against *S. pneumoniae* that may protect against IPD.

In summary, colonisation causes an initial inflammatory response, resulting in cellular infiltration leading to clearance and acquisition of immunity. Primary colonisation results in monocyte/macrophage-mediated clearance and generation of $CD4^+$ memory T cells, resulting in enhanced secondary clearance through recruitment of neutrophils. The balance of Th17 cell versus Treg cell response during carriage is a critical factor in the duration or clearance of colonisation, and may influence the development of the adaptive immune response. A proposed model of the immune response to *S. pneumoniae* colonisation is summarised in figure 1.2.11.

Cell type	Role in carriage (mouse)	Reference
Neutrophils	Insufficient to control colonisation Enhances clearance in secondary colonisation	van Rossum et al., 2005 Zhang et al., 2009
Monocytes/ Macrophages	Sustained recruitment required to clear colonisation	Zhang et al., 2009
B cells/ antibody	Help prevent establishment of colonisation Not required to clear colonisation	Roche et al., 2015 McCool and Weiser 2004
Th17 cells	Promote monocyte/macrophages responses in primary colonisation	Zhang et., 2009
Memory Th17 cells	Enhance neutrophil recruitment in secondary colonisation	Zhang et., 2009
Treg cells	Control density and duration of carriage, TGFβ-dependent	Neill et al., 2014

Table 1.2.11a Roles of immune cells in *S. pneumoniae* carriage in mouse

Cell type	Response to ***S. pneumoniae***	Reference
PBMC $CD4^+$ T cells	Proliferation in response to *S. pn* antigens	Zhang et al., 2007; Mureithi et al., 2009
	Ply does not induce IL-10 and IFNγ production	Zhang et al., 2007
	Predominate over $CD8^+$ T cell responses	Zhang et al., 2007
	Strong Th17 responses with IL-17A production	Lundgren et al., 2012
	IL-22 produced independently of $IL\text{-}17A^+$ cells	Lundgren et al., 2012
NALT $CD4^+$ T cells	Ply induces proliferation of naïve and memory cells	Zhang et al., 2007
	Ply induces IL-10 and IFNγ production	Zhang et al., 2007
	Th17 downregulated in carriage	Jiang et al., 2015
	Th17 numbers increase with age	Mubarak et al., 2016
NALT Treg cells	Higher proportion than in PBMC	Zhang et al., 2011
	Higher proportion in carriage positive than negative	Jiang et al., 2015
	Inhibit $CD4^+$ T cell proliferation, TNFα, IFNγ and IL-17	Zhang et al., 2011
	Antigen-specific effector/memory Treg cells	Zhang et al., 2011
	Suppress $CD4^+$ T cells partly by CTLA4 and PDL-1	Pido-Lopez et al., 2011
	Numbers decrease with age to young adult	Mubarak et al., 2016
	Suppressive ability increases with age	Pido-Lopez et al., 2011
	Control density and duration of carriage	Neill et al., 2014

Table 1.2.11b Comparison of PMBC and NALT in human pneumococcal carriage

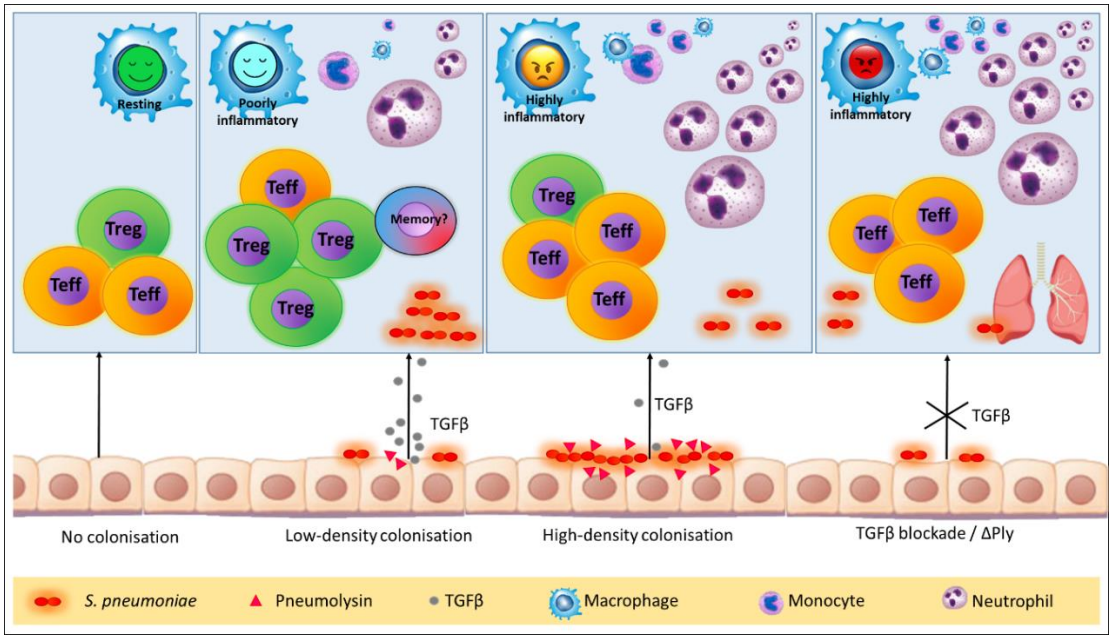

Figure 1.2.11 The immune response to *S. pneumoniae* colonisation

A proposed model of the immune response to *S. pneumoniae* colonisation based on current data from mouse and human studies. *S. pneumoniae* carriage results in increased Treg cells and decreased Th17 cells compared to carriage-negative individuals. Low concentrations of Ply from a low-density *S. pneumoniae* colonisation results in active TGFβ production by nasopharyngeal epithelial cells. This promotes Treg expansion and a controlled infiltration of monocytes, macrophages and neutrophils, delaying bacterial clearance but possibly allowing time for memory responses to develop and preventing bacterial spread. Higher density colonisation causes only transient TGFβ production and no significant Treg expansion, enhancing bacterial clearance but increasing the potential for bacterial spread. Ply is required for epithelial active TGFβ production, and absence of the TGFβ response results in reduced carriage, failure to induce Treg responses, exacerbated neutrophil responses, and transient bacterial translocation to the lungs. This model is based on data from Neill et al., 2014 and Jiang et al., 2015.

1.2.12 Immune response in *S. pneumoniae* pneumonia

Progression from *S. pneumoniae* carriage to pneumonia occurs when pneumococci are aspirated into the lungs in numbers that overwhelm alveolar macrophages, pushing them from maintaining homeostasis to initiating an inflammatory response. *S. pneumoniae* provokes a highly inflammatory response in pneumonia which can cause complications such as septic shock, alveolar consolidation, acute respiratory distress syndrome (ARDS), and invasion of the bacteria through epithelial and endothelial barriers. Within 4 hours, TNFα, IL-1β and IL-6 are present in lung tissue (Bergeron et al., 1998). CXCL8 production is stimulated and attracts neutrophils. Antigen presented by alveolar macrophages or DCs migrate to the lymph node to initiate adaptive responses (Kirby et al., 2009). IL-17A provides protection against pathogens in the lungs by increasing neutrophil recruitment, increasing anti-microbial peptide secretion, and increasing the expression of adhesion molecules (Zhang et al., 2009; Gaffen et al., 2009). In naïve hosts, the development of an adaptive response would take weeks, however, due to priming by previous colonising events, for *S. pneumoniae* this response can occur within days.

The importance of alveolar macrophages in preventing *S. pneumoniae* lung infection is well established, and they are essential for controlling bacterial numbers in the early hours of infection (Green and Kass, 1964; Dockrell et al., 2003; Knapp et al., 2003; Camberlein et al., 2015). *S. pneumoniae* replicate rapidly at early time points to establish lung infection and alveolar macrophages are efficient at controlling the bacteria at a high inoculum (Camberlein et al., 2015). Alveolar macrophage full opsonophagocytic ability is achieved in the presence of specific antibody and complement. As control of infection begins, macrophages can shift to apoptotic and repair phenotypes to promote non-inflammatory resolution. MDMs that have phagocytosed and killed *S. pneumoniae* can undergo apoptosis *in vitro* (Dockrell et al., 2001).

S. pneumoniae replication and host cellular responses in lung homogenates have been tracked in a mouse model of bronchopneumonia using serotype 2 strain D39 (Kadioglu et al., 2000). In this study, *S. pneumoniae* were found to have three phases of growth: an initial decline, followed by a rapid increase peaking at 24-48 hours, then either stasis or decline. *S. pneumoniae* could be detected in the blood at 12 hours. Neutrophil accumulation began after 12-16 hours, which was after the initial bacterial decline, and peaked at 24 hours. Neutrophils were mostly situated in perivascular areas and also the inflamed bronchioles and in bronchiole walls, and decreased by 48 hours. Macrophage numbers remained constant from 0-48 hours in inflamed areas, however more were localised inside inflamed bronchioles and perivascular tissue areas by 24 hours. B and T lymphocyte numbers increased by 24-48 hours. At 24 hours, T lymphocytes were mostly in the tissue surrounding inflamed bronchioles and to a lesser extent in close proximity to the bronchiole walls. At 48 hours, T lymphocytes in the bronchiole walls had decreased whereas those in perivascular tissue and inflamed bronchioles had increased, with more T lymphocytes present across the whole tissue. The accumulation of lymphocytes coincided with the beginning of the decline in *S. pneumoniae* colony forming units (CFU), and the early timing of their presence in the lung may be explained by resident lymphocyte populations. In another study examining the host response in mouse pneumococcal pneumonia, monocytes and lymphocytes were recruited from the blood to the alveoli from 48 hours (Bergeron et al., 1998). Recruited MDMs replenish alveolar macrophages during pneumococcal pneumonia (Taut et al., 2008; Maus et al., 2001).

In naïve mice, *S. pneumoniae* pulmonary infection causes rapid recruitment of T cells resulting in bronchiolar inflammation which is predominantly in areas with dense bacterial presence (Kadioglu et al., 2000). MHC-II knockout mice have been used to investigate the role of $CD4^+$ T cells in *S. pneumoniae* pneumonia (Kadioglu et al., 2004). These mice have 1% $CD4^+$ T cells in the peripheral blood lymphocyte

population, compared to the normal 40-50% in wild types. MHC-II knockout mice had higher *S. pneumoniae* numbers in the lungs and blood compared to wild type mice, and had not cleared the bacteria from the lung and blood by 72 hours, whereas wild type mice cleared the infection by 48 hours. $CD4^+$ T cells migrated across transwells in response to Ply, and collecting the migrated cells and repeating the migration experiment resulted in almost all of the $CD4^+$ T cells migrating for a second time, suggesting that a specific population of the cells migrated to Ply. $CD4^+$ T cells did not require activation in order to migrate, however approximately 44% of these cells activated upon migration as determined by CD25 upregulation. Migration towards *in vivo* passaged *S. pneumoniae* was greater than that for *in vitro* grown *S. pneumoniae*, and migration was not dependent on the cytolytic activity of Ply. However, the $CD4^+$ T cell population migrating to *in vivo S. pneumoniae* would not repeatedly migrate to *in vitro S. pneumoniae*, suggesting that the T cell response to the *in vivo* bacteria was unique to their growth conditions. Overall, this study demonstrates that $CD4^+$ T cells convey an early protective role against pneumococcal pneumonia (Kadioglu et al., 2004). In contrast to these data, $CD4^+$ T cells appeared to be detrimental in a similar mouse pneumonia model possibly related to an excessive inflammatory response (LeMessurier et al., 2010).

Treg cells have been found to have a protective role against invasive pneumococcal pneumonia in mice (Neill et al., 2012). In the study by Neill and colleagues, the responses to intranasal infection with D39 *S. pneumoniae* in BALB/c mice, which are resistant to pneumococcal challenge, and CBA/Ca mice, which are susceptible to respiratory infection, were compared. BALB/c mice had higher *Tgfβ1* expression and had a greater expansion and recruitment of $Foxp3^+$ Treg cells to the lung compared to CBA/ca mice during *S. pneumoniae* pneumonia. Treg recruitment to the lungs was rapid, with significant increase in the first 24 hours, suggesting that these may have been predominantly natural Treg cells, as induced Treg cells may take several days

to differentiate. BALB/c mice had more IL-10^{+} Treg cells than CBA/ca mice, and a population of CTLA4^{+} cells were present in both mouse strains. IFNγ and epithelial cell apoptosis was higher in CBA/ca mice. Blockade of TGFβ reduced Treg cell numbers and made the BALB/c mice more susceptible to bacteraemia, whereas adoptive transfer of Treg cells into CBA/ca mice prolonged their survival. Collectively, these results show an important role for Treg cells in protecting against invasive pneumococcal pneumonia.

Susceptibility to pneumonia is increased by infection with influenza virus through various mechanisms such as disruption of epithelial integrity through tissue damage, upregulation of PAFR, increased CD200R expression, impaired neutrophil function, and increase in type I interferons with subsequent reduced macrophage recruitment (Loosli, 1979; Harford et al., 1949; Hers et al., 1958; Plotkowski et al., 1986; van der Sluijs et al., 2006; Hussell et al., 2009; McNamee and Harmsen, 2006; Nakamura et al., 2011). IFNγ produced by T cells during influenza decreases alveolar macrophage phagocytic ability through downregulation of macrophage receptor MARCO, allowing *S. pneumoniae* to more easily establish subsequent infection (Sun and Metzer, 2008; Ghoneim et al., 2013). Pulmonary IL-10 is increased after influenza infection which may also inhibit *S. pneumoniae* clearance (McNamee and Harmsen, 2006; van der Sluijs et al., 2004). In mouse *Escherichia coli* pneumonia models, recovery from a primary pneumonia induced by *E. coli* or influenza A virus resulted in a more severe secondary *E. coli* pneumonia, with impaired macrophage and DC function associated with increased Treg cells and TGFβ (Roquilly et al., 2017). Another study showed that mice became more susceptible to *Pseudomonas aeruginosa* but not to *Listeria monocytogenes* pneumonia after survival of an initial polymicrobial sepsis (Delano et al., 2013). This suggests that attenuated immune function caused by primary infections predispose to secondary pneumonia dependent on the infecting pathogen.

In summary, alveolar macrophages initiate an inflammatory response when overwhelmed by *S. pneumoniae* in the lung, with pro-inflammatory cytokine presence in the lung within 4 hours. In a mouse model, neutrophils accumulate from 12 hours and peak at 24 hours. Macrophages and T lymphocytes localise to inflamed bronchioles and perivascular areas at 48 hours. The presence of $CD4^+$ T cells has been described as beneficial in some studies and detrimental with excessive inflammation in others. Treg cells protect against invasive pneumococcal pneumonia in mouse models. However, Treg cells are implicated in predisposition to second cases of *E. coli* and *P. aeruginosa* pneumonia.

1.3 Aspects of immunity relevant for this thesis - Macrophage biology

As discussed above alveolar macrophages and recruited monocytes /macrophages play key roles during *S. pneumoniae* infection, controlling bacterial numbers and initiating inflammatory responses as well as presenting antigen to naïve or memory T and B cells. Macrophages can detect pathogens and damage through a wide variety of receptors, triggering signalling pathways and can adapt their phenotype to best aid the host response depending on the specific environmental cues they receive. The sections below describe in detail the key receptors and signalling pathways involved during *S. pneumoniae* interactions with macrophages.

1.3.1 Macrophage receptors

Host cells possessing PRRs recognise PAMPs expressed by pathogens and DAMPs caused by tissue damage. PRRs can be located on the cell surface or on endosomes in the cytosol where they can recognise intracellular pathogens and ingested material. Binding of PRRs and cytokine receptors with their ligands causes signalling through intracellular networks and results in transcriptional and post-translational responses that generate or dampen inflammatory responses. Alveolar macrophages possess a range of PRRs which include TLRs present on the cell surface and phagolysosome membranes. Cytosolic receptors include NOD receptors, retinoic acid-inducible gene-1 (RIG-1) and the inflammasome. Opsonic phagocyte receptors include FcR for antibody and complement receptors. Phagocytic receptors not requiring opsonisation include DC-associated C-type lectin (dectin)-2, DC-SIGN, SRA and MARCO.

1.3.1.1 Toll-like receptors

TLRs are transmembrane proteins with three structural domains: a leucine-rich repeats (LRR) domain, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain. The LRR recognises the pathogen which causes conformational changes and exposes TIR, which initiates signalling by interacting

with signal transduction adaptors. There are ten known human TLRs and thirteen known mouse TLRs. Important roles for TLR2, TLR4 and TLR9 in the immune response against *S. pneumoniae* have been demonstrated. Pneumococcal diacylated lipoproteins are recognised by TLR2-TLR6 heterodimers (Tomlinson et al, 2014). TLR2 neutralisation results in reduced TNFα, IL-6 and CXCL8 production by PBMCs in response to *S. pneumoniae* (Morgensen et al., 2006). Ply is thought to be recognised by TLR4 (Malley et al., 2003; Srivastava et al., 2005). TLR9 recognises unmethylated CpG motifs present in prokaryotic deoxyribonucleic acid (DNA). In a mouse pneumonia model, wild type mice were better able to contain and clear *S. pneumoniae* than TLR9 knockout mice due to impaired macrophage uptake and killing by TLR9 knockout mice (Albiger et al., 2007).

TLR signalling pathways can proceed through the MyD88-dependent pathways or the TIR domain-containing adaptor-inducing IFNβ (TRIF)-dependent pathways. TLRs except TLR3 and endosomal TLR4 signal through MyD88. In the MyD88 pathway, ligand recognition results in TLR dimerization, MyD88, either alone or with TIR adaptor protein (TIRAP), binds to the TIR domain, IL-1 receptor-associated kinase (IRAK)-4 is recruited through the death domain of MyD88, a myddosome complex with IRAK1, IRAK2 and IRAK4 is formed, IRAK1 autophosphorylates, TNF receptor-associated factor (TRAF)-6 activates, which activates TGFβ-activated kinase (TAK)-1/TGF-β-activated kinase (TAB) complex, and phosphorylation of I kappa B alpha (IκBα) by IκB kinase (IKK) occurs. NF-κB essential modulator (NEMO) is a regulatory subunit of the IKK complex. IκBα is an inhibitor of NF-κB, therefore degradation of IκBα allows NF-κB to translocate to the nucleus and induce cytokine gene transcription. NFκB regulates the production of many cytokines, including TNFα, IL-6, IL-1β, IL-2, IL-10, IL-12, CXCL8, MIP-1, MCP-1, CCL5, G-CSF and GM-CSF (Blackwell and Christman, 1997; Saraiva and O'Garra, 2010). The TAK/TAB complex also initiates the MAPK pathway. Through TLR1, TLR2 and TLR4 signalling, TRAF6

can also translocate to the mitochondria and trigger production of ROS (West et al., 2012; Vogel et al., 2007). Invasive pneumococcal disease has been documented in 68% of patients with IRAK4 and MyD88 deficiency (Picard et al., 2010).

The TRIF signalling pathway is thought to be used by TLR3 and TLR4 in mammals. TLR3 recognises double-stranded RNA. Upon recognition, TRIF is recruited and activates TRAF family member-associated NF-kappa-B activator (TANK)-binding kinase (TBK)-1 and RIPK1. The signalling pathway can continue through the TRIF/TBK1 complex by phosphorylating interferon regulatory factor (IRF)-3, which translocates to the nucleus and promotes the production of type 1 interferon. Alternatively, signalling through RIPK1 continues through the same pathway as MyD88 from the TAB1/TAK1/TAB2 complex to promote NFκB and associated cytokine production. TLR4 recognition of PAMPs results in the complex entering the cell by endocytosis where it interacts with TRIF and TIR domain-containing adapter molecule (TRAM), resulting in production of type 1 interferon through IRF3 or NFκB translocation (Sakaguchi et al., 2003). TLR signalling pathways are summarised in figure 1.3.1.1.

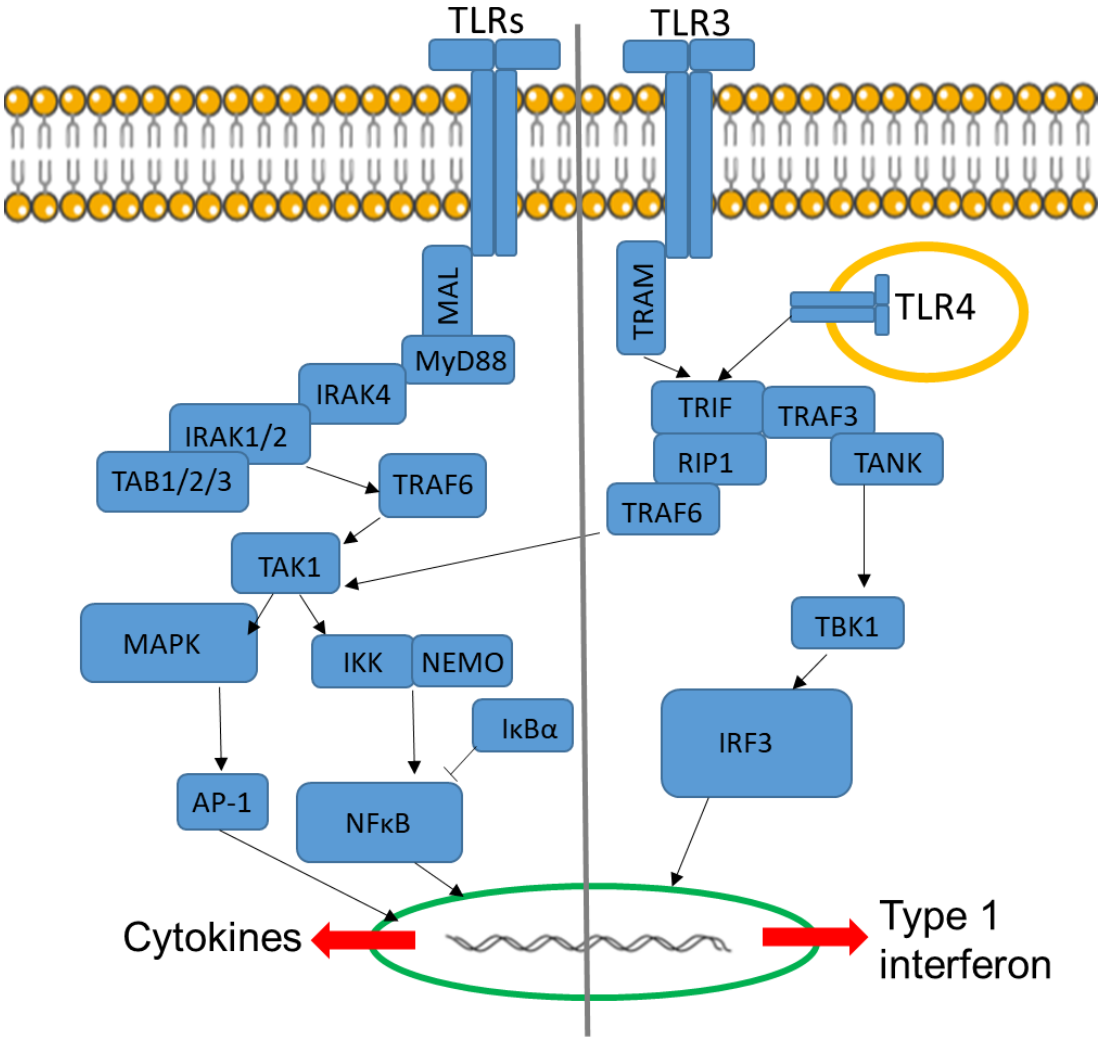

Figure 1.3.1.1 TLR signalling

Binding of most TLRs to their ligands causes recruitment of adaptor proteins MAL and MyD88, signalling cascades resulting in NFκB or AP-1 translocation and production of cytokines. Triggering of TLR3 or endosomal TLR4 causes signalling through the TRAM/TRIF pathway and subsequent IRF3 nuclear translocation to promote type 1 interferon production, or crossover to the NFκB pathway to initiate production of other cytokines.

1.3.1.2 Nucleotide-binding oligomerization domain

NLRs are intracellular PRRs present in the cytosol. Twenty-two have been identified in humans (Chen et al., 2009). NLRs contain a ligand-recognising LRR, a central NOD, and an N-terminal signalling domain, which can consist of a caspase activation and recruitment domain (CARD), pyrin domain, or acidic transactivating domain. NOD1 is expressed by a large number of cell types, whereas NOD2 is primarily expressed on leukocytes and epithelial cells. NOD1 and NOD2 recognise bacterial peptidoglycans and are important in the response against *S. pneumoniae* (Girardin, Boneca et al., 2003; Girardin, Travassos et al., 2003; Inohara et al., 2003; Opitz et al., 2004). Activation of NOD1 and NOD2 causes MAPK signalling and NFκB translocation resulting in cytokine production. NOD2 recognition of peptidoglycan is initiated by lysozyme-dependent digestion of macrophage-phagocytosed *S. pneumoniae*, which activates NFκB (Opitz et al., 2004; Davis et al., 2011). This results in production of CCL2, which recruits monocytes/macrophages, contributing to clearance of colonisation. Muramyldipeptide, a derivative of bacterial peptidoglycan, signals through NOD2 in human DCs to produce IL-23 and IL-1, which then promote IL-17 production (van Beelen et al., 2007).

1.3.1.3 Inflammasome

Inflammasome complexes are assembled upon detection of PAMPs or DAMPs in the cytosol. The inflammasome components NLRP3 and AIM2 recognise *S. pneumoniae*. They activate caspases, which are proteases that promote maturation of IL-1β and IL-18, and induce pyroptotic cell death by forming pores in the plasma membrane. The NLRP3 protein contains a pyrin domain (PYD), which interacts with ASC, and the CARD domain of ASC recruits the CARD domain of pro-caspase-1. Together these components – NLRP3, ASC and pro-caspase-1 – form the NLRP3 inflammasome. The AIM2 inflammasome is composed of AIM2, ASC and pro-caspase-1. NLRP3 inflammasome activation in macrophages requires an

inflammatory stimulus such as TLR activation resulting in pro-inflammatory cytokine production, and recognition of a PAMP/DAMP (Bauernfeind et al., 2009).

Caspase-1 activation and subsequent IL-1β and IL-18 production is Ply-dependent in *S. pneumoniae* infection (Koedel et al., 2002; Shoma et al., 2008). The NLRP3 inflammasome is involved in this response to Ply, and NLRP3 knockout mice are more susceptible to *S. pneumoniae* pneumonia (McNeela et al., 2010). NLRP3 knockout mice can however still produce IL-1β, suggesting the involvement of other inflammasomes. The AIM2 inflammasome recognises cytosolic DNA and also promotes IL-1β production by macrophages infected with *S. pneumoniae* (Fang et al., 2011; Koppe et al., 2012).

1.3.1.4 Cytosolic DNA

S. pneumoniae DNA stimulates type 1 interferon production in macrophages through stimulator of interferon genes (STING), an adaptor molecule implicated in most cytosolic DNA-sensing pathways, and IRF3 (Parker et al., 2011; Koppe et al., 2012). This response required phagocytosis, phagosomal acidification, and cytosolic recognition of DNA. Ply was required but not sufficient to stimulate type I interferon production. Ply may have been responsible for delivery of bacterial DNA into the cytosol following degradation in the phagosome. The resulting type 1 interferon regulates production of CCL5, a chemokine that has an important role in the homing and migration of effector/memory T cells, and is involved in the immune response to *S. pneumoniae* (Palaniappan et al., 2006).

1.3.1.5 C-type lectins

C-type lectins are proteins that bind carbohydrates such as mannose, fructose and glucan structures, and are characterised by the presence of one or more C-type lectin-like domains (CTLDs).

MR is a transmembrane glycoprotein that bind sugars such as mannose and fucose through carbohydrate recognition domains (CRDs). MR is abundantly present on alveolar macrophages and MDMs and enables uptake of non-opsonised microbes. MR is up-regulated by IL-4, IL-13 and IL-10, and down-regulated by IFNγ (Doyle et al., 1994; Harris et al., 1992; Martinez-Pomares et al., 2003; Stein et al., 1992). The membrane-bound receptor can also by cleaved resulting in a soluble form of MR (Martinez-Pomares et al., 1998). MR can bind to capsular polysaccharide of *S. pneumoniae* (Zamze et al., 2002). The signalling pathways used by MR are not known. MR does not contain an immunoreceptor tyrosine-based activation motif (ITAM) or immunoreceptor tyrosine-based inhibition motif (ITIM) but does contain a tyrosine residue in its cytoplasmic tail that is required for endocytosis (Schweizer et al., 2002). Spleen tyrosine kinase (Syk) is phosphorylated upon MR activation by *M. tuberculosis* and co-localises with the MR, suggesting its involvement in signalling (Rajaram et al., 2017). MRC-1 binding Ply downregulates the inflammatory response to *S. pneumoniae* and promotes Treg differentiation from naïve $CD4^+$ T cells (Subramanian et al., 2019).

Dectin-2 is a transmembrane protein consisting of an extracellular carbohydrate recognition domain and signals through an ITAM-containing FcRγ chain. It is expressed by macrophages and DCs (Taylor et al., 2005). The binding of its ligand results in Syk recruitment to the ITAM and activation of CARD9–NF-κB axis. Dectin-2 knockout mice had increased susceptibility to serotype 3 *S. pneumoniae* infection associated with reduced uptake by neutrophils (Akahori et al., 2016). Dectin-2 was essential for IL-12 production, which may contribute to IFNγ production leading to serotype-specific anti-capsular polysaccharide IgG and enhanced opsonisation.

The C-type lectin DC-SIGN and the mouse equivalent specific ICAM-3 grabbing nonintegrin-related 1 (SIGN-R1) binds mannose and ICAM-3 (Geijtenbeek et al., 2000; Park et al., 2001). It is expressed by macrophages, particularly those in the

marginal zone of the spleen. Macrophage SIGN-R1 can bind and internalise *S. pneumoniae* capsular polysaccharide (Kang et al., 2004). SIGN-R1 deficient mice have increased susceptibility to *S. pneumoniae* infection (Lanoue et al., 2004; Koppel, Wieland et al., 2005). SIGN-R1 deficiency was associated with decreased anti-ChoP IgM production, suggesting that recognition of *S. pneumoniae* capsule by macrophage SIGN-R1 promotes B cell IgM production (Koppel, Wieland et al., 2005; Koppel et al., 2008).

Collectins are soluble PRRs that enhance phagocytosis and includes the surfactant proteins SP-A and SP-D which are secreted into the alveoli and bind mannose and glucose residues. They contain a CRD which mediates lectin activity (Kishore et al., 1996). They cause agglutination of microbes, inhibit microbial growth and recruit phagocytes (Jounblat et al., 2004; Madan et al., 1997). SP-A increases phagocytosis of *S. pneumoniae* by alveolar macrophages, and SP-D deficiency reduces neutrophil recruitment to *S. pneumoniae* infection in mice (Sano et al., 2006; Jounblat et al., 2005).

1.3.1.6 Scavenger receptors

Scavenger receptor ligands include modified and non-modified self-molecules, non-opsonized particles and microbial ligands. Class A scavenger receptors comprise a large extracellular domain for ligand recognition, a transmembrane region and a short cytoplasmic domain. This group includes SRA and MARCO.

SRA is constitutively expressed on macrophages and has roles in endocytosis of modified proteins and phagocytosis of apoptotic cells and microbes. SRA can bind *S. pneumoniae* without opsonisation, and SRA deficiency in mice resulted in impaired phagocytosis and reduced clearance of *S. pneumoniae* with increased pro-inflammatory cytokine production (Arredouani et al., 2006).

MARCO expression is constitutive on certain macrophage subsets and is induced by inflammation in others (Elomaa et al., 1995; van der Laan et al., 1997). It can bind *S. pneumoniae* without opsonisation. MARCO has also been demonstrated to be important in lung defence against *S. pneumoniae* in a mouse pneumonia model, in which MARCO deficient mice had reduced phagocytosis by macrophages, decreased bacterial clearance and decreased pro-inflammatory cytokine production compared to wild type mice (Arredouani et al., 2004). In a mouse model of *S. pneumoniae* colonisation, MARCO knockout mice had impaired clearance, cytokine production and leukocyte recruitment (Dorrington et al., 2013). MARCO does not itself trigger inflammation, however it augments TLR2 and NOD2 signalling (Dorrington et al., 2013).

1.3.7 Endocytosis

Endocytic activity in macrophages includes phagocytosis and efferocytosis. Phagocytosis is the cellular uptake of particles larger than 0.5μm within a plasma membrane-derived vacuole called the phagosome (Gordon, 2016). Recognition of particles by non-opsonic receptors such as MR and MARCO, or by opsonic receptors such as FcγR or complement receptors, initiates signalling cascades that induce phagocytosis. Clustering of phagocytic receptors results in phosphorylation of ITAMs that activates Rho-family GTPases, which activate nucleation-promoting factor, allowing for the actin-driven extension of the plasma membrane around the phagocytic target (Levin et al., 2016). The resulting phagosome then undergoes a process of maturation in which it fuses with other membrane-bound intracellular compartments such as endosomes and lysosomes, resulting a mature phagolysome with degradative enzymes. During maturation, the phagosome acidifies. ROS, RNS and NO can form in the phagolysosome, and anti-microbial peptides are delivered into the phagolysosome from cytoplasmic granules.

Efferocytosis is the process by which apoptotic cells are removed. Apoptosis is a non-inflammatory process in which the dying cell is contained and engulfed by a phagocyte. This process differs from phagocytosis, as RhoA activity is suppressed and Rac1 activity coordinates the engulfment of the apoptotic body into an efferosome. Following this, the process is similar to that of phagocytosis as the efferosome matures with gradual acidification and enzymic degradation of its contents. Macrophages are attracted to the dying cells by exposure of phosphatidylserine and release of chemokines by these cells. As a result of efferocytosis, macrophages produce IL-10 and TGFβ, which contributes to resolution of inflammation (Martin et al., 2014).

1.3.8 Macrophage phenotypes

Macrophages can adopt a wide variety of phenotypes dependent on the specific conditions of their environment. The phenotype they develop can be important for roles such as wound healing, immune regulation, and host defence (Mosser and Edwards, 2008). The presence of pro-inflammatory cytokines such as IFNγ and pathogen components such as LPS encourages macrophages to adopt a phenotype to aid host defence (Nathan et al., 1983). These macrophages produce anti-microbial ROS and RNS, high levels of pro-inflammatory cytokines, and promote the generation of a Th1-type immune response. They typically express inducible nitric oxide synthase (iNOS), and the chemokines CXCL9, CXCL10 and CXCL11, which bind to CXCR3 expressed primarily on Th1 cells, $CD8^+$ T cells and NK cells. They restrict the expression of wound-healing markers through SOCS3, which restricts responsiveness to IL-4 (Gordon et al., 2016). The products of these macrophages can result in damage to host tissues.

By contrast, macrophages can adopt a wound-healing role in the presence of IL-4 and/or IL-13 (Stein et al., 1992). They produce growth factors and extracellular matrix components and promote Th2 responses. IL-4 stimulates arginase activity in

macrophages, promoting conversion of arginine to ornithine, a precursor of polyamines and collagen, resulting in extracellular matrix production (Rath et al., 2014). *In vitro*, macrophages primed with IL-4 and/or IL-13 reduced antigen presentation to T cells, produced low levels of pro-inflammatory cytokine, had inefficient ROS and RNS production, and reduced killing of intracellular pathogens, compared with macrophages primed with IFNγ and LPS (Edwards et al., 2006). IL-4 primed macrophages also increase MR expression (Stein et al., 1992). IL-27 induces STAT3 and inhibits macrophage production of pro-inflammatory cytokines such as IL-12/23p40, and IL-4-primed macrophages increase IL-27Rα expression (Rückerl et al, 2006).

Macrophages can also have an anti-inflammatory role which can be promoted by various stimuli such as immune complexes, apoptotic cells, IL-10 and prostaglandin. These macrophages produce large quantities of IL-10 in the presence of these stimuli plus a signalling stimulus such as TLR activation (Edwards et al., 2006). Despite anti-inflammatory IL-10 production, these macrophages retain the ability to produce pro-inflammatory cytokines (Mosser and Edwards, 2008). Macrophages primed by these anti-inflammatory stimuli are not considered to be involved in extracellular matrix production but do have high antigen presentation capacity due to high expression of CD80 and CD86 (Edwards et al., 2006).

Adaptation of the macrophage to its specific environmental cues allows it to adopt roles most appropriate to the response that is required. Their adaptation can come from both innate and adaptive immune cells, for example IFNγ can be produced early in infection by NK cells, and later in infection by Th1 cells. Similarly, basophils and mast cells provide an early source of IL-4, followed by Th2 cells later on. Macrophages are reported as being capable of modifying their roles as the immune response progresses and their environmental cues change, for example IL-4-primed macrophages with wound-healing phenotype activity can be made to produce IL-10

by subsequent addition of immune complexes (Edwards et al., 2006; Gratchev et al., 2006; Stout et al., 2005; Davis et al., 2013). An overview of some of the signalling pathways skewing macrophage inflammatory potential is shown in figure 1.3.8.

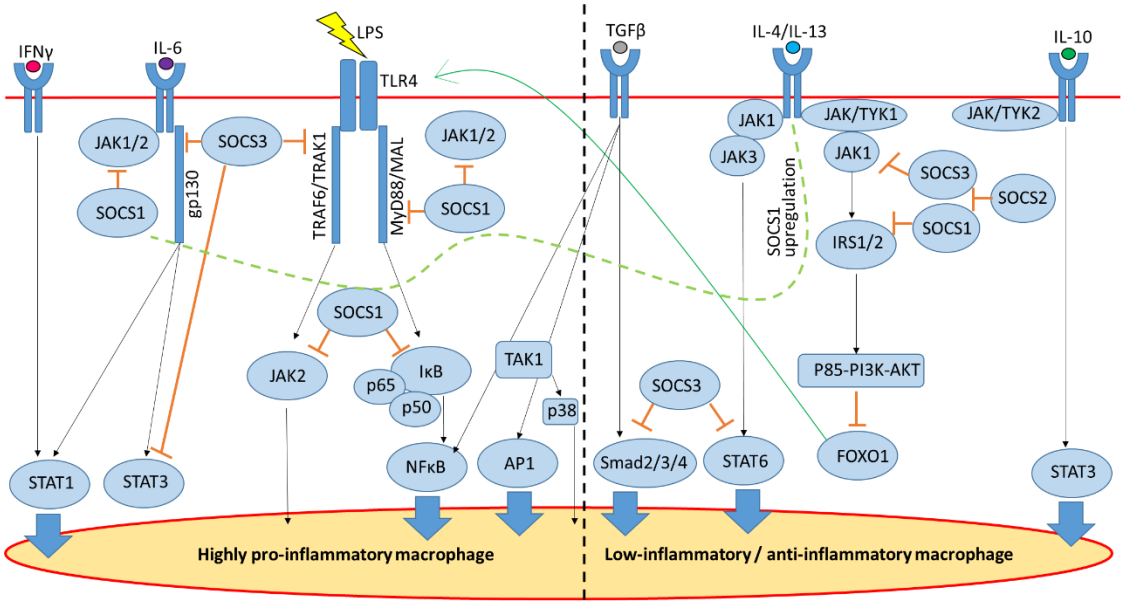

Figure 1.3.8 Signalling pathways in macrophage polarisation

Macrophages can adopt highly pro-inflammatory or low/anti-inflammatory phenotypes depending on the signals they receive. Interaction with pro-inflammatory cytokines such as IFNγ or IL-6 triggers signalling through STAT1 resulting in a highly pro-inflammatory response from the macrophage. Stimulation of TLR4 by ligands such as LPS causes signalling through Janus kinase (JAK)-2 or NFκB translocation resulting in a highly pro-inflammatory response from the macrophage. Interaction with IL-4 or IL-13 results in JAK signalling, STAT6 translocation, upregulation of SOCS1 and prevention of Forkhead box O (FOXO)-1 from upregulating TLR4, all skewing the macrophage to an low/anti-inflammatory phenotype. IL-10 interaction results in STAT3 signalling and low/anti-inflammatory activity. Interaction with TGFβ can result in pro-inflammatory skewing of the macrophage through TAK1 signalling, causeing p38 signalling and NFκB or activator protein 1 (AP1) translocation. Alternatively, TGFβ can reduce the inflammatory activity of macrophages through Smad signalling.

1.3.9 Apoptosis

Apoptosis occurs when the burden of ingested *S. pneumoniae* overwhelms the macrophage (Ali et al., 2003). Apoptosis occurs 16-20 hours after *S. pneumoniae* ingestion and requires NO generation (Marriott et al., 2004). This delay allows maximal time for bacterial killing by other mechanisms, and is achieved by a gradual decrease in expression of the apoptosis-regulator myeloid cell leukemia (Mcl)-1 during exposure to *S. pneumoniae* (Schubert et al., 2001; Marriott et al., 2005). Permeabilisation of the lysosome membrane activates a mitochondrial pathway involving the protease cathepsin D, which activates and allows interaction with Mcl-1 ubiquitin ligase (Mule) instead of heat shock protein (Hsp)-70, and down-regulates eukaryotic elongation factor (eEF)-2, a component required for Mcl-1 translation (Bewley et al., 2011; Bewley, Pham et al., 2011). TLR4 is involved in enhancing the apoptotic response in *S. pneumoniae* infection (Srivastava et al., 2005). Prevention of apoptosis promotes bacterial-driven necrosis and reduces bacterial killing (Marriott et al., 2005; Bewley et al., 2011; Marriott et al., 2004). In mice with impaired apoptosis, *S. pneumoniae* killing is decreased in the lung and is associated with increased bacteraemia and mortality (Fadok et al., 1998; Dockrell et al., 2003; Marriott et al., 2005; Bewley et al., 2011; Srivastava et al., 2005). The inflammatory response is downregulated as apoptotic macrophages are ingested by lung macrophages, reducing the production of pro-inflammatory cytokines, reduction of neutrophil recruitment, and triggering release of anti-inflammatory cytokines such as IL-10 and TGFβ (Marriott et al., 2007; Marriott et al., 2006; Fadok et al., 1998; Voll et al., 1997; McDonald et al., 1999). Apoptosis of *S. pneumoniae*-containing macrophages does not involve Fas ligand, and instead involves a mictochondrial-dependent, caspase-dependent pathway (Dockrell, 2001; Marriott et al., 2004; Dockrell et al., 2003; Dockrell et al., 2001). Neutrophils can aid in inducing macrophage apoptosis by

secreting TNF-related apoptosis-inducing ligand (TRAIL) in response to *S. pneumoniae* infection (Steinwede et al., 2012).

1.4 Aspects of immunity relevant for this thesis - Treg biology

In 1970, Gershon and Kondo discovered that T cells can not only augment but also down-regulate immune responses (Gershon and Kondo, 1970). Sakaguchi and colleagues demonstrated that depletion of $CD4^+CD5^+$ cells from mice resulted in development of autoimmunity, and later discovered CD25 as a marker of $CD4^+$ Treg cells (Sakaguchi et al., 1985; Sakaguchi et al., 1995). In 2001, mutations in *Foxp3* were found to cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome which is characterised expression of multiple autoimmune disorders (Bennett et al., 2001). Foxp3 was then discovered to be essential in Treg development and function (Fontenot et al., 2003; Hori et al., 2003).

1.4.1 Regulatory cell types

The best characterised and most widely studied regulatory cells are the $CD4^+CD25^+Foxp3^+$ Treg cells, which constitute 5-10% of peripheral $CD4^+$ T cells. Several other types of $CD4^+$ regulatory cells have been described. For example, IL-10-producing type 1 regulatory T (Tr1) cells and TGF-β-producing $CD4^+$ Th3 cells also demonstrate regulatory activity (Groux et al., 1997; Gagliani et al., 2013; Chen et al., 1994; Sakaguchi et al., 1995; Apostolou and von Boehmer, 2004; Curotto de Lafaille, 2004). Regulatory $CD8^+$ T ($CD8^+$ Treg) cells have also been described (Gershon and Kondo, 1970; Probst et al., 2005; Endharti et al., 2005). In addition, myeloid-derived suppressor cells (MDSCs), regulatory B (Breg) cells, regulatory γδ T cell (γδ-Tregs), immunosuppressive plasmocytes (ISPC), and a regulatory subset of ILCs all have suppressive functions (Gabrilovich et al., 2009; Wolf et al., 1996; Kang et al., 2009; Mizoguchi et al., 2002; Wang, Xia et al., 2017).

Tr1 cells are identified by $CD4^+CD49b^+LAG\text{-}3^+CD226^+$ induced by chronic activation of $CD4^+$ T cells by antigen in the presence of IL-10 (Gagliani et al., 2013). They do not normally express CD25 or Foxp3, however they do express co-stimulatory

molecules and regulatory factors such as CTLA4, programmed cell death protein 1 (PD-1), human leukocyte antigen – DR (HLA-DR), CD69, CD28, glucocorticoid-induced TNF receptor (GITR) and tumor-necrosis factor receptor TNFRSF9 (Zeng et al., 2015). Their cytokine expression profile is described as IL-10^{++}TGFβ^{+}IFNγ^{+}IL-5^{+}IL-4−IL-$2^{low/neg}$ (Groux et al., Bacchetta et al., 2002). TR1 cells can be induced from $CD4^{+}$ cells *in vitro* by APCs in the presence of IL-21, IL-6, IL-27 and IL-10 (Wang et al., 2011; Zheng et al., 2015).

Th3 cells can differentiate from naïve $CD4^{+}$ T cells and produce large quantities of TGFβ. They were first discovered in mesenteric lymph nodes and produced TGFβ after oral administration of self-antigen (Chen et al., 1994). Foxp3 and CD25 expression in this subset is contentious, as some report their expression and others do not (Ramsdell, 2003; Chien and Chiang, 2017). A specific Th3 transcription factor has not been identified. Th3 cells are reported as expressing LAP (Oida et al., 2003).

There is emerging evidence that Treg cells are also present in various non-lymphoid tissues. These tissue Treg have unique phenotypes and activities dependent on anatomical location, for example in the skin and intestine (Ali et al., 2017; Makita et al., 2007). Treg cells also have a role in tissue repair of infectious injury in the lung (Arpaia et al., 2015).

1.4.2 Regulatory T cell development

There are two sub-types of Treg cells that develop *in vivo* by different pathways. Thymic Treg (tTreg) develop as dedicated regulatory cells while in the thymus, while peripheral Treg (pTreg) develop from naïve $CD4^{+}$ T cells outside of the thymus in response to a stimulus triggering a regulatory phenotype. Collectively, *in vivo*-generated tTreg and pTreg are sometimes termed “natural” Treg (nTreg). Currently, there is no definitive marker to distinguish between tTreg and pTreg. When Treg cells are generated *in vitro* they are termed “induced” Treg (iTreg), however no conditions

have been found to stably commit a T cell to the Treg lineage. All $CD4^+$ T cells develop from thymocytes in the thymus. Thymocytes undergo positive and negative selection during TCR rearrangement. Treg cells receive stronger TCR signals than conventional T cells, which is associated with induction of Treg-specific epigenetic change and gene expression (Moran et al., 2011; Morikawa and Sakaguchi., 2014). In the earliest stage of thymopoiesis, the thymocytes are double-negative $CD4^-CD8^-$, then become double positive $CD4^+CD8^+$, and finally mature into a single positive state expressing either CD4 or CD8. During these processes, a progenitor Treg population expressing a $CD25^+CD4^+$ phenotype develops through high-affinity interactions with self-peptide presented on MHC-II molecules (Hsieh et al., 2004; Jordan et al., 2001). This population can then induce Foxp3 expression by IL-2/IL-15 and exit the thymus as tTreg cells (Lio and Hsieh, 2008). Consequently, tTreg expressing a TCR repertoire biased to self are thought to be important in the prevention of autoimmunity and preventing exaggerated immune responses (Curotto de Lafaille and Lafaille, 2009). Alternatively, pTreg cells can develop from $CD4^+Foxp3^-$ cells that have exited the thymus in response to antigen-stimulation in a process requiring IL-2 and TGFβ *in vitro* (Chen et al., 2003). pTreg cells are thought to be important in tolerance to commensal bacteria (Lathrop et al., 2011). Both tTreg and pTreg are believed to be involved in the maintenance of homeostasis (Curotto de Lafaille and Lafaille, 2009).

1.4.2.1 Foxp3

Foxp3 is critical for Treg function, development and lineage stability (Hori et al., 2003; Gavin et al., 2007). There are four regions of *Foxp3* that are susceptible to epigenetic modification in conserved noncoding sequences (CNS) of the DNA. CNS0 is involved in initiating Treg-SE (specific super-enhancers) activation to induce Foxp3 expression (Kitagawa et al., 2017). The global chromatin organizer Special AT-rich sequence-binding protein-1 (Satb1) binds to CNS0 in immature $CD4^+CD8^+$ double positive thymocytes and modifies the epigenetic status of the *Foxp3* locus to a state that

allows other transcription factors to bind to regulatory elements which is required for Treg development, suggesting that early epigenetic modifications control the expression of Foxp3 and Treg signature genes (Kitagawa et al., 2017). CNS1 is required for TGF-β-induced *Foxp3* induction in peripheral $CD4^+$ T cells but not in thymocytes and contains binding sites for Smad3, NFAT1 and AP-1 (Tone et al., 2008; Zheng et al., 2010). CNS2 is known as Treg-cell-specific demethylation region (TSDR). The CpG motifs in this region are methylated in conventional T cells and demethylated in tTreg cells, and histones near this region are acetylated in Treg cells. CNS2 is activated by TCR expression and IL-2 and has transcription factor binding sites such as runt-related transcription factor 1 (RUNX1), which forms a complex with core-binding factor β (Cbfβ), and its binding to CNS2 maintains a high and stable level of Foxp3 expression in Treg cells (Kim and Leonard, 2007; Kitoh et al., 2009). Demethylated CNS2 is a definitive marker of Treg lineage commitment highlighted by the finding that transcription factors that induce *Foxp3* expression cannot bind to CNS2 without demethylation which results in loss of *Foxp3* expression and that the methylation status of the *Foxp3* locus is actively maintained in T cells (Mouly et al., 2010; Josefowicz et al., 2009). Vitamin C facilitates demethylation of CNS2 of *Foxp3* (Sasidharan Nair et al., 2016). CNS3 is important for Foxp3 induction during thymic and peripheral Treg differentiation and increases Treg frequency (Zheng et al., 2010). Promoter region CpG motifs are partially methylated in naïve $CD4^+$ T cells and are demethylated in Treg cells.

Several transcription factors are involved in development of Treg cells. Forkhead box O (FOXO) transcription factors have an essential role in the pathway leading to development and function of Treg cells (Kerdiles et al., 2010). FOXO regulate thymic and TGFβ-induced Foxp3 expression, bind CNS1 and CNS3 and control Foxp3 promoter activity (Ouyang et al., 2010). They also regulate the expression of additional Treg cell-associated genes such as *Ctla4* and inhibit the acquisition of

effector T cell characteristics by Treg cells (Ouyang et al., 2010). The transcription factor c-Rel can also transactivate the *Foxp3* gene and c-Rel deficiency reduces tTreg cell generation (Ruan et al., 2009). Functions of c-Rel during *Foxp3* transcription include binding and demethylation of CNS2, binding to the promoter and forming a c-Rel enhanceosome over the *Foxp3* locus, and CNS3 binding to trigger *Foxp3* induction through TCR and co-stimulatory signals (Long et al., 2009; Ruan et al., 2009; Zheng et al., 2010). Foxp3 expression is also modulated by Smad3 binding CNS1 upon TGFβ signalling and NFAT binding CNS1 upon TCR signalling (Tone et al., 2008). AP-1 also binds CNS1 to transactivate Foxp3 induction, whereas STAT3 binding to CNS2 silences Foxp3 transcription (Xu et al., 2010). IL-2 signalling plus Stat5 binding to CNS2 prevents other cytokine signalling influencing the Treg identity of the cell and maintains *Foxp3* transcription (Xu et al., 2010). Epigenetic regulation of Treg-signature genes required for suppressive function including demethylation of *Ctla4*, *Il2ra*, *Tnfrsf18* and *Ikzf4* are not dependent on *Foxp3* expression but require TCR signalling (Okhura et al., 2012). *Foxp3* gene expression can be negatively regulated by factors promoting the differentiation of other T cell subsets. IL-4 upregulates GATA-3 (the Th2 master transcription factor) which prevents *Foxp3* expression upon binding to the *Foxp3* promoter region (Mantel et al., 2007; Hadjur et al., 2009). IL-6 signalling inhibits Foxp3 expression and promotes Th17 differentiation of naïve $CD4^+$ T cells (Betelli et al., 2006; Yang et al., 2011). TGFβ-induced Foxp3 induction prevents Th17 transcription factor RORγt activity (Zhou et al., 2008). However, TGFβ and IL-6 signalling combined induces RORγt induction and prevention of Foxp3 expression (Burgler et al., 2010).

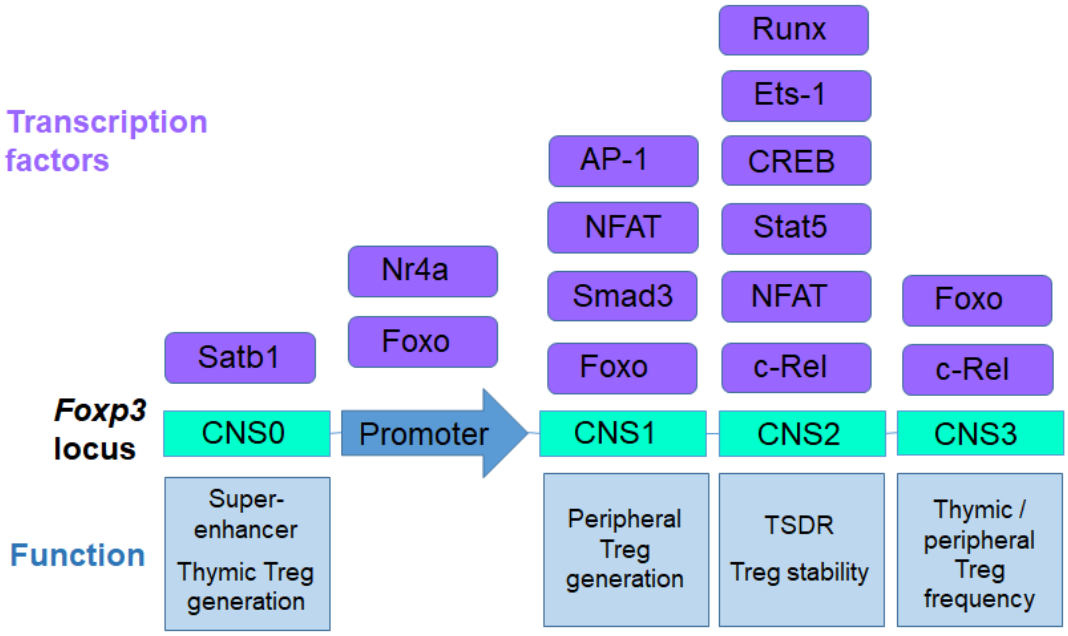

Figure 1.4.2 Transcriptional regulation of the Foxp3 locus

Transcription factors can bind to regulatory regions of the *Foxp3* locus to modulate *Foxp3* expression. CNS0 is required for Treg development in the thymus, whereas CNS1 is involved in peripheral Treg generation. CNS2 is known as TSDR and demethylation of CNS2 is a definitive marker of Treg lineage commitment. CNS3 increases the frequency of tTreg and pTreg (reviewed by and diagram modified from Lee and Lee, 2018).

1.4.2.2 Glucocorticoid-induced TNF-related receptor

Glucocorticoid-induced TNF-related receptor (GITR) is a member of the TNFR superfamily that is a co-stimulatory molecule of T cell subsets (Ronchetti et al., 2004). Treg cells express high levels of GITR, whereas resting Teff cells express low levels that are increased upon activation. GITR is triggered by the ligand GITRL, which is mainly expressed by APCs and endothelial cells (Lacal et al., 2013; Azuma, 2010). GITR co-stimulation activates TCR-triggered T cell proliferation (Kanamaru et al., 2004). GITR is reported in mice and humans to be involved in the differentiation of tTregs, and expansion of both tTregs and pTregs (Ronchetti et al., 2015). Treg progenitors express high levels of three TNFRSF members: GITR, OX40, and TNFR2. Combined neutralization of their ligands prevents Treg development and co-stimulation of GITR and OX40 increases sensitivity to low doses of IL-2. Therefore, Treg progenitors with the highest affinity TCRs and the highest expression of GITR, OX40, and TNFR2 compete more effectively for the respective ligands and are more likely to differentiate into mature Treg cells (Mahmud et al., 2014). As a consequence, GITR expression is high in mature tTregs. In the presence of IL-2 and anti-CD3 antibody, GITR activation promotes proliferation of both Teff and Treg cells; however, $FoxP3^+$ cells exhibit a stronger response compared to that in $FoxP3^-$ cells, therefore Treg proliferation is preferentially enhanced (Liao et al., 2010).

1.4.2.3 Helios

The transcription factor Helios has been reported as a marker of activated and highly suppressive Treg cells (Elkord et al., 2015). In the study by Elkord et al., human $FoxP3^+Helios^-$ Treg cells secreted IL-10, IFN-γ and IL-2, whereas $FoxP3^+Helios^+$ Treg cells secreted IL-10 without IFN-γ and IL-2. These $Helios^+$ Treg also express LAP and glycoprotein A repetitions predominant (GARP) in the steady state, which are upregulated by activation regardless of Foxp3 expression. Helios was originally described as a specific marker of tTreg cells, although evidence to the contrary exists

(Thornton et al., 2010; Gottschalk et al., 2012; Thornton et al., 2018; Elkord, 2016). Helios induces epigenetic silencing of IL-2 expression and thereby regulates the functional stability of Treg cells (Baine et al., 2013). Loss of Helios causes IL-2 expression, increased proliferation, and impaired suppressive ability in Treg cells (Baine et al., 2013). Foxp3 expression is reported to be more stable in Helios$^+$ Treg cells, suggesting a role for Helios in the maintenance of Treg suppressive function (Thornton et al., 2019). Helios$^+$ Treg cells are considered important in immune homeostasis. For example, reduced frequency of Helios$^+$ Treg cells is associated with autoimmune diseases including myasthenia gravis and type I diabetes mellitus (Xu et al., 2012; Du et al., 2013). In mice, Helios$^+$ Treg cells have been found to be more potent suppressors of T cell proliferation and cytokine production than Helios$^-$ Treg cells (Sugita et al., 2015).

1.4.3 Regulatory T cell markers

Currently the most widely used marker for Treg cells is the transcription factor Foxp3. Foxp3 is constitutively expressed by Treg cells. However, Foxp3 can also be expressed by activated T cells, and therefore is not an exclusive marker. Foxp3 requires intracellular staining, making it unsuitable for cell sorting for live cell populations. A commonly used surface-staining strategy to isolate live Treg cells is CD4$^+$CD25$^+$CD127^{low} due to Treg cells expressing high levels of CD25 and low levels of CD127, and the correlation of CD25$^+$CD127^{low} with Foxp3 expression. Difficulty arises in distinguishing Treg from Teff cells upon activation of Teff cells, as they also upregulate CD25 and Foxp3. There is currently no definitive marker of Treg cells, therefore functional assays to prove suppressive ability of the isolated cell population are usually carried out alongside experiments. In addition, there is also no marker to distinguish between thymic-derived or peripherally-induced Treg cells. Expression of several other molecules are also used as markers for Treg cells. Treg cells constitutively express Foxp3, CTLA4 and GITR. Resting Treg cells express higher

levels of CTLA4, GITR, PD-1, PD-L1, CD39, CD73, TNFR2, Helios, LAP and GARP than conventional T cells (Takahashi et al., 2000; Annunziato et al., 2002; Allard et al., 2017; Sugimoto et al., 2006; McHugh et al., 2002; Nakamura et al., 2001; Andersson et al., 2008; Stockis, Colau et al., 2009; Wang et al., 2009)

1.4.4 Regulatory T cell suppressive mechanisms

Treg cells are an essential controller of immune responses including in inflammation, autoimmunity, and allergy. Deficiency in Treg cells in mice and humans results in fatal autoimmunity, associated with inflammatory damage to numerous organs, including the lung, stomach, intestine, pancreas, liver, and skin (Fontenot et al., 2003; Khattri et al., 2003; Kim et al., 2007). A range of mechanisms of Treg-mediated suppression have been described, including cell-contact independent secretion of cytokines, cell-contact dependent association through surface components such as CTLA4, metabolic disruption through IL-2 deprivation and cyclic AMP (cAMP) /adenosine production, and release of cytotoxic mediators. An overview of some of the suppressive mechanisms used by Treg cells is shown in figure 1.4.4.

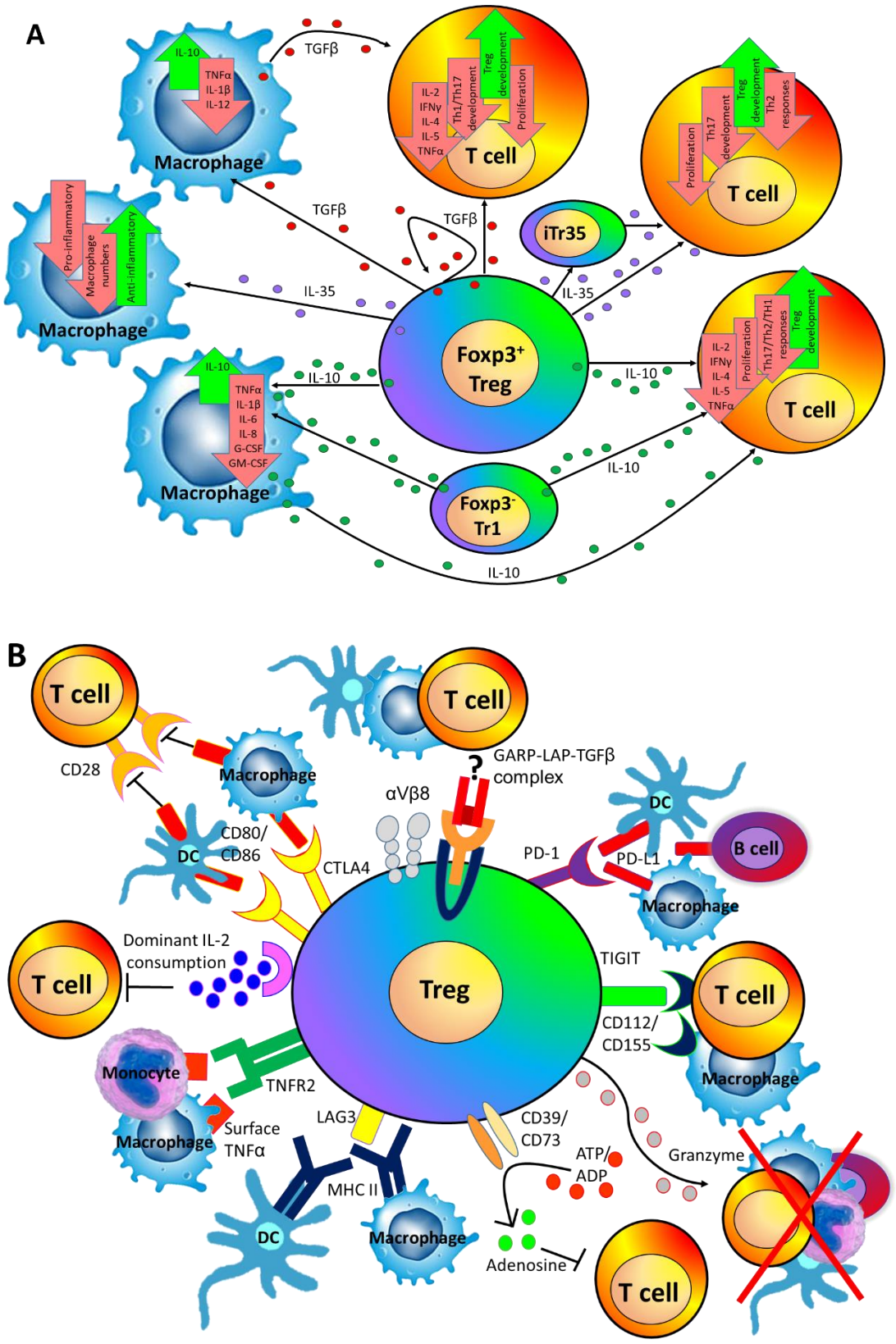

Figure 1.4.4 Treg suppressive mechanisms

A diagram showing some of the mechanisms used by Treg cells involving secreted cytokines (A) and direct cell-contact (B) to suppress immune responses.

1.4.4.1 Interleukin-10 secretion

IL-10 production is considered to be a mechanism of contact-independent suppression used by Treg cells (Vignali et al., 2008). In particular, IL-10 secretion is a major suppressive function of $Foxp3^-$ Tr1 cells. *In vitro*, there is little evidence of $Foxp3^+$ Treg cells producing IL-10, however this contrasts with *in vivo* data, particularly at environmental interfaces (Uhlig et al., 2006; Maynard et al., 2007; Rubtsov et al., 2008). Mice with IL-10-deficient $Foxp3^+$ Treg cells developed spontaneous colitis, increased skin hypersensitivity and increased lung hyperreactivity (Rubtsov et al., 2008). Evidence suggests that both thymic and antigen-specific Treg cells control allergy and asthma in a manner partly dependent on IL-10 (Hawrylowicz and O'Garra, 2005). IL-10 can inhibit $CD4^+$ T cell proliferation and production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α (Couper et al., 2008).

1.4.4.2 Interleukin-35 secretion

IL-35 was first described in 2007 as an anti-inflammatory cytokine specifically produced by Treg cells and has suppressive activity against T cells (Collison et al., 2007). In this study by Collison et al., IL-35 was indispensable for maximal suppressive ability and could inhibit Teff cell proliferation. IL-35 can suppress Th17 differentiation (Niedbala et al., 2007). Intratracheal administration of IL-35 attenuated allergic airway inflammation through reduction of Th2 and antibody responses in mice (Huang et al., 2011). IL-35 has also been reported to reduce macrophage numbers and increase the proportion of anti-inflammatory macrophages compared to inflammatory macrophages in the spleen and skin in a mouse model of psoriasis (Zhang, Lin et al., 2016). IL-35 has been found to improve wound healing and promote the survival of macrophages in a mouse model of myocardial infarction (Jia et al., 2019). Although Treg cells have been considered the primary source of IL-35, this cytokine has been reported to be produced by Breg cells, $CD8^+$ Treg cells and tumour-associated macrophages (Shen et al., 2014; Olson et al., 2012; Lee, Lin et al., 2018).

IL-35 also induces a population of regulatory cells which themselves produce IL-35, termed iTr35 cells (Collison et al., 2010). In mouse models, iTr35 cells were found to be protective in against experimental autoimmune encephalomyelitis (EAE), could restore homeostasis in Foxp3 knockout mice, ameliorate inflammatory bowel disease, and prevent anti-tumour responses (Collison et al., 2010). Early studies failed to detect IL-35 production by human Treg cells (Bardel et al., 2008), although IL-35 has been reported in human samples taken from various patient populations. For example, IL-35 has been reported to be decreased in rheumatoid arthritis patients compared to healthy controls, and IL-35 treatment enhanced Treg regulatory function and decreased pro-inflammatory cytokine levels *in vitro* (Nakano et al., 2015). IL-35 production by human Treg cells in response to viral infections has also been demonstrated (Langhans et al., 2010; Seyerl et al., 2010).

1.4.4.3 Secreted and surface-bound TGFβ

Two molecules involved in the expression and activation of TGFβ are LAP and GARP. LAP is reported to be expressed on the surface of activated Treg cells, but not conventional T cells (Nakamura et al., 2001; Andersson et al., 2008). The transmembrane protein glycoprotein A repetitions predominant GARP is highly expressed on mouse and human Treg cells (Zhou et al., 2013; Noyan et al., 2014). GARP is present at low levels on resting Treg cells and is increased upon TCR activation, whereas GARP expression remains very low on activated Teff cells (Stockis, Colau et al., 2009; Wang et al., 2009). TGFβ-induced Foxp3^{+} human T cells do not express GARP (Wang et al., 2009).

LAP prevents TGFβ binding to its receptor. LAP and TGFβ form inactive complexes on the cell surface (latent TGF-β complexes). GARP anchors the complexes to the cell membrane (Tran et al., 2009; Stockis, Colau et al., 2009). Active TGFβ is released upon cleaving of this complex. Integrin αVβ8 is required in this process and these are present on the Treg cell surface (Stockis, Colau et al., 2017). Therefore, Treg cells

can activate GARP-presented latent TGFβ through αVβ8 on their surface. Recombinant LAP can prevent suppressive ability of $CD4^+CD25^+$ cells from humans and mice (Nakamura et al., 2004). In a mouse colitis model, $CD4^+LAP^+$ cells from normal donor mice were protective against colitis, whereas $CD4^+LAP^-$ cells were not (Nakamura et al., 2004). $CD25^+GARP^+$ cells have increased suppression of Teff proliferation *in vitro* compared to $CD25^+GARP^-$ cells (Wang et al., 2009). $GARP^+$ cells produce abundant IL-10 and TGFβ (Wang et al., 2012; Rappl et al., 2011; Zhong et al., 2016). Naïve human T cells with overexpression of GARP exhibit a Treg phenotype, whereas gene silencing of GARP in Treg cells results in a moderate decrease in suppressive ability (Probst-Kepper et al., 2009; Wang et al., 2008; Wang et al., 2009). Released mature TGFβ can act in an autocrine manner to promote Treg function and can promote the differentiation of pTreg. It also inhibits the differentiation of Th1 and Th17 cells and suppresses proliferation and cytokine secretion of Teff cells (Nakamura et al., 2004). APC functions are downregulated by TGFβ (Tsunawaki et al., 1988). In addition, cell-surface TGFβ has been found to have suppressive roles, although these are not yet fully defined. The findings that $CD4^+CD25^+$ T cells suppress proliferation of $CD4^+CD25^-$ T cells in a contact-dependent manner, that the abolishment of TGFβ prevents suppression, and that TGFβ is expressed on the $CD4^+CD25^+$ cell surface suggests that $CD4^+CD25^+$ T cells can suppress target cells through cell contact through cell surface TGFβ (Nakamura et al., 2001). Several other studies have reported Treg suppression by cell-surface TGFβ (Chen et al., 2003; Oida et al., 2003; Gregg et al., 2004; Shen et al., 2011). Treg-mediated infectious tolerance is reported to be conferred in mice through membrane-bound TGF-β by inducing new $CD4^+FoxP3^+$ T cells that are suppressive *in vivo* and *in vitro* (Andersson et al., 2008). A study using mouse models of autoimmune diabetes found that young mice had significant Treg membrane-bound TGFβ and protection against diseases, whereas older mice had less Treg membrane-bound TGFβ and were not protected against disease, and blockade of membrane TGFβ reversed the protective effect (Gregg et

al., 2004). Antibodies against GARP that block TGFβ activation and inhibit immunosuppression have been produced (Cuende et al., 2015). Integrin αVβ8 has been found to upregulated on activated Treg cells and essential for Treg suppression of T cell responses during inflammation by activating TGFβ but is not required in maintenance of immune homeostasis (Worthington et al., 2015).

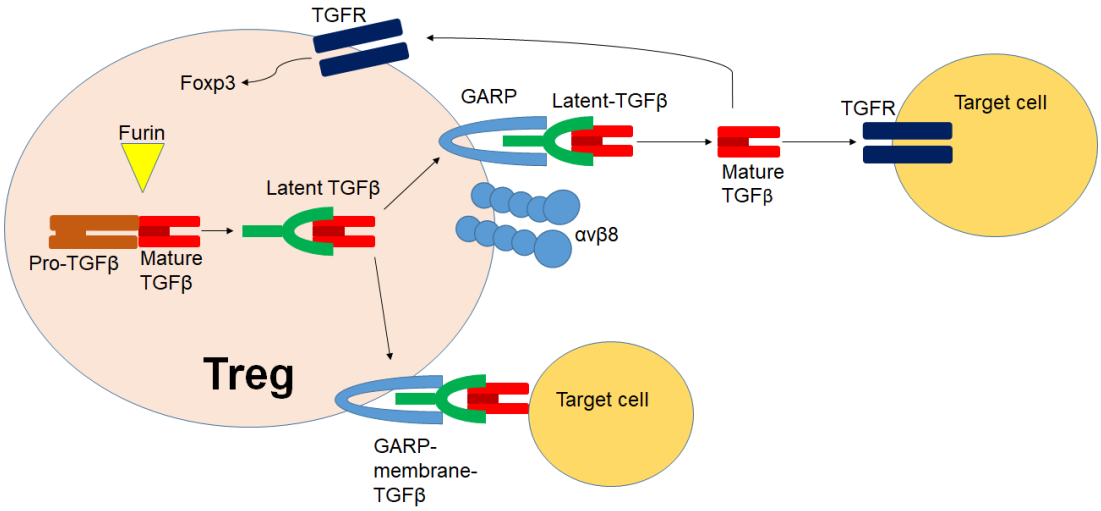

Figure 1.4.4.3 Treg suppression mechanisms involving TGFβ

The pro-TGFβ precursor composed of LAP and mature TGFβ is cleaved by furin to form latent TGFβ and mature TGFβ which remains bound to LAP. GARP transports and anchors latent TGFβ to the surface of the Treg. Integrin αVβ8 aids the release of mature TGFβ from the cell surface. Surface latent TGFβ/GARP complex can also suppress target cells in an as yet unknown mechanism (reviewed by and diagram adapted from Sun et al., 2016).

1.4.4.4 Cytotoxic T-lymphocyte-associated protein 4

Mice deficient in CTLA4 have immune dysregulation and autoimmunity (Waterhouse et al., 1995; Tivol et al., 1995). Deficiency of CTLA4 in Treg cells results in systemic lymphoproliferation, fatal T cell-mediated autoimmune disease, hyper-production of IgE, and potent tumour immunity in mice (Wing et al., 2008). CTLA4 is constitutively expressed on Treg cells in the resting state, although it is also upregulated on Teff cells upon activation (Takahashi et al., 2000). Loss of Treg CTLA4 causes impaired suppressive activity (Peggs et al., 2009). CTLA4 interacts with CD80/CD86 on APCs, and with a higher affinity than CD28, resulting in competition for CD80/CD86 interaction and the relative amount of CD28 binding versus CTLA4 binding determines whether a T cell activates (Azuma et al., 1993; Krummel and Allison, 1995). Where CTLA4 binding predominates, activation of T cells is prevented by inhibition of IL-2 production and cell cycle progression. CTLA-4 stops potentially autoreactive T cells at the initial stage of naive T-cell activation, primarily in lymphoid tissue (Fife and Bluestone, 2008; Krummel and Allison, 1995). CTLA4 exists as a non-functional covalent homodimer and does not undergo any detectable conformation change upon CD80/CD86 (Stamper et al., 2001). The cytoplasmic tail does not contain an ITIM motif and a signalling pathway triggered by CTLA4 binding has not been established (Walker and Sansom, 2015). CTLA4 is reported to downregulate CD80 and CD86 ligands on APCs, leading to reduced CD28 co-stimulation of Teff cells (Qureshi et al., 2011). In this model, CTLA4 captures CD80 and CD86 and internalises them by trans-endocytosis followed by degradation in the lysosome, then CTLA4 can recycle back to the cell surface. The finding that other molecules on DCs were not affected by this process suggests that CTLA4-mediated trans-endocytosis is specific to CD80 and CD86 (Qureshi et al., 2011). CTLA4 has also been proposed to increase T cell motility, which limits contact time between T cells and APCs (Schneider et al., 2006).

1.4.4.5 Programmed death-1

Programmed death (PD)-1 is a member of the B7/CD28 family of co-stimulatory receptors that regulates T cell activation through binding to programmed death ligand (PD-L)-1 and PD-L2 (Keir et al., 2008). PD-1 is expressed on activated T cells, B cells, and myeloid cells. Its ligands are expressed on a wide variety of both immune and non-immune cell types, however they are particularly expressed on APCs and T cells. The PD-1 pathway regulates activated T cells primarily in peripheral tissues at later stages of an immune response (Fife and Bluestone, 2008). PD-1 binding inhibits T-cell proliferation, production of IFNγ, TNFα, and IL-2, and reduces T-cell survival (Keir et al., 2008). If TCR signalling occurs with PD-1 binding, TCR signalling is terminated early and activation reduced (Parry et al., 2005; Bennett et al., 2003). Treg cells express PD-1, however its function on these cells is not fully understood. PD-1^+ Treg cells show stronger suppression function during chronic viral infection (Ha et al., 2016). Splenic DCs have been reported to induce conversion of naïve $CD4^+$ T cells into iTreg in a mechanism requiring PD-L1 in the presence of TGFβ (Wang, Pino-Lagos et al., 2008). It does this by downregulating the threshold of TGF-β-mediated signals (Francisco et al., 2009). Engagement of PD-1 on T cells prevents the expression of the anti-apoptotic protein B-cell lymphoma extra large (bcl-xL), GATA-3, T-box expressed in T-cells (T-bet) and Eomes (Chemnitz et al., 2004; Nurieva et al., 2006). Therefore, a suppressive mechanism of PD-1/PD-L1 is increasing the proportion of Treg cells to Teff cells.

1.4.4.6 Tumour necrosis factor receptor 2

TNFα exists as a transmembrane form and in a soluble form, and signals through TNFR1 and TNFR2. TNFR1 is expressed on almost all cells, whereas TNFR2 expression is more limited. TNFR2 is reportedly expressed at higher levels on Treg cells as opposed to Teff cells (Annunziato et al., 2002). Signalling by soluble TNFα via TNFR1 mainly triggers pro-inflammatory pathways, whereas transmembrane

TNFα binding to TNFR2 usually initiates regulatory and tissue regeneration functions (Yang et al., 2018). It is suggested that initial exposure to TNFα inhibits Treg suppressive functions, which are later restored upon longer exposure, allowing Teff cells to initially establish control over infection by evading Treg suppression, followed by TNF-facilitated expansion and activation of Treg cells (Yang et al., 2018). TNFR2 knockout mice have comparable numbers of Treg cells to wild type mice, however the knockout mice fail to expand Treg upon TCR stimulation, suggesting that TNFR2 mediates the activation of Treg cells but is not required for tTreg to maintain immune homeostasis (Chen et al., 2007). It has been shown that $CD4^+$ T cell transmembrane TNFα and TNFR2, but not soluble TNFα and TNFR1, promote IL-2 production and inhibition of Th17 differentiation (Miller et al., 2015). $Foxp3^+$ Treg cells expressing TNFR2 have been found to have superior suppressive capacity compared to those without TNFR2 (Chen, Subleski et al., 2008). It is also reported that Treg cells can shed TNFR2 in higher amounts for a longer duration than $CD4^+CD25^-$ T cells, which may decrease the concentration of available TNFα for pro-inflammatory responses (van Mierlo et al., 2008).

1.4.4.7 Tim-3, TIGIT and LAG-3

T cell immunoglobulin (Tim)-3 is a negative regulatory molecule expressed by Treg cells as well as other lymphocytes, NK cells, DCs and monocytes. $Tim\text{-}3^+$ Treg cells have been shown to have superior immunosuppressive activity and higher expression of IL-10, granzymes and perforin compared to $Tim\text{-}3^-$ Treg cells (Gautron et al., 2014). $Tim\text{-}3^+$ Treg cells were reported to suppress Th17 cells while $Tim\text{-}3^-$ Treg cells did not (Gautron et al., 2014). Tim and ITIM domain (TIGIT) expression is limited to lymphocytes and has especially high expression in Treg cells. TIGIT binds CD112 and CD155 which are expressed on APCs, T cells, and tumour cells. TIGIT+ Treg cells inhibit Th1 and Th17 cell differentiation and responses but promote Th2 immunity (Joller et al., 2014).

Lymphocyte activation gene (LAG)-3 is a CD4 homologue that is highly expressed on Treg cells and Tr1 cells and is also upregulated on activated T cells and NK cells (Triebel et al., 1990; Anderson et al., 2016). LAG-3 binds to MHC-II with a higher affinity than CD4 and causes ITIM signalling (Huard et al., 1995; Liang et al., 2008). Treg LAG-3 suppresses DC maturation and immunostimulatory capacity (Liang et al., 2008). LAG-3 deficiency results in uncontrolled T cell expansion in mice (Workman et al., 2004; Workman and Vignali, 2003). Blocking LAG-3 on Treg cells prevents Treg cell suppressor function whereas ectopic expression of LAG-3 in non-Treg CD4+ T cells confers suppressive activity (Huang et al., 2004).

1.4.4.8 IL-2 deprivation

The idea of Treg cells using IL-2 depletion as a suppressive function was first proposed in the 1980s (Palacios and Möller, 1981). It was then demonstrated that Treg cells block induction of IL-2 mRNA production by Teff cells and thereby inhibit their proliferation (Thornton and Shevach, 1998). Alternatively, it has been suggested that Treg cells deplete IL-2 rather than inhibit its production, preventing Teff proliferation and leading to apoptosis (Pandiyan et al., 2007). IL-2 is also required for Treg function (Fontenot et al., 2005). Treg cells have been found to accumulate in clusters in the lymph nodes with rare IL-2-positive T cells that are activated by self-antigens, and act to prevent autoimmunity (Liu et al., 2015).

1.4.4.9 Cyclic AMP and adenosine

Treg cells contain high concentrations of cAMP, whereas Teff cells do not have detectable cAMP (Bopp et al., 2007). The mechanism by which Treg cells could utilise cAMP has not yet been discovered. One theory suggests that adenosine, converted from ATP/ADP by Treg cell CD39 and CD73, is released from the Treg and acts to inhibit Teff cell TCR signalling by preventing zeta-chain-associated protein kinase 70 (ZAP70) phosphorylation and AP-1 activation, thus impairing Teff cell IL-2 production,

proliferation and activation (Allard et al., 2017). CD39 knockout mice cannot suppress CD4+CD25- T cell proliferation (Deaglio et al., 2007). Human $CD39^+$ Treg cells suppress IL-2 and IL-17 expression and proliferation of activated Teff cells more efficiently than their $CD39^-$ Treg cells (Fletcher et al., 2009; Jenabien et al., 2013; Herrath et al., 2014). Treg cells may also mediate influx of cAMP to target cells via gap junctions (Bopp et al., 2007). Direct Treg-Teff contact is required for the transfer of cAMP. As Treg cells produce and respond to adenosine this may be an autocrine factor that enhances Treg anti-inflammatory function (Ernst et al., 2010). Increased cAMP in Treg cells enhances suppression of Teffs *in vivo* and *in vitro* (Bopp et al., 2009; Feng et al., 2011). Treg cell cAMP downregulates the expression of DC co-stimulatory molecules CD80 and CD86 (Fassbender et al., 2010). Treg cells are reported to attract DCs towards them in a mechanism requiring adenosine, as $CD39^-$ Treg cells could not attract DCs (Ring et al., 2015). Despite anti-inflammatory mechanisms of adenosine, it can also exacerbate immunopathology. For example, adenosine can influence pro-inflammatory pathways and cause tissue damage in the lung, and is particularly implicated in chronic inflammation, chronic obstructive pulmonary disease (COPD), and asthma (Sun et al., 2006).

1.4.4.10 Cytotoxic mechanisms

$Foxp3^+$ T cells are cytotoxic to cell types including $CD8^+$ T cells, NK cells, and B cells, through granzymes, perforin, or Fas-FasL cytotoxic pathways (Cao et al., 2007; Grossman et al., 2004; Janssens et al., 2003; Zhao et al., 2006). Perforin causes pore-formation in target cell membranes resulting in osmotic lysis. Granzyme B is a protease that can pass through perforin-formed pores into the cytosol and target caspase-3 to initiate DNA fragmentation and apoptosis (Lord et al., 2003). Granzyme B has been reported to be a mechanism of suppression used by Treg cells (Gondek et al., 2005). $CD25^+Foxp3^+$ T cells in mice were found to cause decrease of DCs in tumour-draining lymph node in a manner requiring perforin (Boissonnas et al., 2010).

The ability of Treg cells to regulate lung inflammation during RSV infection in mice depended on their ability to produce granzyme B (Loebberman et al., 2012). In the study by Loebberman et al., lung treg cells, but not draining lymph node Treg cells, from RSV-infected mice expressed granzyme B, and mice with selective loss of granzyme-B in Treg had enhanced cellular infiltration into the lung after infection.

1.4.5 Regulatory T cell effects on antigen presenting cells

The ability of Treg cells to regulate other cells of the adaptive immune system is well documented, however data on Treg effects on innate immune cells are more limited. An indirect effect of Treg depletion is reduced control of Teff activity, resulting in excessive responses from macrophages (Okeke et al., 2014). In addition, there are several reports of Treg cells directly affecting the activity of DCs, neutrophils and macrophages.

DCs are efficient inducers of Treg cells, dependent on signals encouraging immune responses to tilt towards tolerance and the maturation state of the DC (Yamazaki et al., 2003; Lutz and Schuler, 2002). Double positive thymocytes commit to Treg lineage depending on the intensity of their response to self-antigens presented by thymic DCs (Martín-Gayo et al., 2010). In the periphery, sub-immunogenic antigen delivery to DCs results in pTreg differentiation from naïve $CD4^+$ T cells (Kretschmer et al., 2005). IL-10 and TGFβ are implicated in the induction of Treg cells by DCs (Sato et al., 2003; Yamazaki et al., 2007). Inactivation of TGFβ signalling in DCs has been found to cause increased T cell responses in experimental autoimmune encephalomyelitis (Laouar et al., 2008). TGF-β mediates Treg induction by the activating αvβ8 integrin on DCs (Travis et al., 2007). $Foxp3^+$ Treg induction by DCs has been shown to be mediated through PD-L1 expressed on DCs, and inhibition of PD-L1 on DCs cannot induce $FOXP3^+$ Tregs even in the presence of cytokine signals. (Wang et al., 2008). Retinoic acid produced by DCs is also reported to enhance Treg induction and activity (Mucida et al., 2009; Coombes et al., 2007). Treg cells

congregate more readily around DCs than naïve T cells *in vitro*, which is due to Treg cells being more mobile and requires leukocyte function-associated antigen-1 (LFA-1), and subsequently down-regulate DC expression of CD80/86, but not CD40 or MHC-II, in a CTLA4- and LFA-1-dependent manner (Onishi et al., 2008). CD80/86-down-modulation even occurs in the presence of GM-CSF, TNFα, IFNγ, type I IFN, and LPS.

Studies in mouse colitis models gave initial indications that Treg cells could suppress cells of the innate immune system. Maloy and colleagues demonstrated that $CD4^+CD25^+$ cells could inhibit both T cell and innate cell inflammatory responses in mice infected with *Helicobacter hepaticus* in a manner requiring IL-10 and TGFβ (Maloy et al., 2003). In a lymphocyte-deficient mouse model of colitis infected with *Leishmania major*, transfer of $CD4^+CD25^+$ cells alleviated disease, which did not occur in the absence of IL-10, TGFβ or CTLA4 (Liu et al., 2003). Inhibitory effects of human Treg cells on monocytes/macrophages *in vitro* were studied by Taams and colleagues (Taams et al., 2005). In these experiments, Treg-treated monocytes produced minimal cytokines, whereas Teff-treated monocytes produced TNFα, IL-6, IL-1β and IL-10. Upon LPS stimulation, Treg-treated monocytes had inhibited TNFα and IL-6 production, limited upregulation of CD40 and CD80, and downregulated CD86 compared to control monocytes. Further work from Taams and colleagues found that mannose receptor and scavenger receptor expression was increased on Treg-treated monocytes (Tiemmessen et al., 2007). They found that Treg-produced anti-inflammatory cytokines were critical to increased monocyte scavenger receptor expression, increased phagocytosis, and the suppression of the monocyte pro-inflammatory cytokine response, whereas increased mannose receptor expression was entirely cytokine-independent. An additional study found that $CD4^+CD25^+$ T cells suppressed macrophage activation and TNFα and IL-12 production *in vitro*, and $CD4^+CD25^+Foxp3^+$ T cells transferred to lymphocyte-deficient mice reduced injury in

a chronic renal disease model associated with reduced macrophage numbers in a mechanism requiring TGFβ (Mahajan et al., 2006). *In vivo*, Treg cells were found to promote differentiation of macrophages into an anti-inflammatory phenotype at least partially through arginase, IL-10 and TGFβ pathways in mice (Liu et al., 2011). Conversely, $CD4^{+}CD25^{-}$ cells promoted inflammatory macrophage phenotypes. Th2-associated cytokines such as IL-4 and IL-10 induce arginase-1, which has a high affinity for arginase, reducing its availability to be used in NO production. Blocking arginase, IL-10 or TGFβ partially but statistically significantly reversed the effects of Treg on macrophages. Anti-inflammatory macrophages have in turn been found to induce Treg cells from PBMCs mediated by membrane-bound TGFβ (Savage et al., 2008).

1.5 Introduction summary

S. pneumoniae remains a major cause of serious disease worldwide. It is very successful as a nasopharyngeal commensal employing a range of immune-evasion mechanisms. While inflammatory responses are required to clear infections, excessive inflammation can be detrimental to the host.

Tissue-resident macrophages are considered the first to encounter *S. pneumoniae* and orchestrate the immune response. Macrophages detect *S. pneumoniae* through a variety of receptors and have a huge capacity for plasticity, responding with pro-inflammatory, anti-inflammatory and tissue repair roles depending on their environmental stimuli. Treg cells counteract inflammation through cell contact-dependent and contact-independent mechanisms and can influence the responses of APCs as well as other T cell subsets.

The magnitude of the inflammatory response affects the outcome in colonisation and pneumonia. Macrophages are critical for clearance of colonisation (Zhang et al., 2009). Treg numbers are increased in colonisation and prolong carriage (Jiang et al., 2015; Mubarak et al., 2016; Neill et al., 2014). Alveolar macrophages switch from a homeostatic to a pro-inflammatory role if pneumococci are aspirated into the lungs in overwhelming numbers (Dockrell et al., 2003). Excessive inflammatory responses to *S. pneumoniae* in pneumonia can result in serious complications resulting from tissue damage and bacterial invasion. Treg cells and TGFβ are protective against invasive pneumococcal pneumonia in mouse models (Neill et al., 2012). Current data on Treg responses in *S. pneumoniae* infection and the effects of Treg-macrophage interactions are summarised in table 1.6.

To conclude, a balance between production of an effective immune response whilst controlling inflammation is critical for clearance of *S. pneumoniae* and limiting host damage.

Effects of Treg cells in *S. pneumoniae* infection and Treg-macrophage interactions	
Mouse data	**Reference**
Treg cells protect against invasive *S. pn* pneumonia in a TGFβ-dependent manner	Neill et al., 2012
ST3 capsular polysaccharide + pneumolysoid expand Treg cells in AAD models	Thorburn et al., 2013
Elevated TGFβ and Treg cell numbers prolong carriage	Neill et al., 2014
Long term carriage is characterised by:	Neill et al., 2014
> high levels of TGFβ	
> increased Treg cells in the nasopharynx	
> increased presence of alternatively activated macrophages in the nasopharynx	
TGFβ1 blockade prior to colonisation:	Neill et al., 2014
> shortens *S. pn* carriage	
> inhibits Treg responses in the nasopharynx	
> increases neutrophil influx to the nasopharynx	
> causes transient lung invasion	
Human data	**Reference**
NALT Treg cells are increased in *S. pn* colonisation	Zhang et al., 2011
Carriage promotes increased adenoid Treg and decreased Th17 numbers upon *S. pn* stimulation	Jiang et al., 2015
Treg cells in the NALT decrease with age from children to young adults	Mubarak et al., 2016
Adult Treg cells inhibit Teff proliferation to Ply: CTLA4 and PDL-1 implicated	Pido-Lopez et al., 2011
S.pn and Ply promote nasopharyngeal epithelial cell and fibroblast secretion of active TGFβ1	Neill et al., 2014
Increased TGFβ and IL-10 in carriage-positive volunteers	Neill et al., 2014
Treg-macrophage interaction	**Reference**
Indirect effect - Treg depletion increases Teff cell numbers causing increased macrophage activation	Okeke et al., 2014
Treg co-cultured monocyte/macrophages have decreased inflammatory response on LPS challenge	Taams et al., 2005
Treg-derived anti-inflammatory cytokines caused increased:	Tiemmessen et al., 2007
> monocyte/macrophage scavenger receptor expression	
> suppression of the monocyte/macorophage pro-inflammatory cytokine response to LPS	
> phagocytosis by monocyte/macrophages	
Monocyte/macrophage mannose receptor CD206 upregulated by Treg cell contact	Tiemmessen et al., 2007
$CD4^+CD25^+$ T cells suppress macrophage TNFα and IL-12 production *in vitro*	Mahajan et al., 2006
Treg arginase, IL-10 and TGFβ promote differentiation of anti-inflammatory macrophages in mice	Liu et al., 2011
Anti-inflammatory macrophages induce Treg cells from PBMCs by membrane-bound TGFβ	Savage et al., 2008
Infuenza/*E. coli* infection predisposes to second *E. coli* pneumonia by macrophage/DC impairment	Roquilly et al., 2017

Table 1.5 Current data on the effects of Treg cells in *S. pneumoniae* infection and the effects of Treg-macrophage interactions

1.6 Rationale and aims

The literature indicates that inflammatory responses to *S. pneumoniae* need to be robust enough to allow bacterial killing, but controlled such that excessive inflammation does not cause detrimental damage to the host. Macrophages and Treg cells are both key cells in host survival to *S. pneumoniae* infection. It is well established that macrophages contribute to clearance of infection with a broad spectrum of responses, from highly pro-inflammatory to anti-inflammatory mechanisms. Data not specific to *S. pneumoniae* suggest that macrophage responses can be suppressed by Treg cells. However, there is currently no human data on whether Treg cells can modulate macrophage inflammatory responses to *S. pneumoniae*. This data would be useful for several reasons. Firstly, evidence that Treg cells can modulate macrophage responses to *S. pneumoniae* would uncover a previously underexplored interaction between two vital cell types that may be very important to the outcome of infection. This would prompt investigation into whether susceptible individuals have deficiencies in these interactions, for example reduced/increased numbers of Treg cells, impaired Treg function, less/more frequent interactions between the macrophages and Treg cells, or impairment of the specific mechanism of interaction between these cell types. Elucidating the mechanism(s) by which Treg cells may confer suppressive effects to macrophages may indicate future therapeutic targets to decrease or boost inflammatory reponses in pneumococcal infections. Current vaccine design focusses on provoking T cell responses, in which Treg are likely affected, so data on Treg responses in *S. pneumoniae* infection are valuble.

Much of the data on immune responses to *S. pneumoniae* come from mouse models, therefore data on human responses to *S. pneumoniae* infection are valuable due to differences in the immune systems of mice and humans. The *in vitro* work in this project aims to use human peripheral blood cells to identify effects of Treg cells on

macrophage inflammatory responses to *S. pneumoniae*. Co-culture conditions for these cells are first optimised, followed by investigation of suppressive effects on macrophage inflammatory responses when Treg cells are added into macrophage cultures prior to, during, or after *S. pneumoniae* infection – timings which reflect potential Treg-macrophage interactions prior to infection, during infection, and Treg cells arriving at the later stages of infection to promote resolution. Whether cytokine secretion or cell contact-dependent mechanisms of suppression are used by the Treg to suppress macrophage responses is explored.

Cellular recruitment during pneumococcal infection in humans is difficult to examine due to the lack of access to the lung tissue, making development of alternative methods by which to study recruitment and phenotypes of human cells in *S. pneumoniae* desirable. The experiments in this thesis aim to examine T cell recruitment in response to *S. pneumoniae* using a human intradermal model of infection. This is a minimally invasive technique allowing investigation of T cell recruitment timing, subtypes, activation status, tissue-homing markers and memory/effector status, as well as cytokine milieu, granulocyte and agranulocyte presence, and blood flow as an indicator of the magnitude of the inflammatory response. The possibility of lung-resident Treg cells already present in human lungs ready to modulate macrophage responses pre-infection or early in infection is examined by IF staining of normal human lung tissue for $CD4^+Foxp3^+$ cells, along with $CD4^+Foxp3^-$ T cells.

The aim of this thesis is to address the hypothesis:

Human Treg cells can suppress macrophage inflammatory responses to *S. pneumoniae in vitro* and recruit to the presence of intradermal *S. pneumoniae.*

The specific aims of the thesis are as follows:

1. Determine if human Treg co-culture suppresses MDM inflammatory responses to *S. pneumoniae* infection
    i. Establish an MDM, Treg/Teff cell and *S. pneumoniae* co-culture for use in experiments
    ii. Measure pro-inflammatory cytokine concentration and phagocytic ability of MDMs co-cultured with Treg cells added before, during or after infection
    iii. Compare the effects of MDM/Treg cell co-culture on the MDM inflammatory response to *S. pneumoniae* with another pneumonia pathogen, *Acinetobacter baumannii*
2. Investigate the mechanism(s) used by Treg cells to modulate MDM inflammatory responses to *S. pneumoniae* infection
    i. Determine whether addition of IL-10 or TGFβ suppress MDM pro-inflammatory cytokine production to *S. pneumoniae*
    ii. Ascertain if Treg cells modulate MDM responses by anti-inflammatory cytokine production and/or through cell-contact
    iii. Investigate Treg surface TGFβ as a mechanism of suppression of MDM responses
3. Examine Treg/T cell recruitment to the presence of *S. pneumoniae* using a human intradermal challenge model
    i. Quantify the blood flow at the site of challenge using laser Doppler scanning as an indicator of the magnitude of inflammation
    ii. Examine the marker expression of the recruited T cells using flow cytometry
    iii. Measure cytokine concentrations at the site of challenge
4. Determine if Treg cells are present in normal human lung using immunofluorescent staining and confocal microscopy

Chapter 2: Materials and Methods

2.1 Bacterial strains and growth

2.1.1 *S. pneumoniae*

Media and reagents for bacterial culture and storage were prepared as follows:

- Todd Hewitt broth medium (THY, Sigma Aldrich, T1438) supplemented with 0.5% yeast extract (Sigma Aldrich, Y1625)
- 10% glycerol (VWR 24388.260) diluted in distilled water

The TIGR4 strain of *S. pneumoniae* was used in experiments except where otherwise stated. TIGR4 is a highly virulent capsular serotype 4 isolated from the blood of a 30-year-old male patient in Kongsvinger, Norway. Some experiments used D39 (which is an important invasive serotype 2 strain), and the clinical isolates 6B and 23F (isolated from children under 5 years old in Finland, 2002). TIGR4 and D39 were a gift from Professor Jeffrey Weiser, University of Pennsylvania, and 23F and 6B were a gift from Professor Brian Spratt, Imperial College London. A list of all strains used and their origin is shown in table 2.1.1. For culture of frozen stocks, *S. pneumoniae* were streaked on to a Columbia agar blood (CBA) plate (SGL, 8022) using an inoculating loop (Thermo Scientific, 251586) and incubated overnight at 37°C in 5% CO_2. A colony from the plate was added to 15mls of autoclaved THY medium in a 50ml falcon tube (Greiner, T2318-500EA) and the cap loosely replaced. The bacteria were incubated at 37°C until reaching an optical density (OD) of 0.4 which gives approximately $1x10^8$ CFUs per ml. For long term storage of these stock cultures 1ml aliquots were frozen at -80° in autoclaved 10% glycerol. Stock concentrations were determined by defrosting an aliquot, centrifuging at 13 000x g for 10 minutes and re-suspending in 1ml PBS (Sigma Aldrich, D8537). Triplicate 10µl spots of Log_{10} serial

dilutions were plated on CBA plates and incubated overnight at 37°C in 5% CO_2 and the resulting colonies counted. For experiments requiring CFU calculation, triplicate 10µl spots of Log_{10} serial dilutions of supernatant were plated on CBA plates and incubated overnight at 37°C in 5% CO_2 then the colonies counted.

Table 2.1.1. *S. pneumoniae* strains used in experiments

Name	Strain	Capsular Serotype	Phenotype	Origin / source
TIGR4	TIGR4	4	Wild-type	J. Weiser
D39	D39	2	Wild-type	J. Weiser
23F	OXC-1417-23F	23F	Wild-type	B. Spratt
6B	M158	6B	Wild-type	B. Spratt

2.1.2 *A. baumannii*

A. baumannii used in experiments was sequence type 2 (ST2), which corresponds to a common circulating type also referred to as IC-2 or GC-2 (International clone or Global clone 2), and was a kind gift from Dr. Richard Stabler, London School of Hygiene and Tropical Medicine. Stocks were grown by Dr. Giuseppe Ercoli, UCL. *A. baumannii* was streaked onto CBA plates and incubated overnight at 37°C in 5% CO_2. A colony was taken from the plate with an inoculating loop and transferred to 15mls of autoclaved brain heart infusion broth (Oxoid, CM1135). The bacteria were incubated at 37°C in 5% CO_2 with shaking until reaching an OD of 0.5. 1ml aliquots were frozen at -80° in autoclaved 10% glycerol. Stock concentrations were determined by defrosting an aliquot, centrifuging at 13 000x g for 10 minutes and re-suspending in 1ml PBS. Triplicate 10µl spots of Log_{10} serial dilutions were plated on

CBA plates and incubated overnight at 37°C in 5% CO_2 and the resulting colonies counted. For experiments requiring CFU calculation, triplicate 10µl spots of Log_{10} serial dilutions of supernatant were plated on CBA plates and incubated overnight at 37°C in 5% CO_2 and the resulting colonies counted.

2.2 Cell isolation

2.2.1 Peripheral blood mononuclear cell isolation

Experiments using PBMC isolation from healthy volunteers were approved by the UCL Research Ethics Committee (ref. 6032/001) and informed consent was obtained from all participants.

60ml blood samples were taken from healthy volunteers. 50mls of the blood was placed in a 50ml falcon tube with 300µl heparin (88145, UCL Hospitals NHS Foundation Trust). The remaining 10mls of blood were added to a serum separation tube, centrifuged at 3000x g for 20 minutes, the resulting serum collected and heat-inactivated at 56°C for 30 minutes. The heparinised blood was diluted by addition of 25mls sterile PBS. Approximately 19mls of the diluted blood was carefully layered onto 15mls of Ficoll-Paque (GE Healthcare, 17144002) in 4x 50ml falcon tubes and centrifuged at 800x g for 25 minutes with no brake. The visible buffy coat containing the PBMCs was aspirated using a Pasteur pipette and transferred into another 50ml falcon tube. The tube was topped up to 50mls with sterile PBS and centrifuged at 800x g for 10 minutes. The supernatant was discarded and the pellet re-suspended in 50mls sterile PBS and centrifuged at 400x g for 5 minutes and repeated twice.

2.2.2 Monocyte-derived macrophage (MDM) differentiation

Media for MDM cell culture were prepared as follows:

- Monocyte adhesion medium: Roswell Park Memorial Institute 1640 medium with L-glutamine (RPMI), Fisher Scientific, 11560406) supplemented with 5% human AB serum (Sigma Aldrich, H4522-20ML)
- MDM differentiation medium: RPMI with 10% heat-inactivated autologous serum and 20ng/ml macrophage colony stimulating factor (M-CSF) (R&D Systems, 216-MC-005)
- MDM culture medium: RPMI with 10% heat-inactivated autologous serum

The protocol used to differentiate MDMs is an established method from the lab of Prof. Mahdad Noursadeghi, UCL. PBMCs were isolated as described in part 2.2.1. The PBMC pellet was re-suspended at $1x10^7$ cells/ml in monocyte adhesion medium. Cells were seeded onto 24-well or 48-well culture plates by addition of 200µl of the suspension was added to each well of a 48-well or 400µl added to each well of a 24-well cell culture plate. Plated cells were incubated at 37°C in 5% CO_2 for 1 hour to allow monocyte adherence. The non-adherent cells were then removed by washing three times in PBS. 400µl (48-well plate) or 800µl (24-well plate) of MDM differentiation medium was added to each well and incubated for 3 days at 37°C in 5% CO_2. Media was then removed, the cells were washed three times in PBS, and 400µl (48-well plate) or 800µl (24 well plate) of MDM culture medium (without M-CSF) was added and incubated at 37°C 5% CO_2 for a further 3 days. The estimated MDM number on day 6 of culture is $1x10^5$ MDMs per well of a 48-well plate and $2x10^5$ per well of a 24-well plate based on work from the Noursadeghi lab. A summary of volumes and cell yields can be seen in table 2.2.2.

Table 2.2.2 Estimated MDM yield and medium volumes

Plate Size	Seeding volume	Culture medium volume	Estimated MDMs per well
24-well	400µl	800µl	$2x10^5$
48-well	200µl	400µl	$1x10^5$

2.2.3 Treg and Teff cell isolation by Fluorescence Activated Cell Sorting (FACS)

Media and buffers for FACS were prepared as follows:

- FACS buffer: Sterile PBS supplemented with 0.5% fetal bovine serum (FBS, Thermo Fisher Scientific, 26140079) and 0.4% 2mM EDTA (ThermoFisher Scientific, 15575020)
- PBMC culture medium: RPMI with 10% FBS and 1% penicillin-streptomycin (Fisher Scientific, 11480425)
- FACS collection medium: RPMI with 50% foetal calf serum (FCS) and 1% penicillin-streptomycin

PBMCs were isolated by Ficoll-Paque density gradient as described in part 2.2.1. PBMCs were incubated overnight in 10 ml of PBMC culture medium at 37°C in 5% CO_2. PBMCs were counted using a haemocytometer (KOVA, 22-270141) and trypan blue (Sigma, T8154-20ML) then transferred to a 50ml falcon tube. The falcon tube was topped up to 50mls with FACS buffer and centrifuged at 400x g for 5 minutes. The supernatant was discarded and the pellet stained with 4µl anti-human CD4 Alexa Fluor 700 (eBioscience, 56-0042-82), 3µl anti-human CD25 APC Cy7 (BD Biosciences, 557753), 3µl and anti-human CD127 PE Cy5 (BD Biosciences, 560733) per 10^6 cells. Cells were incubated on ice in the dark for 30 minutes then washed once by topping the falcon tube up to 50ml in FACS buffer and centrifuging at 400x g for 5 minutes. Cells were re-suspended in 300µl FACS buffer and transferred to a sterile 5ml polystyrene sort tube through the cell strainer cap (Corning, 352235). Single stain compensations were set up by adding 1µl of each antibody to a separate sort tube each containing 1 drop of OneComp ebeads (eBioscience, 01-1111-41) and 250µl FACS buffer. Beads were incubated for 20 minutes at room temperature in the dark. After incubation, the single stained beads were washed in FACS buffer at 400x

g for 5 minutes and re-suspended in 300µl FACS buffer. $CD4^{+}CD25^{+}CD127^{low}$ cells (Treg) and $CD4^{+}CD25^{-}CD127^{high}$ cells (Teff) were sorted using the FACS Aria (BD Biosciences) by Jamie Evans, UCL. Treg and Teff cells were collected in separate sort tubes containing FACS collection medium. The gating strategy can be seen in figure 2.2.3.

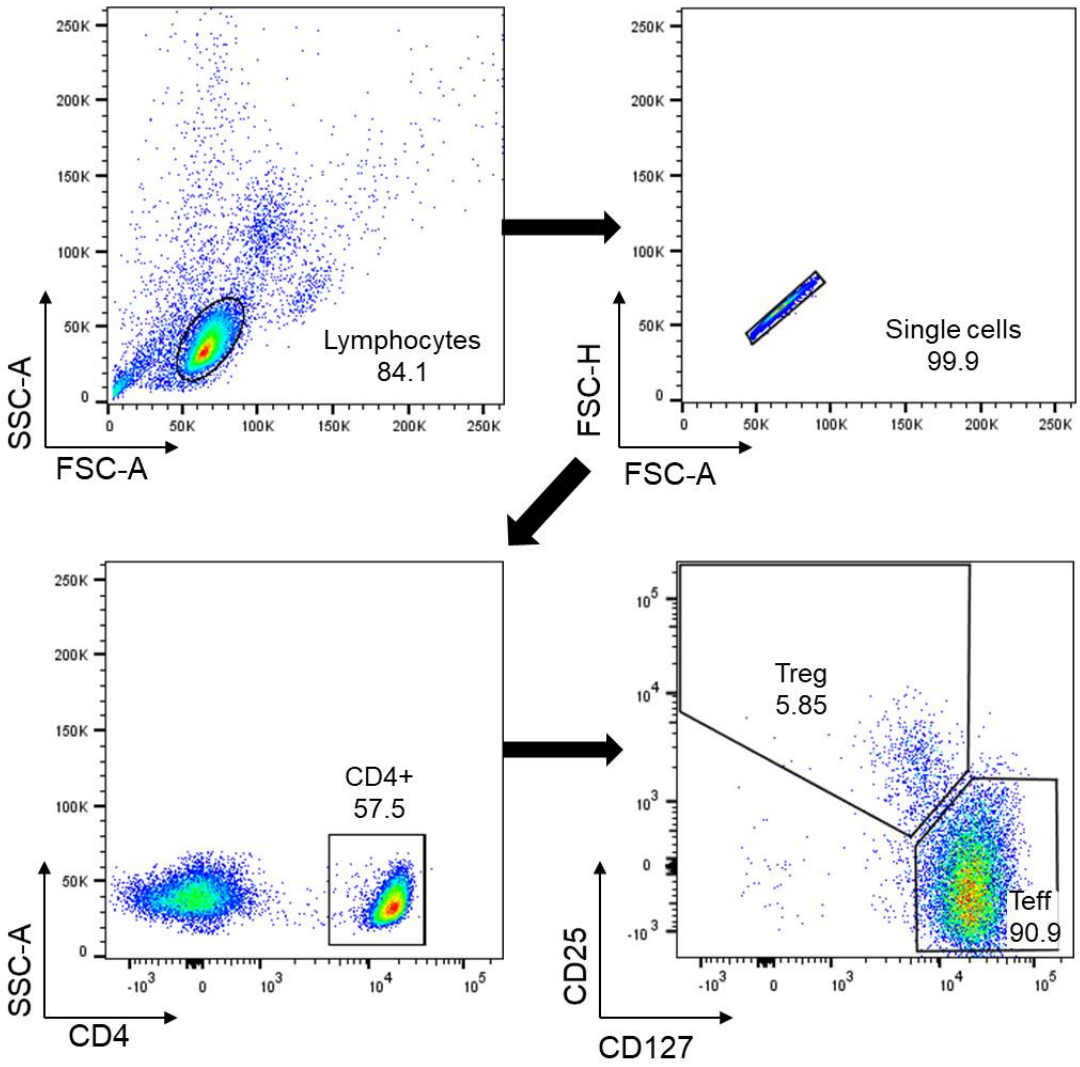

Figure 2.2.3 Gating strategy for FACS sorting Treg and Teff cells

PBMCs were stained with 4µl anti-CD4, 3µl anti-CD25 and 3µl CD127 per 10^6 cells and incubated on ice in sterile conditions to isolate live Treg and Teff cell populations by FACS (FACS Aria, BD). $CD4^+$ single cells were separated into $CD4^+CD25^+CD127^{low}$ (Treg) and $CD4^+CD25^-CD127^{high}$ (Teff) cell population collected in separate tubes. The protocol typically yields 10^5 Treg and 10^6 Teff cells.

2.2.4 $CD4^+$ T cell isolation

Media and buffer for $CD4^+$ T cell isolation were prepared as follows:

- Monocyte adhesion medium: RPMI supplemented with 5% human AB serum
- Cell separation buffer: sterile PBS with 0.5% FBS and sterile 0.4% 2mM EDTA

PBMCs were isolation from blood by Ficoll-Paque density gradient as described in 2.2.1. Monocytes were depleted by adhesion to cell culture plates by incubating the PBMCs re-suspended in monocyte adhesion medium at $3.3x10^6$ cells in 300µl per well of a 48-well plate for 1 hour at 37°C in 5% CO_2. The resulting non-adherent cells were collected in a 50ml falcon by washing the wells with sterile PBS 3 times. The monocyte-depleted cells were washed 3 times by topping up the falcon tube to 50mls with sterile PBS, centrifuging at 400x g for 5 minutes and discarding the supernatant. The resulting cells were purified by negative magnetic separation for $CD4^+$ T cells using Magnisort Human CD4 T Cell Enrichment Kit (eBioscience, 8804-6811) as per the manufacturer's instructions. Cells were suspended at $1x10^7$ cells per 100µl in cell separation buffer in sterile sort tubes. 20µl of the supplied MagniSort enrichment antibody cocktail, which is a mixture of biotinylated antibodies for markers of cells other than $CD4^+$ T cells, was added per tube and incubated for 10 minutes at room temperature. The list of antibodies in the antibody cocktail can be seen in table 2.2.4. The cells were then washed by topping up to 4mls in cell separation buffer and centrifuging at 400x g for 5 minutes then discarding the supernatant. The cells were then re-suspended in 100µl of cell separation buffer and 20µl of the supplied streptavidin-coated MagniSort negative selection beads were added per tube and incubated for 5 minutes at room temperature. The volume was then brought up to 2.5mls in cell separation buffer and placed in the Magneto (eBioscience, MAG-4902-10) magnet and incubated for 5 minutes at room temperature. Without removing the tube from the magnet, the contents of the tube containing the purified $CD4^+$ T cells

were poured into a fresh sort tube. The resulting cells had a purity of ~93% $CD4^+$ T cells taken from the mean $CD4^+$ expression from 5 individual tests (figure 2.2.4).

Table 2.2.4 Antibodies contained in the supplied enrichment antibody cocktail

Antibody	Depletes
Anti-human CD8 Biotin	$CD8^+$ T cells
Anti-human CD11b Biotin	Monocytes/macrophages
Anti-human CD14 Biotin	Monocytes/macrophages
Anti-human CD16 Biotin	NK cells
Anti-human CD19 Biotin	B cells
Anti-human CD20 Biotin	B cells
Anti-human CD36 Biotin	Platelets
Anti-human CD56 Biotin	NK cells
Anti-human CD123 Biotin	Dendritic cells
Anti-human CD235a Biotin	Red blood cells
Anti-human γδ TCR Biotin	γδ T cells

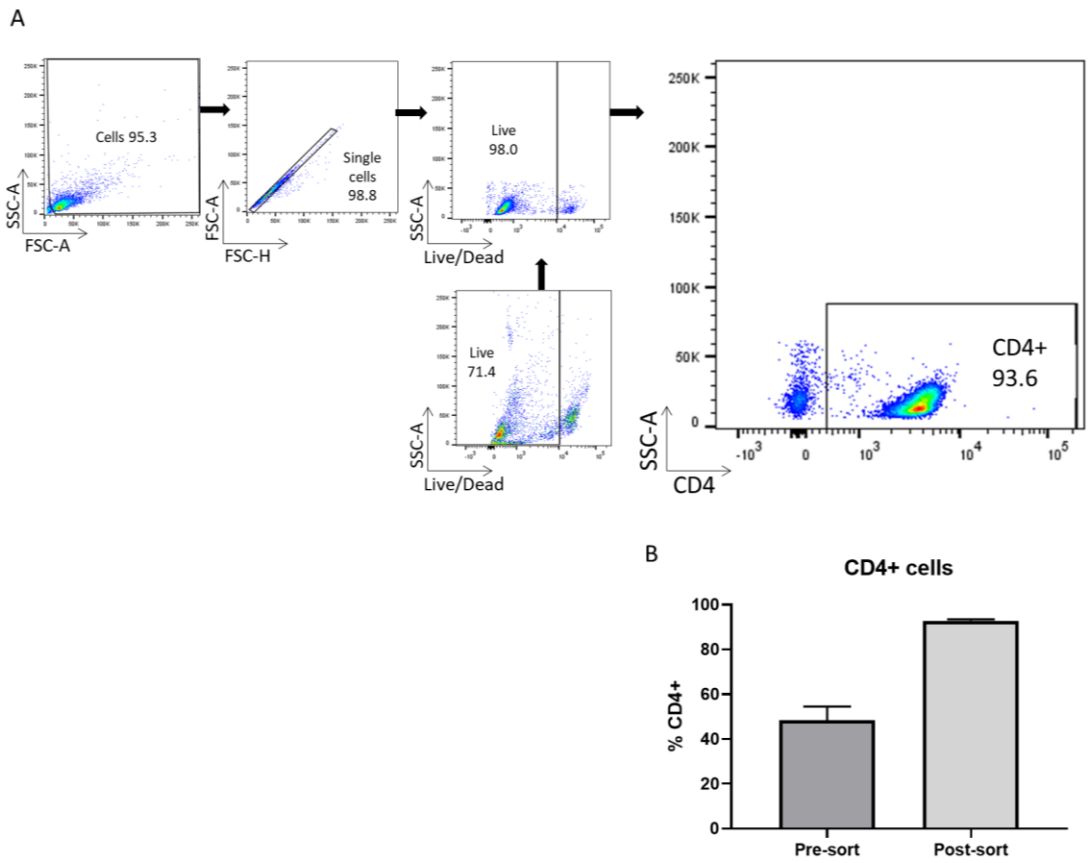

Figure 2.2.4 Purity of the CD4⁺ T cell population after magnetic negative selection

CD4⁺ T cells were purified as described and the resulting CD4⁺ population purity was determined by flow cytometry compared to pre-sort PBMCs. Single, live cells were analysed for CD4 expression. A representative example of post-sort purified CD4⁺ T cells including the gating strategy is shown in A. The percentage purity from 5 tests are shown in B. This protocol yields CD4⁺ T populations of approximate 93% purity.

2.3 Flow cytometry

Staining buffer used in the following protocols was PBS with 0.5% FBS and 0.4% 2mM EDTA. Live/dead stain (Invitrogen, L23105) was prepared as per the manufacturer's instructions and was used at 1:1000 dilution in staining buffer. For the live/dead control, a sample of live cells was stained and combined with a sample of heat-killed stained cells in a 50:50 ratio. For all other markers, compensation controls were prepared by adding 1µl of antibody and 1 drop of OneComp eBeads to 250µl staining buffer, incubating for 20 minutes at room temperature in the dark, washing by topping up to 1ml with staining buffer and centrifugation at 400x g for 5 minutes, then re-suspending in 300µl staining buffer containing 2% paraformaldehyde. All antibody panel combinations were compensated by calculation through the FACSDiva software on the flow cytometer. Where required, fluorescent minus one (FMO) controls were used by staining cells as per the below protocols with the required panel minus 1 antibody, repeated for each marker. An example of FMO controls for a panel can be seen in figure 2.3.

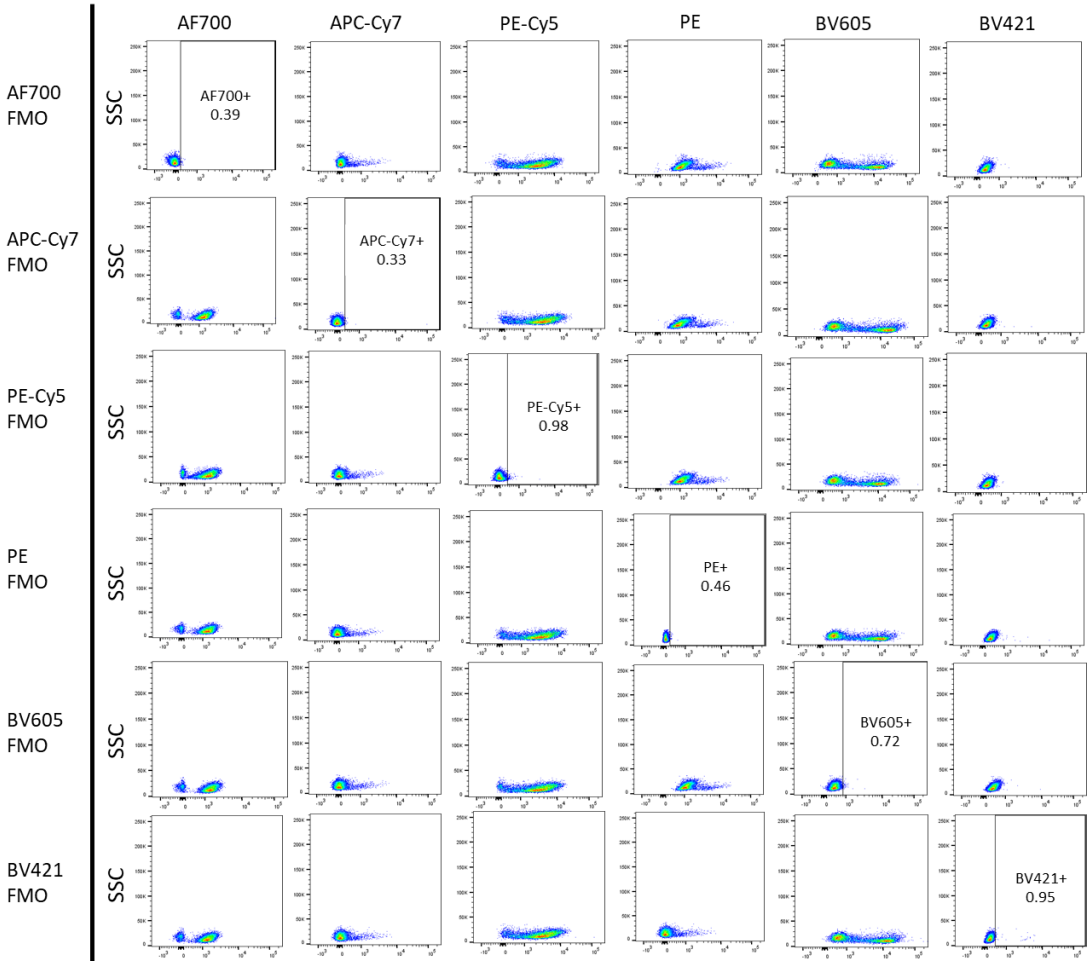

Figure 2.3 An example of FMO controls on a panel of antibodies used in flow cytometry experiments

$CD4^+$ T cells purified from PBMCs were stained with a panel of antibodies used in flow cytometry experiments each with a sample containing the whole panel minus one antibody, and the positive expression of each fluorochrome is gated in the FMO samples. Where stated, FMO controls were stained in addition to the experimental samples and used to determine gating boundaries on cell populations.

2.3.1 Gating strategy

The first 3 stages in all flow cytometry analyses included selection of whole cells, removal of dead cells, and removal of doublets. Removal of cell debris was determined by selection of all cells except those with very low side scatter area (SSC-A) and forward scatter area (FSC-A). Doublets were removed by excluding outliers on the forward scatter height (FSC-H) and FSC-A profile. Live cells were selected as those that did not take up the live/dead stain, with gating taken from a control sample with 50% live cells and 50% heat-killed cells showing visible live and dead populations. This strategy can be seen in figure 2.3.1.

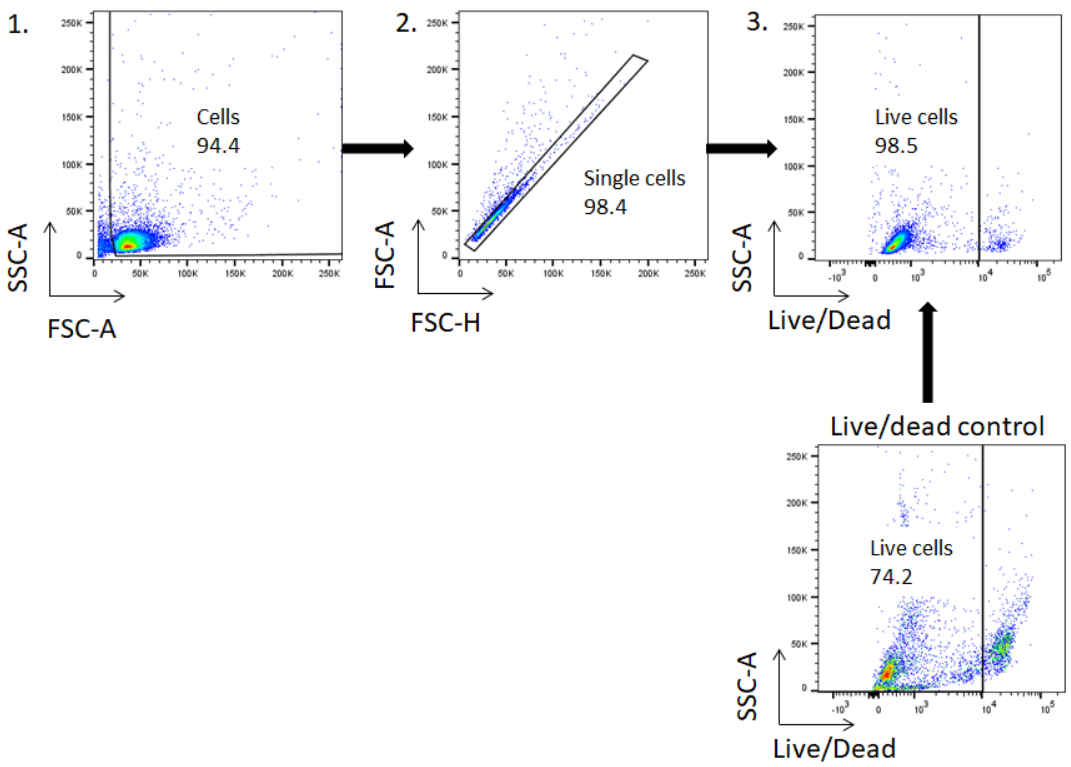

Figure 2.3.1 Gating strategy removing cell debris, dead cells and doublets used in all flow cytometry analyses

In all experiments, cell debris, dead cells and doublets were removed as shown in this example prior to analysis of the experimental markers. Cell debris was excluded based on the low side- and forward scatter (area). Doublets were excluded based on outliers from single cells with a uniform FSC-A scatter against FSC-H scatter. Live cells were selected based on those that did not bind the live/dead stain, as could be determined based on a control containing both live and dead cells stained with the same marker. The resulting single, live cells were used for further analysis.

2.3.2 Antibody panels

A full list of antibodies is provided in table 2.3.2. Cells were stained with the following antibody combinations:

- Post-sort analysis: CD4 AF700, CD25 APC-Cy7, CD127 PE-Cy5, Foxp3 PE
- Treg cell suppression of Teff cell proliferation: Live/dead stain, CD4 AF700, CD25 APC-Cy7, CD127 PE-Cy5, Foxp3 PE, Ki67 FITC
- Cytokine production by FACS-sorted Treg/Teff cells: CD4 AF700, CD25 APC-Cy7, CD127 PE-Cy5, Foxp3 PE, IL-2 BV785, IL-10 BV421, IFNγ PE-Cy7, IL-17A BV421 (separate samples were stained for IL-10 and IL-17A)
- Measurement of LAP: Live/dead stain, CD4 AF700, CD25 APC-Cy7, CD127 PE-Cy5, LAP PE, Foxp3 AF488
- $CD4^+$ cell differentiation: Live/dead stain, CD4 AF700, CD25 APC-Cy7, CD127 PE-Cy5, CD45RA BV605, Foxp3 PE
- Differentiated CD4 cell cytokine production: Live/dead stain, CD4 AF700, CD25 APC-Cy7, CD127 PE-Cy5, CD45RA BV605, Foxp3 PE, IL-2 BV785, TNFα BUV395, IFNγ PE-Cy7, IL-10 BV421, IL-17A BV421 (separate samples were stained for IL-10 and IL-17A)
- Blister fluid cells from the site of intradermal UV-killed *S. pneumoniae*: Live/dead stain, CD3 BUV395, CD8 PerCP-Cy5.5, CD4 APC, CD25 PE, CD127 BV421, CD45RA BV605, CCR6 BB515. Cells were stained with the volumes stated in table 2.3.2 regardless of cell count. Whole blood as a comparator to the blister fluid cells were stained with the same panel.

Table 2.3.2 Antibodies used for flow cytometry

Marker	Fluorochrome	Manufacturer	Volume per 10^6 cells	Type of staining
Live/dead	Blue	Invitrogen, L23105	1:1000 dilution	Surface
CD3	BUV395	BD Biosciences, 563548	2.5µl	Surface
CD8	PerCP-Cy5.5	BD Biosciences, 560662	2.5µl	Surface
CD4	AF700	eBioscience, 56-00-48	5µl	Surface
CD4	APC	BD Biosciences, 555349	10µl	Surface
CD25	APC-Cy7	BD Biosciences, 557753	2.5µl	Surface
CD25	PE	BD Biosciences, 555432	10µl	Surface
CD127	PE-Cy5	eBioscience, 15-1278	2.5µl	Surface
CD127	BV421	BD Biosciences, 562437	2.5µl	Surface
CD45RA	BV605	BD Biosciences, 562886	2.5µl	Surface
CCR6	BB515	BD Biosciences, 564479	2.5µl	Surface
LAP	PE	BD Biosciences, 562260	5µl	Surface
Ki67	FITC	BD Biosciences, 556026	20µl	Intracellular
Foxp3	PE	BD Biosciences, 560082	5µl	Intracellular
Foxp3	AF488	BD Biosciences, 560887	5µl	Intracellular
IL-17A	BV421	BD Biosciences, 562933	5µl	Intracellular
IL-10	BV421	BioLegend, 501421	5µl	Intracellular
IFNγ	PE-Cy7	BD Biosciences, 560924	5µl	Intracellular
TNFα	BUV395	BD Biosciences, 563996	5µl	Intracellular
IL-2	BV785	BioLegend, 500347	5µl	Intracellular

2.3.3 Surface staining

Cells were re-suspended in 100µl of staining buffer per 10^6 cells containing surface staining antibodies according to the panels stated in part 2.3.2 with volumes stated in table 2.3.2. Cells were incubated at 4°C for 25 minutes in the dark. Cells were topped up to 1ml with staining buffer and centrifuged at 400x g for 5 minutes and the supernatant discarded. Where staining for surface markers only was required, cells were re-suspended in 300µl staining buffer with 2% paraformaldehyde. Otherwise cells were prepared for intracellular staining as described in part 2.3.4. Cells were analysed within 3 days post-staining using the LSR II (BD Biosciences) flow cytometer and FlowJo software (Version 10).

2.3.4 Intracellular staining

Surface stained cells were re-suspended in 100µl of fixation/permeabilisation solution (BD Biosciences, 00-5523-00) and incubated overnight at 4°C in the dark. Cells were then topped up to 1ml in permeabilisation buffer (BD Biosciences, 00-5523-00) and centrifuged at 400x g for 5 minutes. The supernatant was discarded and the cells re-suspended in 100µl permeabilisation buffer containing the required intracellular staining antibodies according to the panels and volumes stated in part 2.3.2 and were incubated at 4°C in the dark for 1 hour. Cells were topped up to 1ml in permeabilisation buffer and centrifuged at 400x g for 5 minutes. The supernatant was discarded and the cells were re-suspended in 300µl of staining buffer with 2% paraformaldehyde. Cells were analysed within 3 days post-staining using the LSRII (BD Biosciences) flow cytometer and FlowJo software (Version 10).

2.4 Suppression assays

Media used in these experiments were as follows unless otherwise described:

- Culture medium: RPMI supplemented with 1µg/ml anti-CD3 antibody HIT3a clone (eBioscience, 16-0039-85), 1% penicillin-streptomycin, and 10% heat-inactivated autologous serum
- Infection medium: RPMI supplemented with 10% autologous serum, 2.5% HEPES (Sigma Aldrich, H3375-25G) and 1µg/ml anti-CD3 antibody

Experiments used cell types isolated from the same donor. The majority of experiments in this thesis used a ratio of 3 MDMs to 1 Treg/Teff cell, with Treg/Teff cells incubated on MDMs for 14 hours prior to removal of the Treg/Teff cells and infection of the MDMs with a multiplicity of infection (MOI) of 2 *S. pneumoniae* per MDM. Some experiments substituted *S. pneumoniae* for *A. baumannii* at the specified MOI.

2.4.1 Pre-infection co-culture of MDMs with Treg/Teff cells

MDMs were cultured in a 48-well plate as described in part 2.2.2. On day 5 of MDM culture, Treg and Teff cells were FACS-sorted as described in part 2.2.3 and were re-suspended in culture medium at $1.67x10^5$ cells/ml. Media from day 5 MDMs was removed and $3.3x10^4$ Treg or Teff cells were added per well (giving a 1:3 Treg/Teff:MDM ratio based on the typical yield of $1x10^5$ MDMs per well). Wells were topped up to 300µl with culture medium. Cells were incubated for 14 hours at 37°C in 5% CO_2. The non-adherent Treg/Teff cells were then removed from the adherent MDMs by washing with sterile PBS. The MDMs were then either infected with 2×10^5 *S. pneumoniae* (MOI 2 *S. pneumoniae* per MDM) in 100µl of infection medium, or uninfected groups had 100µl medium added. Wells were topped up to 300µl with infection medium. Cells were incubated for 6 hours at 37°C in 5% CO_2. 1% penicillin-streptomycin was added at 6 hours and a 50µl supernatant sample was taken for

cytokine analysis. The cells were incubated until 72 hours post-infection at 37°C in 5% CO_2 with a 50µl supernatant sample taken at 24 hours and the remaining supernatant taken at 72 hours. Supernatant samples were stored at -20°C until cytokine analysis.

2.4.2 MDM-Treg/Teff co-culture during infection

Treg and Teff cells were incubated on MDMs for 14 hours as described in 2.4.1. Following the 14 hour incubation, the Treg and Teff cells were then removed from the MDMs by washing with PBS and placed into separate falcon tubes. The Treg and Teff cells were centrifuged at 400x g for 5 minutes, the supernatant discarded and the cells re-suspended in 400µl of infection medium. 100µl of the cell suspension was added to fresh MDMs (4 wells of Treg cells and 4 wells of Teff cells). MDMs were infected with 2 x 10^5 *S. pneumoniae* (MOI 2 *S. pneumoniae* per MDM) in 200µl infection medium. Cells were incubated at 37°C in 5% CO_2 for 6 hours before addition of 1% penicillin-streptomycin. Cells were incubated until 72 hours post-infection at 37°C in 5% CO_2 with 50µl supernatant samples taken at 6 and 24 hours post-infection. The remaining supernatant was collected at 72 hours post-infection. Supernatant samples were stored at -20°C until cytokine analysis.

2.4.3 Post-infection addition of Treg/Teff cells to MDMs

Treg and Teff cells were incubated on MDMs for 14 hours as described in 2.4.1. Following the 14 hours incubation, all wells except the Treg/Teff cell incubation wells were washed 3 times in sterile PBS then infected with 2 x 10^5 *S. pneumoniae* (MOI 2 *S. pneumoniae* per MDM) in 300µl infection medium. Cells were incubated at 37°C in 5% CO_2 for 6 hours, after which 1% penicillin-streptomycin was added to each well. The Treg and Teff cells were then removed from the MDMs by washing with PBS and placed into separate falcon tubes. The Treg and Teff cells were centrifuged at 400x g for 5 minutes, the supernatant discarded and the cells re-suspended in infection

medium. $3.3x10^4$ Treg or Teff cells were added to the previously infected MDMs in 50µl (giving a 1:3 Treg/Teff:MDM ratio based on the typical yield of $1x10^5$ MDMs per well) and incubated until 72 hours post-infection. A 50µl supernatant sample was taken at 24 hours post-infection and the remaining supernatant collected at 72 hours post-infection. Supernatant samples were stored at -20°C until cytokine analysis.

2.4.4 Transwell-separated MDM-Treg/Teff co-culture

MDMs were cultured in a 24-well plate as described in part 2.2.2. On day 5 of MDM culture, Treg and Teff cells were FACS-sorted as described in part 2.2.3 and were re-suspended in culture medium at $1.67x10^5$ cells/ml. Media from day 5 MDMs was removed and wells were topped up to 500µl with culture medium. $6.6x10^4$ Treg or Teff cells were added to 3µm pore transwell inserts (Appleton Woods, CC419) and placed above the MDM wells (giving a 1:3 Treg/Teff:MDM ratio based on the typical yield of $2x10^5$ MDMs per well). Cells were incubated for 14 hours at 37°C in 5% CO_2. After the 14 hour incubation, the transwell insert containing the Treg/Teff cells was removed and MDMs were infected with 4×10^5 *S. pneumoniae* (MOI 2 *S. pneumoniae* per MDM) in 600µl of infection medium. Cells were incubated for 6 hours at 37°C in 5% CO_2. 1% penicillin-streptomycin was added at 6 hours and a 100µl supernatant sample was taken for cytokine analysis. The cells were incubated until 72 hours post-infection at 37°C in 5% CO_2 with a 100µl supernatant sample taken at 24 hours and the remaining supernatant taken at 72 hours. Supernatant samples were stored at -20°C until cytokine analysis.

2.4.5 MDM-Treg/Teff supernatant stimulation of MDMs

MDMs were cultured in a 48-well plate as described in part 2.2.2. Media from day 5 MDMs was removed and replaced with 300µl of supernatant taken from a previous MDM-Treg/Teff 14 hour incubation from the same donor. Cells were incubated for 14 hours at 37°C in 5% CO_2. Following incubation, the MDMs were washed with sterile

PBS. The MDMs were then either infected with 2 x 10^5 *S. pneumoniae* (MOI 2 *S. pneumoniae* per MDM) in 100µl of infection medium, or uninfected groups had 100µl medium added. Wells were topped up to 300µl with infection medium. Cells were incubated for 6 hours at 37°C in 5% CO_2. 1% penicillin-streptomycin was added at 6 hours and a 50µl supernatant sample was taken for cytokine analysis. The cells were incubated until 72 hours post-infection at 37°C in 5% CO_2 with a 50µl supernatant sample taken at 24 hours and the remaining supernatant taken at 72 hours. Supernatant samples were stored at -20°C until cytokine analysis.

2.4.6 LAP neutralisation / ALK5 inhibition in MDM-Treg/Teff co-culture

These experiments were carried out as in 2.4.1 with the addition that the MDM+Treg /Teff cells were incubated in the presence of either 2µg/ml anti-LAP neutralising antibody (R&D Systems, AF-246-NA) or 1µg/ml ALK5 inhibitor (Sigma Aldrich, SB-525334) for the 14 hour incubation following FACS sorting.

2.4.7 Antibiotic protection assay

Treg and Teff cells were incubated on MDMs for 14 hours as described in 2.4.1. Following the 14 hours incubation, the non-adherent Treg/Teff cells were removed from the adherent MDMs by washing with sterile PBS. MDMs were infected with 1x10^6 *S. pneumoniae* (MOI 10 *S. pneumoniae* per MDM) in RPMI for 4 hours. The supernatants were then removed and plated for extracellular *S. pneumoniae* growth as described in 2.1.1 and the MDMs washed 3 times with sterile PBS. MDMs were incubated in RPMI containing 200µg/ml gentamicin for a further 1 hour. The supernatants were then removed and the MDMs washed 3 times with sterile PBS. MDMs were lysed by addition of sterile water for 1 minute and the lysate plated for growth of intracellular *S. pneumoniae* as described in 2.1.1.

2.4.8 Exogenous IL-10 and TGFβ addition to MDMs

MDMs were cultured in a 48-well plate as described in part 2.2.2. On day 6 of culturing, the MDMs were washed three times in PBS. Medium was replaced with either RPMI alone, RPMI + 1ng/ml IL-10, RPMI + 100ng/ml IL-10, RPMI + 1ng/ml TGFβ, or RPMI + 50ng/ml TGFβ, in a total 200µl volume and incubated for 1 hour at 37°C (porcine TGFβ1, R&D Systems, 101-B1-001; IL-10, Miltenyi Biotech, 130-093-947). After incubation, 100µl of *S. pneumoniae* was added at $1x10^7$ CFUs /ml (MOI 10 *S. pneumoniae* to 1 MDM) in RPMI. Cells were then incubated at 37°C for 6 hours, then the supernatants were collected for analysis of cytokine concentrations by ELISA.

2.4.9 Treg suppression of Teff proliferation

Activating medium was prepared by supplementing RPMI with 10% heat-inactivated autologous serum, 1% penicillin-streptomycin, 2µg/ml anti-CD3 antibody (eBioscience, 16-0039-85) and 2µg/ml anti-CD28 antibody (eBioscience, 16-0289-85). Treg and Teff cells were FACS-sorted as described in part 2.2.3. Treg and Teff cells were washed in PBS by centrifuging at 400x g then re-suspended in activating medium at $3.3x10^5$ cells per ml. 100µl of Treg cell suspension were added to 300µl Teff cell suspension in wells of a 48-well plate. Additional wells contained $3.3x10^4$ Treg cells alone and $1x10^5$ Teff cells alone in a total of 400µl activating medium. Cells were incubated for 5 days at 37°C in 5% CO_2. Cells were then collected and stained for Foxp3 and Ki67 as described in part 2.3.

2.4.10 Treg/Teff cytokine detection in MDM co-culture

Treg and Teff cells were incubated on MDMs for 14 hours as described in 2.4.1. Following incubation, 10ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, P1585), 1.5µg/ml ionomycin (Sigma Aldrich, I9657) and 1:100 GolgiStop (BD Biosciences, 554724) were added to each well of MDM-Treg/Teff cell co-culture in a

total volume of 200µl per well and incubated at 37°C in 5% CO_2 for 6 hours. Treg and Teff cells were then recovered from the MDMs by washing 3 times with sterile PBS, centrifuging at 400x g and stained for intracellular cytokines as described in part 2.3.

2.5 $CD4^+$ T cell differentiation and cytokine detection

Culture medium was prepared by supplementing RPMI with 10% heat-inactivated autologous serum, 2.5% HEPES buffer and 1µg/ml anti-CD3 antibody. MDMs were grown in 48-well plates as described in part 2.2.2. After monocyte adherence of the MDMs, the non-adhered cells were collected and frozen in 95% FCS with 5% dimethyl sulphoxide (DMSO, Sigma Aldrich, C6164) at -80°C. On day 6 of MDM differentiation, these none-adhered PBMCs were rapidly defrosted in a water bath at 37°C and were purified for $CD4^+$ T cells by the method described in 2.2.4. The $CD4^+$ purified cells were re-suspended in culture medium at 300µl per the number of wells of MDMs. The MDMs were washed in sterile PBS and 300µl of $CD4^+$ T cell solution added per well, or 300µl of cell suspension was added to wells without MDMs. Cells were either infected with $2x10^5$ *S. pneumoniae* (MOI 2 *S. pneumoniae* per MDM) in 100µl of culture medium, or an additional 100µl of medium alone was added and incubated for 6 hours at 37°C in 5% CO_2. After 6 hours, 1% penicillin-streptomycin was added to each well and the cells were incubated at 37°C in 5% CO_2 for a further 24 hours. For experiments examining differentiation only, $CD4^+$ T cells were at this point recovered from the MDMs by washing 3 times with sterile PBS and centrifuging at 400x g for 5 minutes. The pellets were re-suspended in staining buffer and stained for markers as described in part 2.3. For experiments examining $CD4^+$ T cell cytokine production, 2µl of GolgiStop was added to each well and incubated for a further 6 hours. $CD4^+$ T cells were then recovered from the MDMs by washing 3 times with sterile PBS and centrifuging at 400x g for 5 minutes. The pellets were re-suspended in staining buffer and stained for markers as described in part 2.3. Samples of the $CD4^+$ T cells stained immediately after $CD4^+$ purification were used for comparison to the resulting differentiated cells. Samples of the differentiated cells were also stained as FMO controls.

2.6 UV-killed *S. pneumoniae* intradermal injection model

Healthy volunteers aged 18-50 years old were recruited for this study. Experiments were approved by the UCL Research Ethics Committee (ref. 7577/001) and informed consent was obtained from all participants. These experiments are adapted from the methods described by Motwani *et al.,* 2016.

2.6.1 Preparation of UV-killed *S. pneumoniae*

S. pneumoniae were grown in autoclaved THY broth medium at 37°C until reaching an OD of 0.4 (approximately $1x10^8$ CFUs /ml). *S. pneumoniae* were washed by centrifugation at 13 000x g for 10 minutes, the supernatant discarded, and bacteria were re-suspended in sterile PBS in a sterile petri dish. The bacteria were killed by exposure to ultraviolet light (UV) for 1 hour. The UV-killed *S. pneumoniae* were collected and washed in sterile saline at 13 000x g for 10 minutes. Aliquots of $1.5x10^8$ UV-killed *S. pneumoniae* in 1 ml of sterile saline were frozen at -80°C in autoclaved 10% glycerol until required for injection. The bacteria were confirmed dead by University College London Hospitals Microbiology department.

2.6.2 Intradermal injection of UV-killed *S. pneumoniae* and induction of suction blisters

$7.5x10^5$ UV-killed *S. pneumoniae* in 100µl of sterile saline were injected intradermally into the forearms of healthy volunteers by a medical professional. Each volunteer had one injection in each arm. At the specified time points, a suction chamber with a 10mm diameter hole was connected to a negative pressure instrument (NP-4, Electronic diversities Ltd., MD, USA) and secured at the site of injection. The negative pressure was applied gradually until a blister was visibly fully formed, at which point the pressure was gradually returned to the baseline (figure 2.6.2). The blister was pierced with a 23G needle (Fisher Scientific, NN-2332R) and the fluid collected using a 200µl pipette. The blister area of the forearm of the volunteers was cleaned with 0.5%

Cetrimide spray (Savlon) and a large protective dressing applied (Mepore). The blister fluid was centrifuged at 400x g for 5 minutes, the supernatant collected for cytokine analysis, and the cells re-suspended in ACK lysis buffer (Lonza, 10-548E) for 1 minute. The cells were washed in PBS by centrifugation at 400x g and re-suspended in 100µl staining buffer (PBS with 0.5% FBS and 0.4% 2mM EDTA). The cell count was determined using a haemocytometer with Trypan blue dead cell exclusion. The cells were then surface stained for flow cytometry as described in 2.3. Dr Daniel Marks, Riccardo Wysoczanski and Dr. Ricardo Jose at UCL aided in experimental design, intradermal injecting, and conducting of experiments.

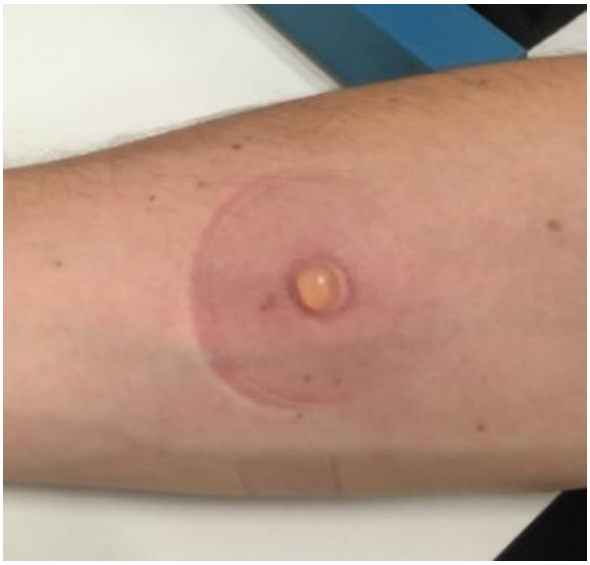

Figure 2.6.2 Suction blister raised by negative pressure applied to the forearm

Blisters were raised by negative pressure at the site of intradermal injection of UV-killed *S. pneumoniae* to collect the cells that had recruited in response to the presence of the bacteria. The fluid was collected when the blister had formed as shown.

2.6.3 Laser Doppler imaging

Healthy volunteers with UV-killed *S. pneumoniae* intradermally injected into the forearms had laser Doppler scans taken of the injection area at the specified time points immediately prior to blister induction. The forearm was placed at a fixed distance from the scanner of the Laser Doppler Imager (moor LDI-HIR, Moor Instruments Ltd, UK) focused at the injection site and scanned to produce a colour-coded image. The resulting images were analysed using moorLDI software (Version 5). The blood flow was quantified by multiplying the number of pixels by the mean blood flow signal and is referred to as arbitrary “perfusion units”. A set level of background pixels below a fixed threshold (below 300 perfusion units) were removed from each image which allowed comparison of only the inflamed areas.

2.7 Immunofluorescence staining

2.7.1 Cytospins of PBMCs

Media, buffers and reagents were prepared as follows:

- Blocking buffer: 10% normal goat serum (NGS, Abcam, ab7481), 1% bovine serum albumin (BSA, Sigma Aldrich, A2153) and 0.01% Tween 20 (Sigma Aldrich, P1379) in PBS.
- Washing buffer: 0.01% Tween in PBS
- Primary antibody solution: rabbit anti-human CD4 (Abcam, ab133616) and mouse anti-human Foxp3 (eBioscience, 236A/E7) and were diluted 1:100 in PBS with 0.01% Tween 20 and 1% BSA.
- Secondary antibody solution: 1:1000 Alexa Fluor 488 goat anti-mouse IgG (Thermo Fisher, A28175), 1:1000 goat anti-rabbit IgG Alexa Fluor 546 (Thermo Fisher, A11035) and 1:10 000 DAPI (Abcam, ab228549) diluted in PBS with 0.01% Tween 20 and 1% BSA

PBMCs were re-suspended at $5x10^6$ cells/ml in RPMI supplemented with 30% FCS. 200µl of the cell suspension was pipetted on to a chamber slide and mounted with the paper pad and cuvette into the metal holder and loaded into the cytocentrifuge. The slides were centrifuged at 800 rpm for 3 minutes then allowed to air dry. Slides were fixed for 5 minutes in methanol then washed twice by submerging in a container of wash buffer with a magnetic stir bar and placing on a magnetic stirrer. A hydrophobic barrier was drawn with a PAP pen (Abcam, ab2601) around the visible cell cluster. The slides were placed in a slide tray lined with damp tissue paper. 200µl of blocking buffer was pipetted on to the cells inside the hydrophobic circle and incubated for 1 hour. Slides were then washed 3 times in washing buffer on the magnetic stirrer. 200µl of primary antibody solution was pipetted on to the cells and incubated at 4°C overnight. The primary antibody solution was removed by tapping

off the liquid and the slides were washed 3 times in washing buffer on the magnetic stirrer. 200µl of the secondary antibody solution was pipetted onto the cells and incubated for 1 hour at room temperature in the dark. The secondary antibody solution was removed by tapping away the liquid and washed 3 times with washing buffer on the magnetic stirrer. The residual liquid was removed by tapping the slide and 30µl of Fluoromount (Invitrogen, 00-4958-02) was dropped onto the cells, followed by addition of a coverslip. The edges of the coverslips were sealed by addition of clear varnish and allowed to dry at room temperature in the dark for 45 minutes. Cells were imaged by confocal microscopy at 60x magnification with Olympus TIRF and images processed with ImageJ software with the kind help of Dr. Giuseppe Ercoli, UCL.

2.7.2 Immunofluorescence staining of human normal lung sections

Media, buffers and reagents were prepared as follows:

- Washing buffer: 0.3% Triton X-100 (Sigma Aldrich, 11332481001) in PBS
- Antigen-retrieval buffer: 10mM sodium citrate and 0.05% Tween 20 in distilled water at pH 6
- Blocking solution: 1% BSA, 5% normal donkey serum (NDS, Abcam, ab7475), 0.1% Triton X-100 in PBS
- Primary antibody solution: rabbit anti-human CD4 (Abcam, ab133616) and mouse anti-human Foxp3 (eBioscience, 236A/E7) diluted 1:100 in blocking solution
- Secondary antibody solution: 1:1000 Alexa Fluor 488 goat anti-mouse IgG (Thermo Fisher, A28175) and 1:1000 goat anti-rabbit IgG Alexa Fluor 546 (Thermo Fisher, A11035) diluted in PBS with 5% NDS and 0.1% Triton X-100
- DAPI solution: 1:10000 dilution in PBS with 5% NDS and 0.1% Triton X-100

Sections of normal human lung were a kind gift from Professor Robin McAnulty, UCL. The paraffin-embedded sections were chilled on ice before cutting on a microtome

into 5µm slices and floated in ultrapure water heated to 45°C to flatten out. The slices were collected and placed onto microscope slides and dried overnight at 37°C. Slides were then de-waxed in an autostainer and placed in a beaker of tap water.

Slides were rinsed in PBS then washed by submerging the slides into a container of wash buffer and addition of a magnetic stir bar and placing on a magnetic stirrer for 5 minutes. Wash buffer was then discarded and replaced and repeated 3 times. Slides were then arranged in a container of antigen retrieval buffer with a blank slide at the front of the slides and microwaved at 800 watts for 5 minutes. The slides were allowed to cool for 1 hour then rinsed in PBS.

A slide tray was lined with tissue soaked in deionised water and the slides placed on the tray. A circle was drawn around the section on the slide with a hydrophobic PAP pen. 200µl of blocking solution was pipetted inside the circle and incubated for 1 hour at room temperature. The blocking solution was then flicked off the slide and replaced with 200µl of primary antibody solution. The slides were incubated overnight at 4°C in the dark.

The liquid was flicked off the slides and slides were washed 3 times in washing buffer as described. 200µl of secondary antibody solution was added and incubated for 3 hours at room temperature in the dark. The liquid was then flicked off followed by addition of 200µl of DAPI solution and incubated for 20 minutes at room temperature in the dark. The slides were washed 3 times as described, a drop of Fluoromount was added to the slides and covered with a cover slip. The edges of the coverslips were sealed by addition of clear varnish and allowed to dry at room temperature in the dark for 45 minutes. Cells were imaged by confocal microscopy at 60x magnification with Olympus TIRF and images processed with ImageJ software with the kind help of Dr. Giuseppe Ercoli, UCL.

2.8 Enzyme-linked immunosorbent assay (ELISA)

All cytokine concentration analysis was carried out using kits from R&D Systems as per the manufacturer's instructions. The kits used were TNFα (DY210), IL-6 (DY206), IL-1β (DY201), IL-10 (DY217B), IL-17 (DY317), IL-22 (DY782), TGFβ (DY240), IL-8 (DY208). Buffers and reagents for ELISA were prepared as follows:

- Wash buffer: PBS with 0.05% Tween 20
- Reagent diluent (all kits except TGFβ): 1% BSA in PBS
- Reagent diluent for TGFβ kit: 1.4% Reagent diluent (R&D Systems, DY997) and 0.05% Tween 20 in PBS
- Blocking buffer (for all kits except TGFβ): 1% BSA in PBS
- Blocking buffer for TGFβ kit: 5% Tween 20 in PBS
- Substrate solution: tetramethylbenzidine (TMB, Invitrogen, 002023)
- Stop solution: 2N sulphuric acid

Coating antibody diluted in PBS was added to the wells of a 96-well plate (Fisher Scientific, 13-882-220) and incubated overnight at room temperature. Plates were then washed 3 times in wash buffer, tapped on tissue paper to remove residual liquid and wells were blocked by addition of blocking buffer for 1 hour. Blocking buffer was then removed and the plate washed 3 times in washing buffer. Samples were diluted in reagent diluent and added to wells in duplicate. For TGFβ analysis, samples were activated by addition of 1N hydrochloric acid for 10 minutes then neutralized by addition of 1.2N sodium hydroxide. A standard curve of 7x two-fold concentrations and a blank of reagent diluent only was included. The plate was incubated for 2 hours then washed 3 times in wash buffer. Detection antibody diluted in reagent diluent was added to the wells for 2 hours. The plate was then washed 3 times in wash buffer and streptavidin-HRP added for 20 minutes. The plate was washed 3 times in wash buffer and substrate solution was added and incubated in the dark for approximately 20

minutes or until colour change was observed, at which point 2N sulphuric acid was added to the wells. The absorbance was read on a Versamax plate reader at 450nm and 540nm. Concentration of cytokines in the samples were calculated by comparison against the standard curve.

2.9 Cytotoxicity assay

Cell cytotoxicity in *S. pneumoniae*-infected MDM cell culture was measured using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, 88953). This assay measures lactate dehydrogenase, which is released upon damage to the cell membrane and is used as a measurement of cell death. LDH present in the supernatant catalyses reactions resulting in a red formazan product that can be measured by absorbance at 490nm. The level of formazan is directly proportional to the level of LDH and therefore cell cytotoxicity.

The assay was carried out as per the manufacturer's instructions. Controls corrected for background LDH presence in the culture medium, spontaneous LDH release by MDMs and by *S. pneumoniae* cultured separately, and for volume changes caused by reagent addition. The maximum possible LDH release by MDMs was determined by lysing the MDMs using the supplied lysis buffer. The supplied reaction mixture (lyophilizate) was added to supernatant samples and controls then incubated for 30 minutes before addition of the supplied stop solution. The absorbance was measured at 490nm and 680nm.

To correct the acquired values, the mean culture medium background LDH value was subtracted from each of the mean LDH presence in the samples, spontaneous MDM LDH release, and the mean *S. pneumoniae* LDH release. The mean volume correction control was then subtracted from the mean MDM maximum LDH release control. The percent cytotoxicity was then calculated using the following calculation:

% cytotoxicity = (sample LDH – *S. pneumoniae* LDH – MDM LDH) / (MDM maximum LDH – MDM spontaneous LDH) x 100

2.10 Statistics

Statistical analyses appropriate to the nature of the data were conducted using GraphPad Prism Version 8 software. Normality tests were first used to determine whether a parametric or non-parametric test would be used. Where the assumptions of the parametric test were met and 2 groups were being compared, data were analysed using paired or unpaired t-tests. Where 2 groups were compared that did not meet pass the normality tests, Mann-Whitney tests were used for unpaired data or the Wilcoxon matched-pairs signed rank test was used for paired data. Where more than 2 groups were being compared, one-way analysis of variance (ANOVA) was used if the data were normally distributed otherwise the Kruskal-Wallis test was used. For paired data with more than 2 groups, the repeated-measures one-way ANOVA was used if the data were normally distributed or the Friedman test was used when data were not normally distributed. Post-tests were conducted on analyses with more than two groups. When comparing groups against a control in normally distributed, unpaired data, the Dunnett's multiple comparisons test was used. If these groups were all being compared against each other, Tukey's multiple comparisons test was used. For non-parametric analyses, Dunn's multiple comparisons test was used. If data had 2 grouped variables, two-way ANOVA was used with Sidak's multiple comparisons post-test. Error bars show standard deviation (SD) of the mean.

Statistical significance is denoted as follows:

*p<0.05
**p<0.01
***p<0.001
****p<0.0001

Chapter 3: Establishing co-culture conditions to investigate *in vitro* effects of Treg on macrophage inflammatory responses to *S. pneumoniae*

3.1 Chapter 3 Introduction

Treg effects on macrophage inflammatory responses to *S. pneumoniae* have not been previously investigated, therefore culture conditions to investigate these potential interactions have not been established. This chapter of experiments aimed to optimise co-culture conditions to investigate whether Treg cells can suppress *in vitro* macrophage inflammatory responses to *S. pneumoniae*. Combining MDMs, Treg cells and *S. pneumoniae* together in culture required:

- the separation of a highly purified and suppressive Treg population from peripheral blood
- purification of autologous monocytes differentiated into macrophages
- selection of a strain of *S. pneumoniae* to provoke a robust MDM inflammatory response
- a culture medium optimised to allow survival and activity of all three cell types
- establishing the optimal incubation times and cell ratios

Culture conditions such as the ratios of MDMs to Treg/Teff cells, whether additional activating antibodies are required for the Treg/Teff action, and the timings of co-culture between the MDMs, Treg/Teff cells and *S. pneumoniae* to preserve cell survival and allow time for effector mechanisms to work required examination. Different strains of *S. pneumoniae* provoke different intensities of inflammatory response, for example the serotypes associated with invasive disease such as TIGR4 are documented as promoting highly inflammatory response from immune cells,

whereas the more effective colonisers such as 23F promote less inflammatory responses. The inflammatory responses of these strains were compared in order to determine which strain would provide the most informative results, as a robust enough macrophage inflammatory response is first required in order to see any suppressive effects by Treg cells on this response. The production of the inflammatory cytokines TNFα and IL-6 were used as readouts for measuring the strength of the pro-inflammatory response of MDMs to *S. pneumoniae* infection, as these are the main cytokines produced by macrophages in response to *S. pneumoniae* infection (Tomlinson et al., 2014). The experiments all used MDMs as opposed to alveolar macrophages, as MDMs are utilised in the response to both colonisation and lung infection, and are a primary cell type which are more likely to more closely represent *in vivo* cell behavious and phenotypes compared to using a cell line. MDMs were differentiated using M-CSF, as macrophage development *in vivo* is thought to be driven principally by this cytokine (Hume, 2006). Treg cells were isolated based on their $CD25^{+}CD127^{low}$ expression and Teff cells were isolated as $CD25^{-}CD127^{high}$ expression to act as a comparator, therefore the purities of these populations was examined to ensure that the risk of contamination between these cell types was minimal, and the results highly likely to reflect the effect caused by the intended population. The suppressive ability of the Treg population on the proliferation of the Teff population was examined as an indicator that the isolated Treg cells are a suppressive population which may be able to suppress MDMs. The findings of this chapter describe the data used for selecting the optimal conditions and protocol for further *in vitro* Treg-MDM-*S. pneumoniae* co-culture experiments, and these conditions are used in the experiments described in further chapters of this thesis.

3.2 Determining the purity of sorted Treg and Teff populations

Experiments comparing inflammatory responses of MDMs with or without Treg requires a method to separate purified populations of Treg cells and purified Teff to act as a comparator. Currently the best marker available for $CD4^+$ Treg is Foxp3 along with high expression of CD25 and low expression of CD127. However, as Foxp3 is intracellular, this marker cannot be used to sort live populations of Treg cells. Therefore, sorting for Treg cells was based on $CD4^+CD25^+CD127^{low}$ expression, and Teff cells were also collected as $CD4^+CD25^-CD127^{high}$. To determine the purity of the sorted cell populations, the CD4, Foxp3, CD25 and CD127 expression of the pre-sorted PBMCs and the post-sorted Treg cells and Teff cells were compared. Expression of these markers was examined after selection of lymphocytes, exclusion of doublets and dead cells, and gating the $CD4^+$ population. Post-sort $CD4^+$ purity was approximately 98%, whereas pre-sorted PBMCs contained approximately 45% $CD4^+$ cells (figure 3.2 A and B). The mean Foxp3 expression of the pre-sorted $CD4^+$ cells was 9%, whereas post-sort the mean Foxp3 expression for Treg and Teff cells was 82% and 4%, respectively (figure 3.2 C and D). Pre-sort, the mean percentage of $CD4^+$ cells falling into the $CD25^+CD127^{low}$ and $CD25^-CD127^{high}$ gates were 11% and 84%, respectively, whereas post-sort a mean of 90% of the sorted Treg cells fell into the $CD25^+CD127^{low}$ gate, with 4% falling in the $CD25^-CD127^{high}$ gate (figure 3.2 E and F). A mean of 97% of the post-sorted Teff cells fell into the $CD25^-CD127^{high}$ gate, with a mean of 3% falling into the $CD25^+CD127^{low}$ gate (figure 3.2 E and F). Collectively, these data indicate that the sorting strategy produced Treg populations of an approximate purity of over 80% based on Foxp3 expression and 90% based on surface $CD25^+CD127^{low}$ expression, and Teff populations of approximate purity of over 95% based on either $Foxp3^-$ or $CD25^-CD127^{high}$ expression.

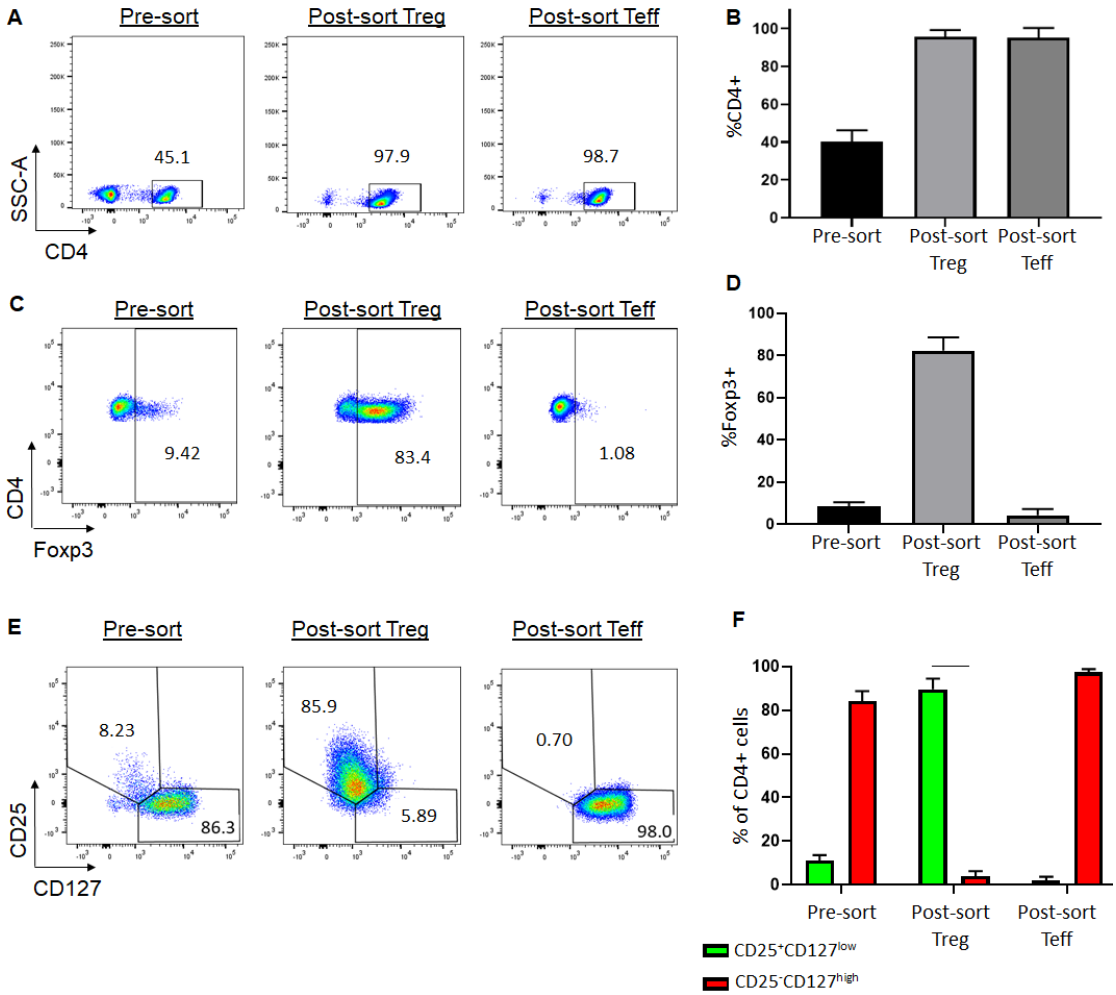

Figure 3.2 CD4, CD25, CD127 and Foxp3 expression of pre-sort PBMCs and post-sort Treg and Teff populations

Sorted Treg/Teff cells and pre-sort PBMCs stained for CD4, CD25, CD127 and Foxp3 were analysed by flow cytometry. Flow cytometry plots from 1 representative donor (A) and data from biological replicates from 3 donors (B) show CD4 expression of pre-sort PBMCs and post-sort Treg cells and Teff cells. Foxp3 expression of pre-sort $CD4^+$ cells and post-sort Treg cells and Teff cells are shown from 1 representative donor (C) and from biological replicates from 3 donors combined (D). Percentages of $CD4^+$ cells falling into the $CD25^+CD127^{low}$ gate and the $CD25^-CD127^{high}$ gate are shown for pre-sort PBMCs, post-sorted Treg cells and post-sorted Teff cells from 1 representative donor (E) and from biological replicates from 3 donors combined (F). Graphs B, D, and F represent mean percent expression +/- SD of the mean.

3.3 Confirming the sorted Treg population has suppressive capability

A definitive marker for Treg cells has not yet been found. Therefore, regulatory ability is confirmed by testing whether the presence of Treg cells suppresses proliferation or cytokine production by Teff cells. Hence, FACS-sorted $CD4^{+}CD25^{+}CD127^{low}$ Treg cells and $CD4^{+}CD25^{-}CD127^{high}$ Teff cells were cultured either separately or together in a ratio of 3 Teff cells to 1 Treg for 5 days in medium containing the T cell activating antibodies anti-CD3 and anti-CD28. Treg cells and Teff cells were then stained for Ki67 expression as a marker of proliferation and analysed by flow cytometry. Cells were additionally stained with Foxp3 in order to exclude the majority of the Treg cells from the Ki67 analysis and thus prevent a diluting effect of Ki67 in the Teff+Treg group by the Treg cells (figure 3.3 A). A reduction of expression of Ki67 by $Foxp3^{-}$ Teff cells cultured with Treg cells is indicated by a negative shift in fluorescence compared to Teff cells cultured alone as shown from one representative from the Teff group and one representative from the Teff+Treg group (B). Calculating the median fluorescence intensity (MFI) from 4 technical replicates each from the Teff group and from the Teff+Treg group showed a statisitically significant decrease in Ki67 expression by Teff cells co-cultured with Treg cells (C). This result suggests that the $CD25^{high}CD127^{low}$ cells isolated by this FACS protocol may suppress Teff cell proliferation, but more data is required to confirm this. These data are limited in that this experiment was only conducted on cells from 1 donor, and should be repeated with cells from additional donors to improve the reliability of this result. Additionally, these data could be improved by incorportation of a fluorescent tracer such as carboxyfluorescein succinimidyl ester (CFSE) or cell trace violet to track the proliferation of the Teff cells.

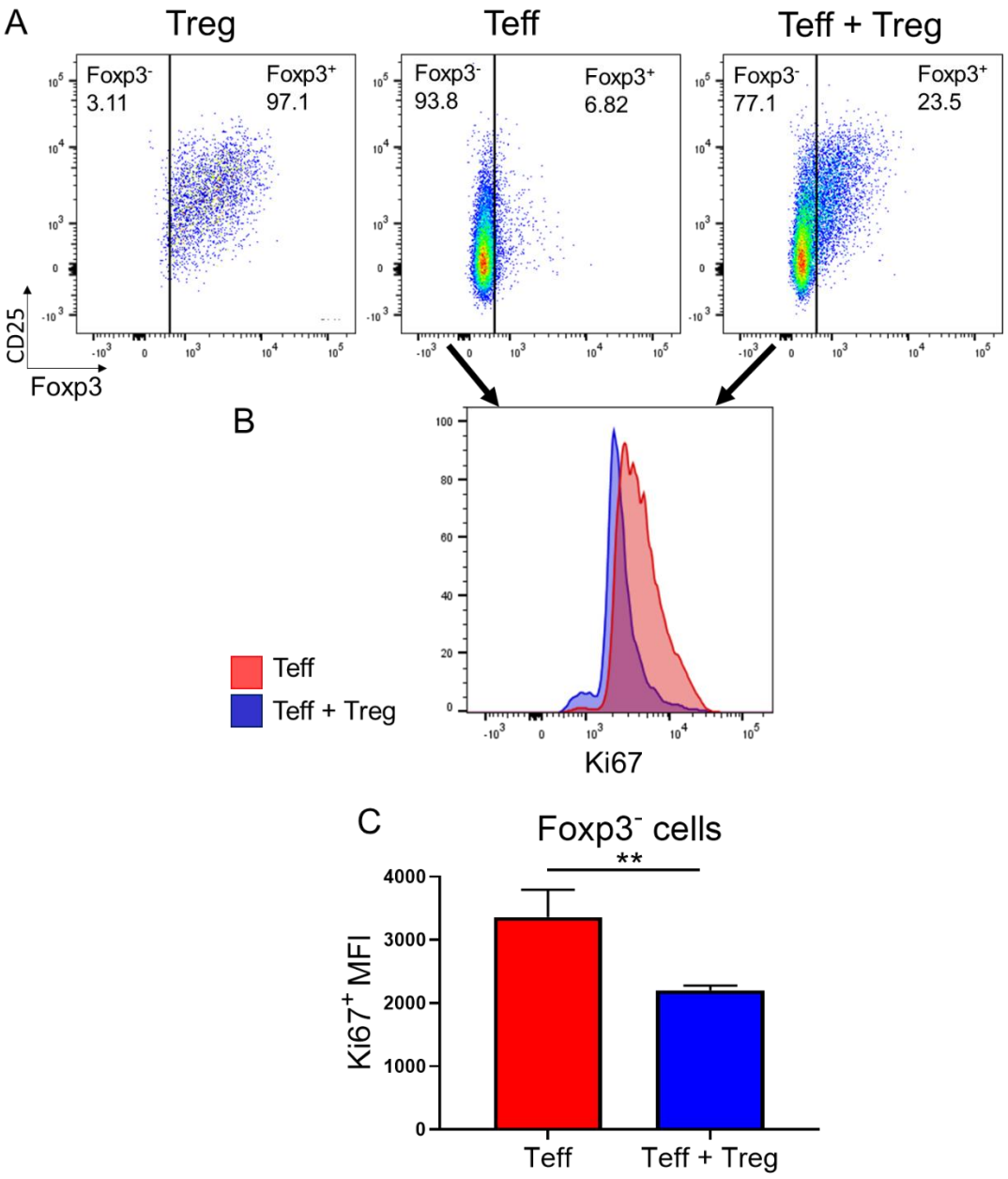

Figure 3.3 Ki67 expression of Teff cells cultured with or without Treg cells

Treg cells and Teff cells were cultured either alone or together at a ratio of 3 Teff cells to 1 Treg for 5 days with anti-CD3 and anti-CD28 activating antibodies before intracellular staining for Ki67 and Foxp3 and analysis by flow cytometry. Single, live $CD4^+$ cells were separated into $Foxp3^-$ and $Foxp3^+$ populations to remove the Treg cells from further anaylsis with gating based on visible cell populations (A). Ki67 expression by $Foxp3^-$ populations in the Teff and Teff+Treg groups is shown as a histogram (B). The MFI of Ki67 expression by the $Foxp3^-$ cells in the Teff and Teff+Treg groups from 4 technical replicates (4 wells per condition containing cells from one donor) was calculated (C). Graph C shows the mean MFI values with error bars showing +/- SD of the mean and is analysed by upaired t test (**p<0.01)

3.4 Comparing strains of *S. pneumoniae* on MDM TNFα and IL-6 production

To investigate whether Treg cells can reduce the inflammatory response of MDMs to *S. pneumoniae* required choosing a strain that would cause a robust inflammatory response from MDMs. Monocytes were isolated from healthy donors and grown for 6 days in the presence of M-CSF to differentiate into macrophages. The MDMs were infected for 6 hours in the presence of MOI 10 *S. pneumoniae* (10 bacteria to 1 MDM) of either serotype 4 (TIGR4), 6B, 23F or D39 strains, then the supernatants collected and analysed by ELISA for the pro-inflammatory cytokines TNFα and IL-6. All *S. pneumoniae* strains induced a TNFα responses and both TIGR4 and D39 induced biologically significant IL6 responses. However, the MDMs infected with TIGR4 produced the highest concentrations of TNFα (figure 3.4 A) and IL-6 (figure 3.4 B) compared to uninfected MDMs, and this strain was selected for use in subsequent experiments.

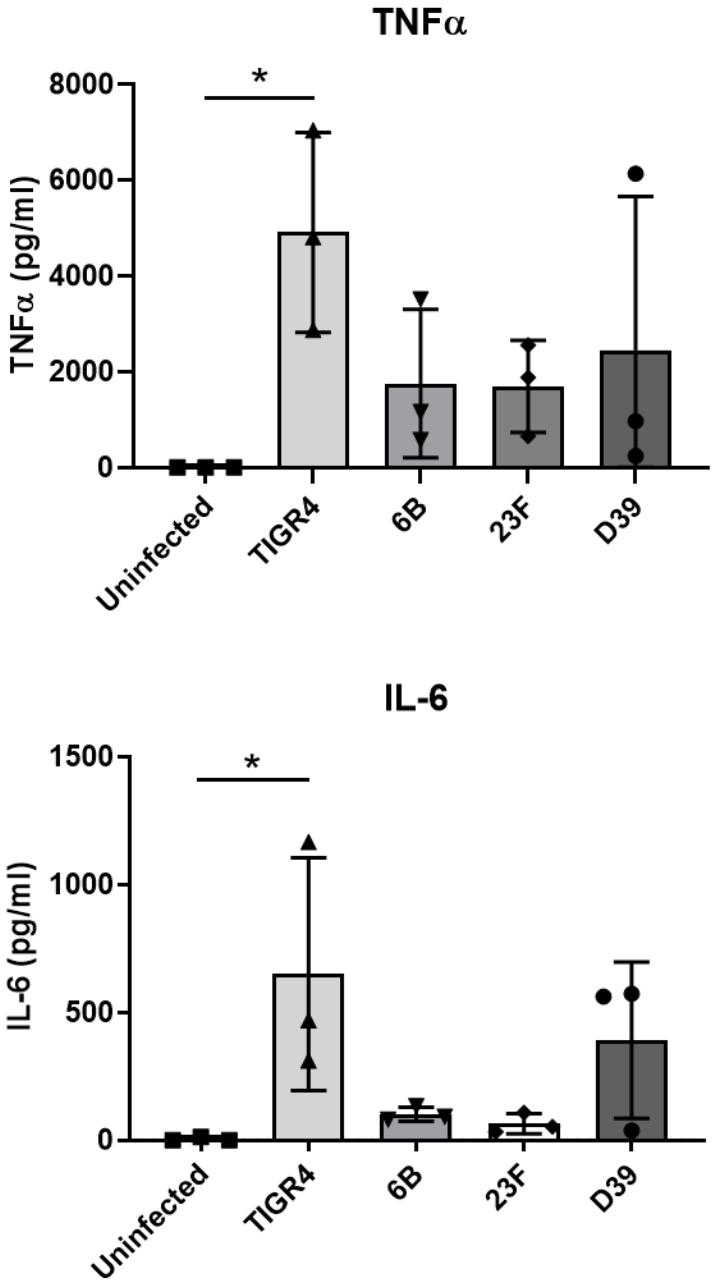

Figure 3.4 Supernatant cytokine concentration from MDMs cultured with *S. pneumoniae* strains

TNFα (A) and IL-6 (B) concentrations in supernatants from MDMs incubated for 6 hours in the presence or absence of MOI 10 *S. pneumoniae* of either TIGR4, 6B, 23F or D39 strain. Graphs show the means from 3 biological repeats on MDMs taken from 3 donors +/- SD of the mean. Statistical analysis was by Kruskal-Wallis test ($p<0.05$ A and B) with Dunn's multiple comparisons test (*$p<0.05$).

3.5 Determining the maximum incubation time of *S. pneumoniae* on MDMs

In order to create a successful co-culture between MDMs, Treg/Teff cells and *S. pneumoniae*, the optimal incubation time between the cell types needed to be determined. The co-culture experiments required a robust inflammatory response from the MDMs whilst allowing their survival. The time of incubation with MDMs, *S. pneumoniae* and Treg cells was also important, as some Treg-associated cytokines such as IL-10 might only start to be detected 24 hours after activation of the cell. Moreover, too high an inflammatory environment could prevent Treg cells from having any anti-inflammatory effect on the MDMs.

To see whether the bacteria survived and replicated in the medium used in MDM/Treg experiments, $1x10^7$ TIGR4 *S. pneumoniae* were incubated in RPMI supplemented with 10% FCS for 6 hours, with supernatant samples taken and plated at 0, 30, 60, 180 and 360 minutes for counting of CFUs. Although no statistically significant difference was observed between any of the groups, it was noted that by 6 hours in an individual sample bacterial CFU could either increase or decrease (figure 3.5). For this reason, it was decided that in order to maintain consistency of bacterial exposure to MDM across experiments, the remaining bacteria would be killed using penicillin-streptomycin after 6 hours. Bacteria were confirmed dead 30 minutes after addition of the antibiotics by lack of CFU growth from neat supernatant plated on CBA plates and incubated overnight (data not shown). A 6-hour infection would allow time for the MDMs to begin production of TNFα, IL-6 and IL-1β (the intended readout for the experiments), and supernatant samples were obtained beyond 6 hours to account for the possibility that Treg cells may take over 24 hours to suppress responses.

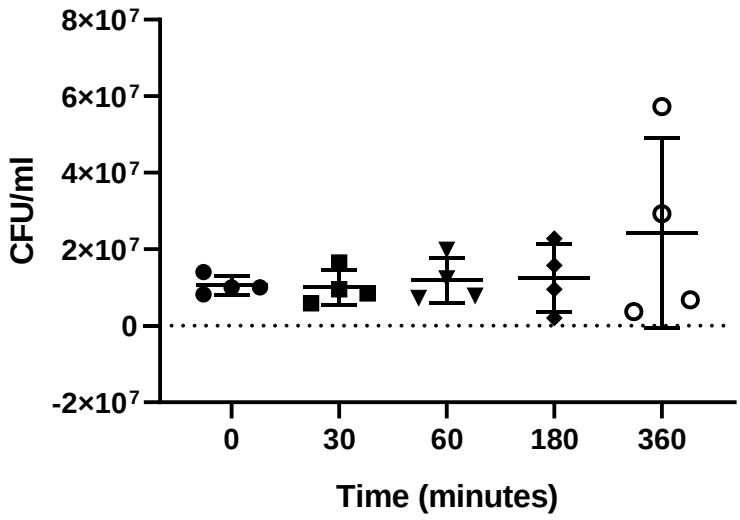

Figure 3.5 *S. pneumoniae* colony forming units over 6 hours

The CFU numbers from an inoculum of $1x10^7$ of TIGR4 *S. pneumoniae* were measured over 6 hours in MDM/Treg culture medium. Data are from 4 biological replicates with error bars representing +/- SD of the mean. No statistically significant differences between groups were observed with analysis by one-way ANOVA with Dunn's multiple comparisons test.

3.6 MDM survival after infection with increasing MOI of *S. pneumoniae*

To provide an indication of the level of MDM survival beyond the 6 hour infection with *S. pneumoniae*, an LDH assay to determine cell cytotoxicity was performed on supernatant samples taken from MDMs infected with an increasing MOI of *S. pneumoniae*, with bacteria killed after 6 hours infection by addition of penicillin and streptomycin. The results showed that 24 hours after infection mean MDM cell death compared to a 100% lysis positive control sample was 9% for MDMs infected with MOI 0.1, 18% for MDMs infected with MOI 1, and 20% for MDMs infected with MOI 10 (figure 3.6 A). After 48 hours, MDMs infected with MOI 0.1 had a mean of 31% cell death, 36% for MDMs infected with MOI 1, and 37% for MDMs infected with MOI 10 (figure 3.6 B). MDM death was statistically significant compared to the uninfected MDM control at both time points for all MOIs, and statistically less significant when MDMs were infected with MOI 0.1 compared to both MOI 1 and MOI 10 up to 24 hours. However, by 48 hours post-infection, there was no statistically significant difference between cell death of MDMs infected with either MOI 0.1, 1, or 10. MDMs activated with the TLR2 agonist PAM_2CSK_4 did not show any statistically significant cell death compared to the uninfected MDM control at either 24 hours or 48 hours.

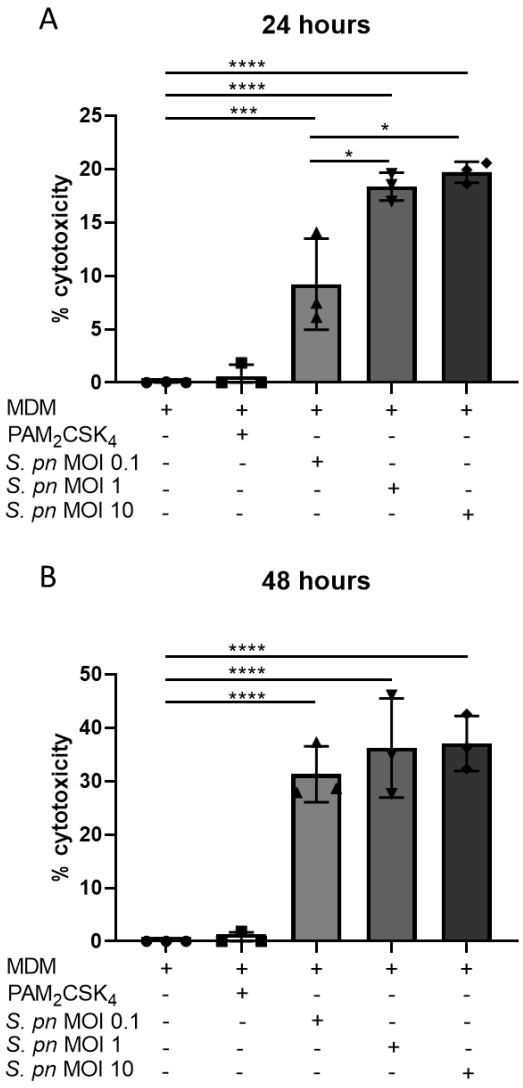

Figure 3.6 MDM cell cytotoxicity at 24 hours and 48 hours after a 6 hour infection with increasing MOI of *S. pneumoniae*

MDMs were infected for 6 hours with increasing MOI of TIGR4 *S. pneumoniae* then incubated until 24 hours (A) and 48 hours (B) post-infection, at which time supernatant samples were analysed for the presence of LDH. The concentration of LDH as read by optical density was converted to percent cytotoxicity of MDMs by dividing the experimental values by a total cell lysis control (induced by cell lysis buffer provided in the kit), with the optical density of uninfected MDM LDH release and the optical density of *S. pneumoniae* cultured alone eliminated. Graphs show means of 3 biological replicates (MDMs from 3 donors) +/- SD and analysed by one-way ANOVA (p<0.0001 A and B) with Dunn's multiple comparison test (*p<0.05, ***p<0.001, ****p<0.0001).

3.7 Effect of Treg cells on MDM TNFα and IL-6 production upon infection with an MOI of 10 *S. pneumoniae* in anti-CD3 and anti-CD28 antibody-supplemented medium

Previous experiments to decide a strain of *S. pneumoniae* to be used in subsequent experiments and to look at their survival in MDM/Treg culture medium used an MOI 10 *S. pneumoniae* to 1 MDM. Experiments checking the suppressive ability of Treg cells to Teff cells used medium supplemented with activating anti-CD3 and anti-CD28 antibodies, with a ratio of 3 Teff cells to 1 Treg. Therefore, an initial attempt to see whether Treg cells can suppress MDM responses to *S. pneumoniae* used MOI 10 bacteria to MDMs, a ratio of 1 Treg or Teff to 3 MDMs, with anti-CD3 and anti-CD28 antibodies in the culture medium. Teff cells were substituted for Treg cells as a comparator to show whether or not any effect was specific to the Treg subset, or could also be achieved by Teff cells. Sorted Treg/Teff cells were incubated with MDMs for 14 hours before infection with MOI 10 *S. pneumoniae* for 6 hours, at which point remaining bacteria were killed using penicillin and streptomycin. Supernatant samples were taken 24 hours after infection and TNFα and IL-6 concentrations as the readout of MDM inflammatory responses were analysed by ELISA. These experiments resulted in very high TNFα levels, but no statistically significant decrease in the MDM TNFα (figure 3.7 A) or IL-6 (figure 3.7 B) responses were seen in the presence of Treg cells. IL-6 was increased in the supernatant of infected MDM-Teff co-culture compared to infected Treg-MDM co-culture (figure 3.7 B). The experiment was repeated with a similar result. From this result, it was hypothesised that there may be too high an inflammatory environment for the Treg cells to have any suppressive effect on the MDMs, so further experiments examined the effect of decreasing the MOI of *S. pneumoniae*, or omitting the activating antibodies from the culture medium.

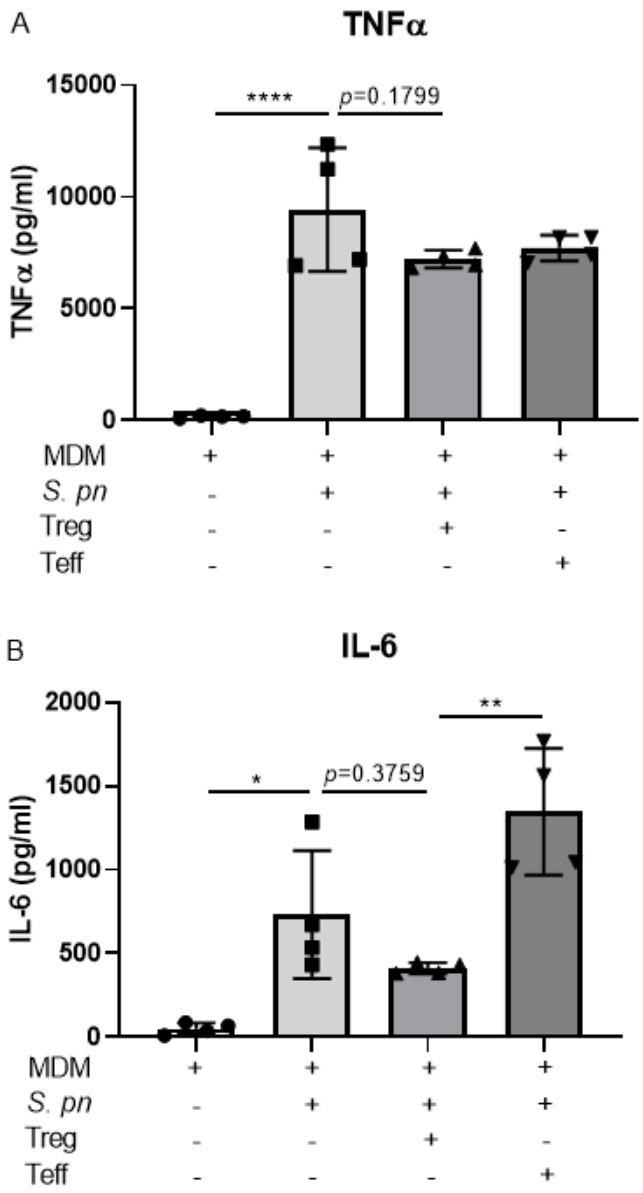

Figure 3.7 TNFα and IL-6 concentration in supernatants after MOI 10 *S. pneumoniae* infection of MDMs cultured in the presence or absence of Treg/Teff cells

Treg/Teff cells were incubated on MDMs for 14 hours then infected with MOI 10 TIGR4 *S. pneumoniae* for 6 hours before addition of penicillin-streptomycin. Supernatant samples were taken 24 hours post-infection for analysis by ELISA for TNFα (A) and IL-6 (B). Graphs show means of 4 technical replicates (4 wells of each condition with cells taken from 1 donor) +/- SD. Data were analysed by one-way ANOVA (A $p<0.0001$; B $p<0.001$) with Tukey's multiple comparisons test (*$p<0.05$; **$p<0.01$; ****$p<0.0001$).

3.8 Effect of omitting anti-CD28 from the culture medium on the inflammatory response of MDMs to MOI 10 *S. pneumoniae*

Treg cells could not suppress MDM inflammatory responses when infected with MOI 10 *S. pneumoniae* in medium containing anti-CD3 and anti-CD28 activating antibodies. The possibility that the Treg cells were being destabilised and losing suppressive capability by over-stimulation by anti-CD28 antibody in addition to also receiving this signal from the MDM's surface CD80/CD86 was investigated by omitting the anti-CD28 antibody from the culture medium. Sorted Treg/Teff cells were incubated on MDMs overnight in medium containing anti-CD3 antibody with or without anti-CD28 antibody, then infected with MOI 10 *S. pneumoniae* for 6 hours. Supernatant samples were taken 24 hours post-infection and analysed for TNFα and IL-6 concentration. Although no statistically significant differences could be seen on the TNFα (figure 3.8 A) and IL-6 (figure 3.8 B) production by MDMs co-cultured with Treg/Teff cells when omitting anti-CD28 antibody from the medium, it was observed that there was generally less variation between the internal replicates with tighter SD, and there was also a trend to lower inflammatory cytokine levels in the Treg and Teff groups. Omission of anti-CD28 antibody appeared likely to be a more favourable condition for Treg function, as infected MDMs in co-culture with Treg cells came closer to showing statisitical significance in reduction of TNFα and IL-6 compared to infected MDMs without co-culture in the absence of anti-CD28. The experiment was repeated once with cells from a different donor with similar results. Therefore, going forward, the anti-CD28 antibody was omitted from the culture medium.

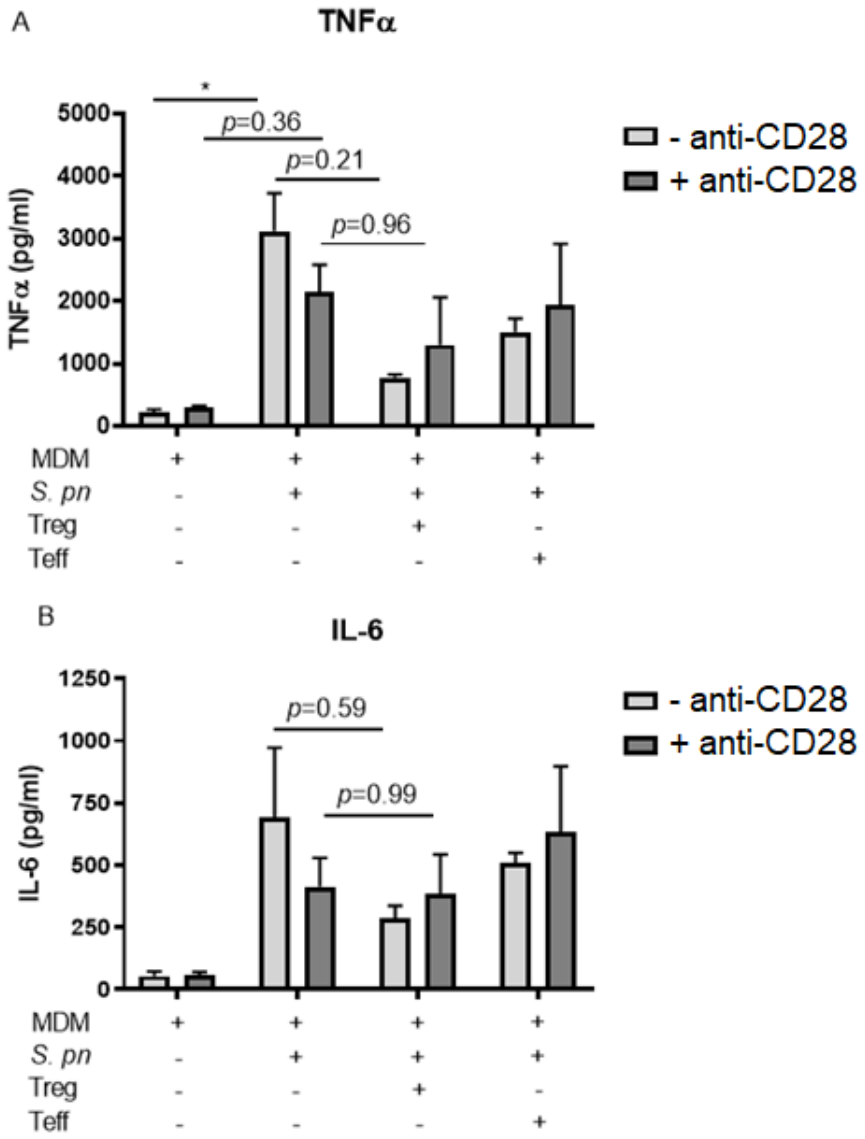

Figure 3.8 TNFα and IL-6 production by *S. pneumoniae*-infected MDMs cultured with or without Treg/Teff cells in the presence or absence of anti-CD28 antibody

MDMs were cultured in the presence or absence of Treg/Teff cells in RPMI supplemented with anti-CD3 antibody with or without anti-CD28 antibody and infected with MOI 10 TIGR4 *S. pneumoniae* for 6 hours. Supernatant samples were taken 24 hours post-infection and analysed for TNFα (A) and IL-6 (B) concentration. Graphs show means of 4 technical replicates (4 wells per condition with cells from 1 donor) with error bars showing +/- SD of the mean. Data were analysed by two-way ANOVA (interaction factor not significant in A or B; row factor "condition" $p<0.01$ in A and B; column factor "-/+ anti-CD28" not significant in A or B) with Tukey's multiple comparisons test (*$p<0.05$).

3.9 Effect of reducing the MOI of *S. pneumoniae* on Treg suppressive ability

Despite the small but statistically not significant decrease and less internal variation in concentrations of inflammatory cytokines by *S. pneumoniae*-infected MDMs in the presence of Treg cells upon removing the anti-CD28 antibody from the medium, no statistically significant suppression of this response was observed. TNFα has been shown to impair Treg function (Valencia et al., 2006). The possibility that high concentrations of pro-inflammatory cytokine were preventing Treg from effectively suppressing MDM responses was considered, which may be lowered by using less bacteria. To investigate whether this was related to bacterial MOI, MDMs were cultured in the presence or absence of a ratio of 3 Treg/Teff cells to 1 MDM for 14 hours in medium containing anti-CD3 antibody but not anti-CD28 antibody, then infected for 6 hours with a reduced number of *S. pneumoniae* at MOI 1 (1 bacterium to 1 MDM). Due to the possibility that the lowered number of bacteria could cause no response from the MDMs, the positive control of PAM_2CSK_4, a TLR2 agonist, was added to the MDMs at the same time as the other groups were infected with *S. pneumoniae*. Supernatant samples were then taken 24 hours later for TNFα and IL-6 ELISAs. While the PAM_2CSK_4 positive control confirmed that the MDMs were capable of producing TNFα and IL-6, the inflammatory response of the MDMs to MOI 1 *S. pneumoniae* was too small to compare whether Treg cells could have a suppressive effect, with the *S. pneumoniae* infected MDM group showing no statistically significant difference to the uninfected MDM control group for both TNFα production (figure 3.9 A) and IL-6 production (figure 3.9 B). This experiment was repeated once producing a similar low response of the MDMs to MOI 1 *S. pneumoniae*. Therefore, the experiment was repeated increasing the MOI to 2 *S. pneumoniae* per MDM. This resulted in a more robust MDM inflammatory response at 24 hours. The TNFα (figure 3.9 C) and IL-6 (figure 3.9 D) was increased compared to the uninfected MDM control, and the infected MDMs produced these cytokines at

similar levels to the PAM_2CSK_4 positive control. MDMs co-cultured with Treg cells resulted in a statistically significant decrease in TNFα (figure 3.9 C) and IL-6 (figure 3.9 D) production upon infection with MOI 2 *S. pneumoniae* compared to MDMs cultured alone. The experiment was repeated twice with cells taken from 2 additional donors, showing similar results.

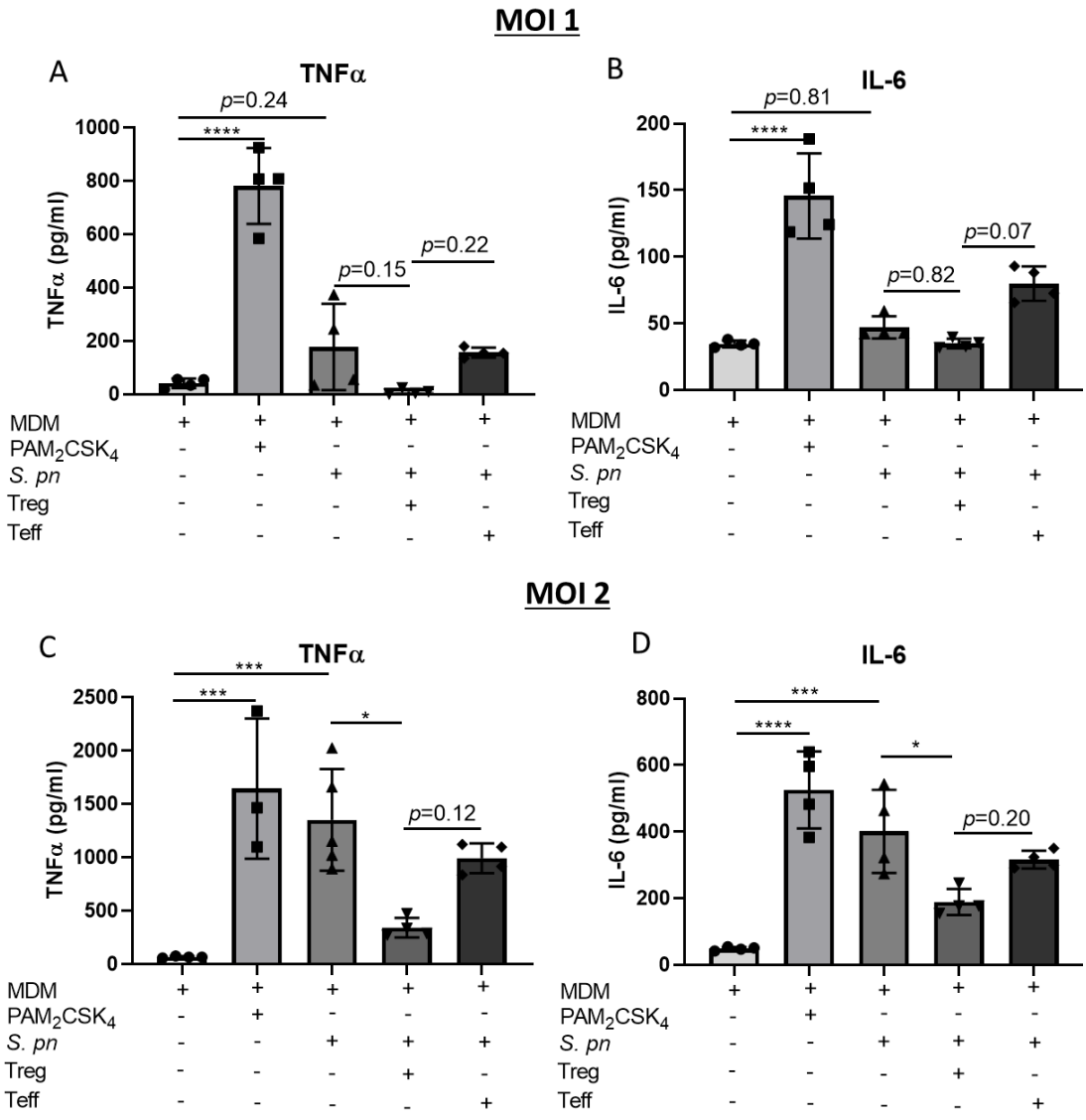

Figure 3.9 TNFα and IL-6 production by MDMs infected with MOI 1 or MOI 2 *S. pneumoniae*, in the presence or absence of Treg/Teff cells

MDMs were infected with TIGR4 *S. pneumoniae* at either MOI 1 (A and B) or MOI 2 (C and D) in the presence or absence of Treg/Teff cells for 6 hours then cultured until a further 24 hours post-infection at which time supernatant samples were taken for ELISA analysis for TNFα and IL-6. Graphs show the mean of 4 technical replicates (4 wells per condition with cells from 1 donor) per group +/- SD. Data are analysed by one-way ANOVA (A, B, C and D $p<0.0001$) with Tukey's multiple comparisons test (*p,0.05, ***$p<0.001$, ****$p<0.0001$).

3.10 Effect of MDM-Treg/Teff incubation on MDM TNFα and IL-6 production in the absence of *S. pneumoniae* infection

To ensure that the process of incubating Treg/Teff cells on MDMs does not itself trigger MDM inflammatory responses, Treg/Teff cells were incubated on MDMs for 14 hours then the supernatants were analysed by ELISA for TNFα and IL-6. While MDM-Treg co-culture showed little difference in TNFα (figure 3.10 A) or IL-6 (figure 3.10 B) production compared to MDMs alone, MDM-Teff co-culture showed slight but statistically not significant increases these cytokines. To compare the level of TNFα and IL-6 generated by co-culture of uninfected MDMs and Teff cells with those levels that are seen upon infection with *S. pneumoniae*, the experiment was repeated for Teff groups adding a 6 hour infection with MOI 2 after the 14 hour incubation, before ELISA of the supernatants for TNFα and IL-6. Concentrations of TNFα (figure 3.10 C) and IL-6 (figure 3.10 D) produced by MDMs infected with *S. pneumoniae* showed statistically significant increase compared to Teff cells co-cultured on MDMs without infection. Therefore, although overnight co-culture of MDMs with Teff cells resulted in approximately 100pg/ml increase in TNFα and 5pg/ml IL-6 compared to MDM alone, these were small effects compared to the approximate 1500pg/ml TNFα and 300pg/ml IL-6 generated when the MDMs were infected with *S. pneumoniae*.

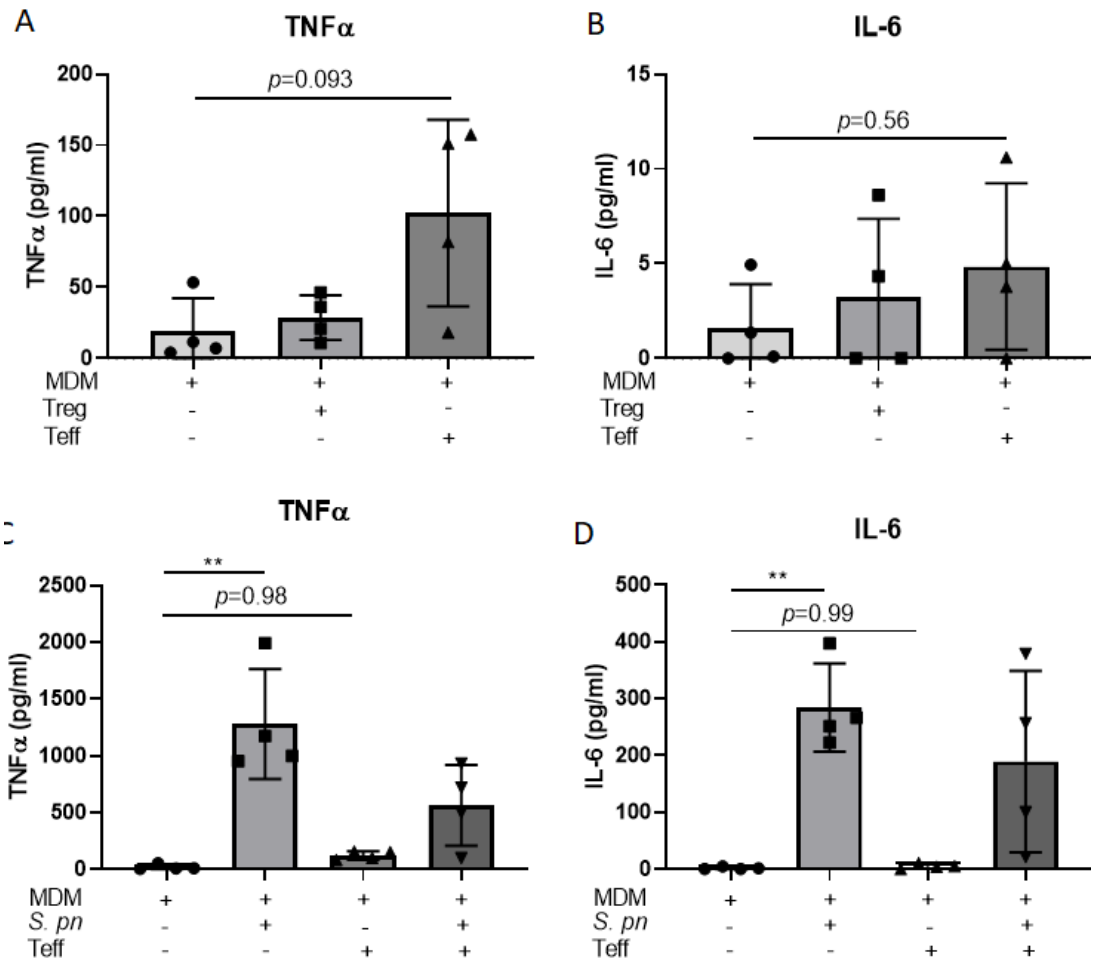

Figure 3.10 TNFα and IL-6 production by MDMs co-cultured with Treg/Teff cells with or without *S. pneumoniae* infection

MDMs were cultured with Treg or Teff cells for 14 hours then supernatants analysed for TNFα (A) and IL-6 (B) production, with or without a 6 hour infection with MOI 2 TIGR4 *S. pneumoniae* (C and D). Graphs show the means of 4 biological replicates from cells from 4 donors +/- SD of the mean. Statistical analysis was by one-way ANOVA (A and B not statistically significant, C and D $p<0.01$) with Tukey's multiple comparisons test (**$p<0.01$).

3.11 Chapter 3 Summary

The experiments conducted in this chapter were designed to produce a co-culture system in which the effects of Treg cells on MDM inflammatory responses initiated by infection with *S. pneumoniae* could be investigated. The conditions determined by these experiments are used in later work in this thesis exploring the effects that Treg cells have on macrophage inflammatory responses to *S. pneumoniae*.

The TIGR4 strain of *S. pneumoniae* was chosen to be used in this co-culture system as this strain induced the most statistically significant inflammatory response in MDMs. This is perhaps not surprising, as TIGR4 is an invasive strain that provokes a robust inflammatory response *in vivo*, whereas 23F is a strain more associated with successful colonisation and therefore evasion of the host immune response. Despite choosing to use TIGR4 for all subsequent experiments in this thesis, it would be valuable to repeat these studies using different strains of *S. pneumoniae*, especially comparing more invasive strains with those found predominantly in nasopharyngeal carriage. In addition, comparison of TIGR4 with strains containing less surface-associated Ply may provide some mechanistic information regarding differences in inflammatory responses of the macrophages to these bacteria (Price and Camilli, 2009).

The length of time that MDMs should be infected for was determined by the observation that the bacterial CFU number became inconsistent after 6 hours incubation. Hence, in order to ensure as far as possible that all experiments had the same *S. pneumoniae*-MDM exposure, it was decided that live bacteria should be incubated on MDMs for no longer than 6 hours. In addition, the 6 hour infection also allowed the MDMs to produce detectable levels of the inflammatory cytokines TNFα, IL-6 and IL-1β; the intended readouts for future experiments. Due to the possibility that MDM-Treg incubations longer than 6 hours are likely to be required for the suppressive effects of Treg cells to be seen, the remaining bacteria were killed by

addition of penicillin-streptomycin at 6 hours so that supernatant samples could be taken beyond this time. Addition of these antibiotics was likely to have caused a large release of bacterial factors such as Ply as the bacterial cells lysed (Brown et al., 2017). This could have been confirmed by determining the Ply concentration in the supernatants. This is likely to have caused an increase in the inflammatory response of the macrophages. The effects of this could be reduced by removal of the supernatant and washing the macrophages in PBS. This would be useful when examining resolution of the initial response by the macrophages and whether they return to a homeostatic state after the infection is cleared. Release of bacterial factors through lysis caused by penicillin may also be prevented by using a macrolide antibiotic such as erythromycin, which can effectively penetrate the macrophages but does not induce lysis (Brown et al., 2017; Ercoli et al., 2018).

The optimal number of *S. pneumoniae* to infect the MDMs was MOI 2 bacteria to each MDM. Although MDM cell death was found to be statistically significant compared to uninfected MDMs after incubation with a range of MOI of *S. pneumoniae*, approximately 82% of the MDMs survived at 24 hours, and 64% survived at 48 hours at MOI 1. It is unlikely that this MDM survival rate could be improved, as even at MOI 1 and 0.1, which were too low to stimulate a major inflammatory response, after 48 hours similar levels of cell death were observed as seen with an MOI of 10. MDM death may be affecting the inflammatory responses of the remaining surviving MDMs. This may be reduced by washing MDMs after taking each time point supernatant sample.

A protocol to collect a highly pure population of Treg cells with suppressive capability was established, along with a highly pure Teff population to serve as a comparator group in the experiments. Although many published Treg assays include the activating antibody anti-CD28 in the culture medium, as was the case in the suppression assay conducted in Figure 3.3, results were more reliable without adding

this antibody, therefore anti-CD28 is not used in subsequent MDM experiments in this thesis. Finally, results confirmed that the co-culture of Treg/Teff cells on MDMs were not themselves triggering a statistically significant inflammatory cytokine production from the MDMs in the absence of infection.

The experiment in 3.9 combined the optimal conditions as found from the experiments in this chapter. However, although results were internally statistically significant for the technical replicates for each of 3 separate experiments in 3.9, when combined they did not produce a statistically significant result, perhaps due to large donor variation. To avoid this in further experiments using this protocol, more donors will be tested per experiment.

To conclude, this chapter suggests that the protocol to investigate Treg effects on MDM inflammatory responses to *S. pneumoniae* should:

1. Use MDMs cultured in the presence or absence of 1 Treg/Teff to 3 MDMs
2. Infect MDMs with an MOI 2 TIGR4 *S. pneumoniae* for 6 hours before addition of penicillin-streptomycin
3. Use medium that contains anti-CD3 antibody but not anti-CD28
4. Use more than 3 repeats necessary to account for donor variation.

Chapter 4: *In vitro* effects of Treg cells on MDM inflammatory responses to *S. pneumoniae*

4.1 Chapter 4 Introduction

This chapter uses the conditions optimised in chapter 3 to investigate the effects of Treg-MDM co-culture in greater depth. This section examines the effects on pro-inflammatory cytokine production by *S. pneumoniae*-infected MDMs when:

- varying the timing of Treg cell addition to MDMs
- varying Treg to MDM ratios for a dose-response effect
- increasing the time between Treg removal from the MDMs and addition of *S. pneumoniae*, to determine if macrophages retain a suppressed phenotype which may affect their response to future infection

In addition, the chapter investigated:

- replication and phagocytosis of *S. pneumoniae* infecting MDMs co-cultured in the presence of Treg cells
- the effect that pre-infection Treg-MDM co-culture has on MDM inflammatory responses to another bacterial pneumonia pathogen, *A. baumannii*

Experiments separating the timing of addition of Treg cells to the culture into pre-infection, during infection and post-infection were carried out. These timings reflect potential modulation of macrophages by Treg cells prior to infection, control of macrophage inflammatory responses during infection, and resolution of inflammation after infection. Pre-infection modulation of macrophage responses may occur in those susceptible to pneumococcal pneumonia, for example those recovering from a previous pneumonia or influenza infection. Modulation of macrophage responses

during infection may occur as both macrophages and Treg cells recruit to the location of infection. Treg cells may also help promote resolution post-infection by arriving at the site of infection as the infecting organism is clearing. The relative ability for Treg cells to modulate MDM responses was compared across these timings to identify which scenario results in the largest suppressive effect on macrophages. Such information may be important when considering intervention treatment in susceptible groups, for example those recovering from a prior respiratory infection may benefit from therapy boosting their immune response to prevent a second, more serious infection. Treg experiments were carried out in parallel with Teff cells to compare their relative effects. Suppressive ability of Treg cells and Teff cells are compared to identify whether these cell types adopt different roles (i.e. suppressive or provoking inflammation) when interacting with macrophages prior to and in the absence of infection compared to during infection. As statistically significant suppression is more likely to be observed at high ratios of Treg cells to macrophages, which may not be relevant ratios *in vivo*, the numbers of Treg cells present in the co-cultures were decreased to in order to identify if a dose-dependent decrease in suppression could be observed. CFU numbers in the supernatants are examined to determine if the presence of Treg or Teff cells may increase or decrease bacterial burden. Phagocytosis of *S. pneumoniae* by macrophages is briefly examined, however requires further investigation. Whether the MDMs can retain their suppressed ability to respond to *S. pneumoniae* when Treg cells are removed from the culture for an extended time provides an indication as to whether any effects *in vivo* may be long-lasting, promoting pre-disposition to infection over an extended period of time, and may help narrow down the mechanism by which macrophages adopt decrease reactivity to infection, for example if epigenetic modification is involved. Experiments examining the effect of Treg-macrophage co-culture prior to infection were repeated with *A. baumannii* to determine if the suppressive effect was seen with an alternative pathogen.

4.2 Effect of Treg/Teff addition to MDMs simultaneously with *S. pneumoniae* infection

The aim of the experiments in this section was to investigate whether simultaneous addition of Treg cells and *S. pneumoniae* to MDMs resulted in a reduced inflammatory response by the MDMs in the presence of Treg cells compared to MDMs infected alone. Treg/Teff cells were added to MDMs at a ratio of 1 Treg/Teff to 3 MDMs, and were simultaneously infected with an MOI of 2 *S. pneumoniae* per MDM. The bacteria were killed after 6 hours by addition of penicillin-streptomycin, and supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for measurement of the concentrations of TNFα, IL-6 and IL-1β by ELISA.

There was a statistically significant decrease in TNFα concentration at 6 hours, 24 hours and 72 hours (figure 4.2 A) post-infection by MDMs cultured with Treg cells compared to MDMs cultured alone. There were also lower IL-6 concentrations for MDMs infected with *S. pneumoniae* in the presence of Treg cells at 24 hours and 72 hours post-infection, with no differences 6 hours post-infection (figure 4.2 B). No statistically significant differences in IL-1β concentrations were seen in infected MDMs with or without addition of Treg or Teff cells (figure 4.2 C). Addition of Teff cells to MDMs simultaneously with infection had variable effects on cytokine concentration. In some cases, addition of Teff cells was associated with a trend towards increased cytokine concentrations, particularly TNFα (figure 4.2 A), whereas in other cases a trend towards decreased cytokine concentrations was observed, particularly IL-1β (figure 4.2 C). Overall, there were no statistically significant differences in TNFα, IL-6 or IL-1β concentrations measured in the supernatants of MDM+Teff groups compared to MDM+Treg groups or MDMs alone (figure 4.2 A, B and C).

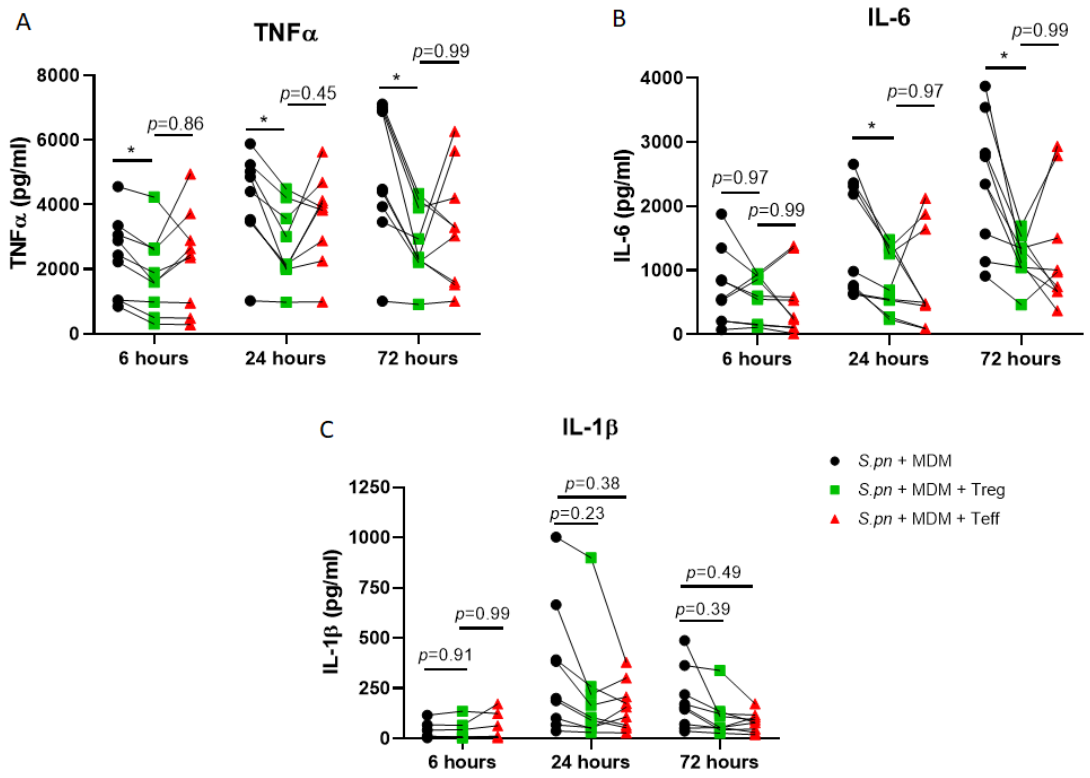

Figure 4.2 Pro-inflammatory cytokine production by MDMs infected with *S. pneumoniae* in the presence or absence of Treg or Teff cells during infection

MDMs were infected with MOI 2 TIGR4 *S. pneumoniae* with or without addition of Treg cells or Teff cells. Supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for measurement of TNFα (A), IL-6 (B) and IL-1β (C) concentrations by ELISA. Dots show concentration of cytokine for each donor and lines match donors across the conditions (total 9 donors). Statistical analysis was by repeated measures two-way ANOVA with Geisser-Greenhouse correction and matched values for both time and condition (row factor "time" $p<0.05$ A and C, $p<0.0001$ B; column factor "condition" $p<0.05$ A and C, $p<0.01$ B; interaction factor $p<0.01$ A and C, $p<0.01$ B) with Tukey's multiple comparisons test (*$p<0.05$).

4.3 Effect of post-infection addition of Treg/Teff cells to *S. pneumoniae*-infected MDMs

Further experiments investigated whether post-infection addition of Treg cells to *S. pneumoniae*-infected MDMs can reduce the inflammatory response of these MDMs compared to infected MDMs without Treg addition. MDMs were infected with an MOI of 2 *S. pneumoniae* to 1 MDM for 6 hours, then the bacteria were killed by addition of penicillin-streptomycin. Alongside antibiotic addition, Treg or Teff cells were added to the MDMs at a ratio of 1 Treg/Teff to 3 MDMs. Supernatant samples were taken 24 hours and 72 hours post-infection for measurement of pro-inflammatory cytokines by ELISA.

At 24 hours post-infection there was a statistically significant reduction in TNFα concentration detected in infected MDMs with Treg addition compared to infected MDMs alone, however this difference was not statistically significant at 72 hours (figure 4.3 A). No statistically significant differences in TNFα concentration were seen in MDMs infected in the presence of Teff cells (figure 4.3 A). At 24 hours and 72 hours post-infection no statistically significant difference in IL-6 concentration was observed in infected MDMs with Treg addition compared to infected MDMs alone, however IL-6 concentration detected in MDMs in the presence of Treg cells differed significantly from IL-6 concentration in MDMs with Teff addition at 24 hours (figure 4.3 B). Addition of Treg or Teff cells post-infection did not result in any significant differences in IL-1β concentrations (figure 4.3 C).

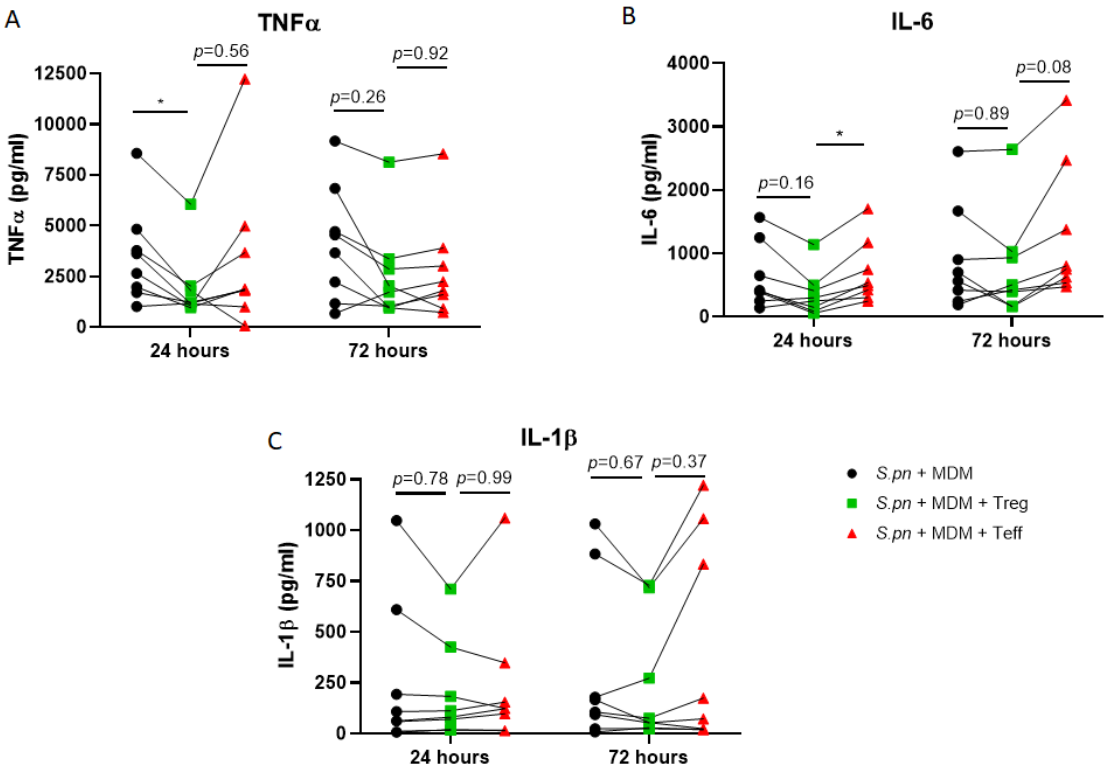

Figure 4.3 Pro-inflammatory cytokine production by *S. pneumoniae*- infected MDMs with post-infection addition of Treg or Teff cells

MDMs were infected with MOI 2 TIGR4 *S. pneumoniae* for 6 hours, followed by addition of penicillin-streptomycin, or addition of penicillin-streptomycin and Treg cells or Teff cells. Supernatant samples were taken at 24 hours and 72 hours post-infection for measurement of TNFα (A), IL-6 (B) and IL-1β (C) concentrations by ELISA. Dots show concentration of cytokine for each donor and lines match donors across the conditions (total 8 donors). Statistical analysis was by repeated measures two-way ANOVA with Geisser-Greenhouse correction and matched values for both time and condition (row factor "time" not significant in A and C, $p<0.05$ B; column factor "condition" not significant in A and C, $p<0.01$ B; interaction factor not significant in A and C, $p<0.05$ B) with Tukey's multiple comparisons test (*$p<0.05$).

4.4 Effect of culturing MDMs in the presence or absence of Treg or Teff cells prior to infection with *S. pneumoniae*

The aim of the experiments in this section was to see if culturing MDMs in the presence of Treg or Teff cells before infection would affect MDM production of inflammatory cytokines upon subsequent infection with *S. pneumoniae*. Treg or Teff cells were incubated on MDMs at a ratio of 1 Treg/Teff cell per 3 MDMs for 14 hours and then removed and the MDMs infected with an MOI of 2 *S. pneumoniae* per MDM for 6 hours. The remaining bacteria were killed at 6 hours by addition of penicillin-streptomycin and allowed to incubate until 72 hours post-infection. Supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for measurement of the concentrations of TNFα, IL-6 and IL-1β by ELISA.

There was a statistically significant reduction in TNFα concentration at 24 hours and 72 hours post-infection by MDMs that had been incubated with Treg cells prior to infection compared to MDMs cultured alone (figure 4.4 A). There were also statistically significant reductions in TNFα concentration at 6 hours and 24 hours post-infection by MDMs that had been incubated with Treg cells prior to infection compared to MDMs that had been incubated with Teff cells, and the TNFα concentrations at 72 hours were close to a statistically significant decrease in MDMs that had been incubated with Treg cells prior to infection compared to MDMs that had been incubated with Teff cells at p=0.07 (figure 4.4 A). Similarly, there was a statistically significant reduction in IL-6 concentration at 24 hours and 72 hours post-infection by MDMs that had been incubated with Treg cells prior to infection compared to MDMs cultured alone (figure 4.4 B). There was also a statistically significant reduction in IL-6 concentration at 72 hours post-infection by MDMs that had been incubated with Treg cells prior to infection compared to MDMs that had been incubated with Teff cells (figure 4.4 B). There was also a statistically significant reduction in IL-1β concentration at 24 hours and 72 hours post-infection by MDMs that had been

incubated with Treg cells prior to infection compared to MDMs cultured alone, however no statistically significant differences in IL-1β concentrations when comparing MDMs that had been cultured with Treg cells compared to MDMs that had been cultured with Teff cells at any time point (figure 4.4 C).

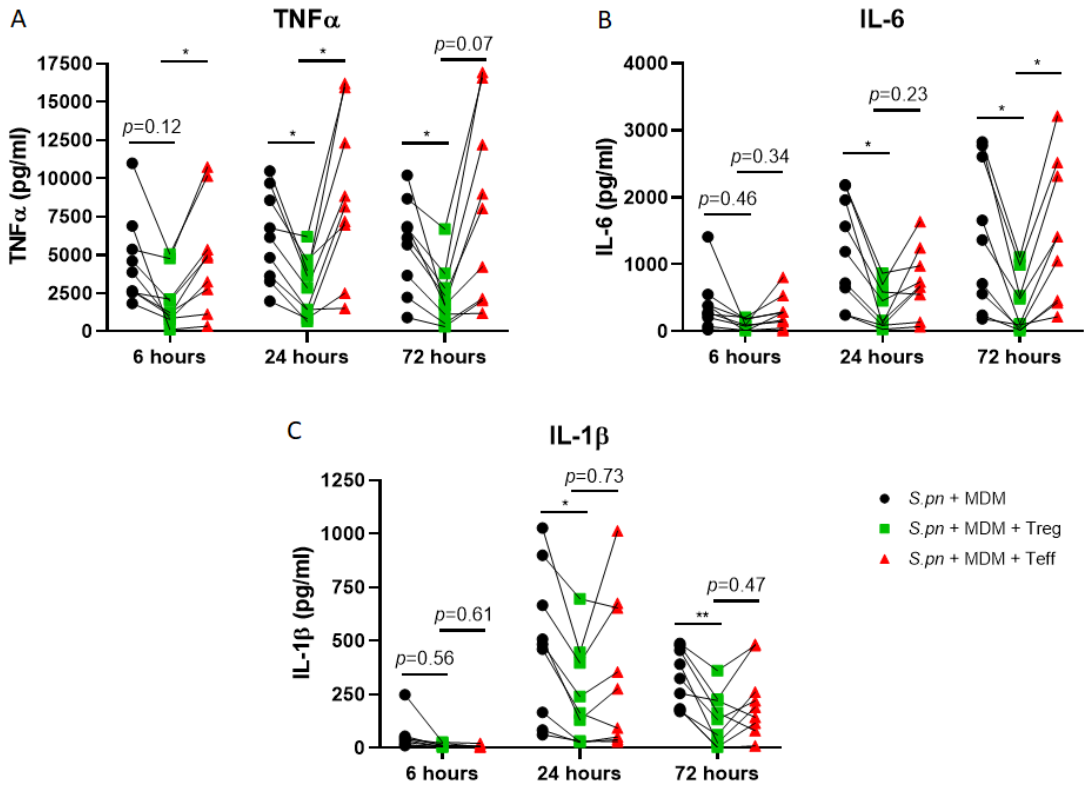

Figure 4.4 Pro-inflammatory cytokine production of MDMs cultured in the presence or absence of Treg or Teff cells before infection of the MDMs with *S. pneumoniae*

MDMs were cultured either alone or in the presence of Treg cells or Teff cells for 14 hours, after which the Treg/Teff cells were removed and the MDMs infected with MOI2 TIGR4 *S. pneumoniae*. Supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for measurement of TNFα (A), IL-6 (B) and IL-1β (C) concentrations by ELISA. Dots show concentration of cytokine for each donor and lines match donors across the conditions (total 9 donors). Statistical analysis was by repeated measures two-way ANOVA with Geisser-Greenhouse correction and matched values for both time and condition (row factor "time" $p<0.05$ A and B, $p<0.01$ C; column factor "condition" $p<0.01$ A, $p<0.001$ B and C; interaction factor not significant in A, $p<0.01$ B, $p<0.05$ C) with Tukey's multiple comparisons test (*$p<0.05$ **$p<0.01$).

4.5 Comparing the timing of Treg addition to MDMs on pro-inflammatory cytokine production upon *S. pneumoniae* infection

This section combines the results of parts 4.2, 4.3 and 4.4 to compare the reduction in inflammatory response by the presence of Treg cells added to *S. pneumoniae*-infected MDMs either pre-infection, during infection or post-infection. Absolute cytokine concentrations measured were converted into a percentage of the MDM + *S. pneumoniae* group in order to reduce donor variability from the overall effects of Treg/Teff addition and individual time points were examined.

Cytokine concentrations were statistically most significantly decreased when MDMs had been incubated with Treg cells pre-infection at all time points excluding the 24 hour TNFα result, which had equal statistical significance when Treg cells were added to the culture during infection (figure 4.5). Addition of Treg cells to the culture during infection also caused statistically significant decrease in TNFα at 6 hours and 72 hours, however this was not as statistically significant as the decreased resulting from pre-infection incubation of Treg cells on MDMs (figure 4.5 A and C). Post-infection addition of Treg cells to infected MDMs showed a trend towards decreasing TNFα production by MDMs at 72 hours at p=0.0795 (figure 4.5 C). Of note, the post-infection group had 2 fewer donors than the pre- and during- infection groups, which could have affected statistical significance.

IL-6 production by MDMs that had been co-cultured pre-infection with Treg cells was statistically significantly lower than MDMs infected alone at all time points, whereas Treg cells added simultaneously with *S. pneumoniae* infection showed trends towards reducing IL-6 production compared to MDMs infected alone at p=0.11 and p=0.0758 at 24 hours and 72 hours, respectively (figure 4.5 D-F). Post-infection addition of Treg cells did not reduce IL-6 production at any time point (figure 4.5 D-F).

IL-1β production was reduced when MDMs had been co-cultured with Treg cells prior to infection compared to MDMs infected alone at all time points (figure 4.5 G-I). In addition, IL-1β production was reduced when Treg cells were added to MDMs simultaneously with *S. pneumoniae* infection compared to MDMs infected alone at 24 hours and 72 hours, however the difference was less compared to Treg co-culture pre-infection (figure 4.5 G-I). Post-infection addition of Treg cells did not reduce IL-1β production at any time point (figure 4.5 G-I).

Overall, the results showed that co-culture of Treg cells with MDMs suppressed pro-inflammatory cytokine responses to *S. pneumoniae*, with addition of Tregs prior to infection resulting in the most statistically significant decreases.

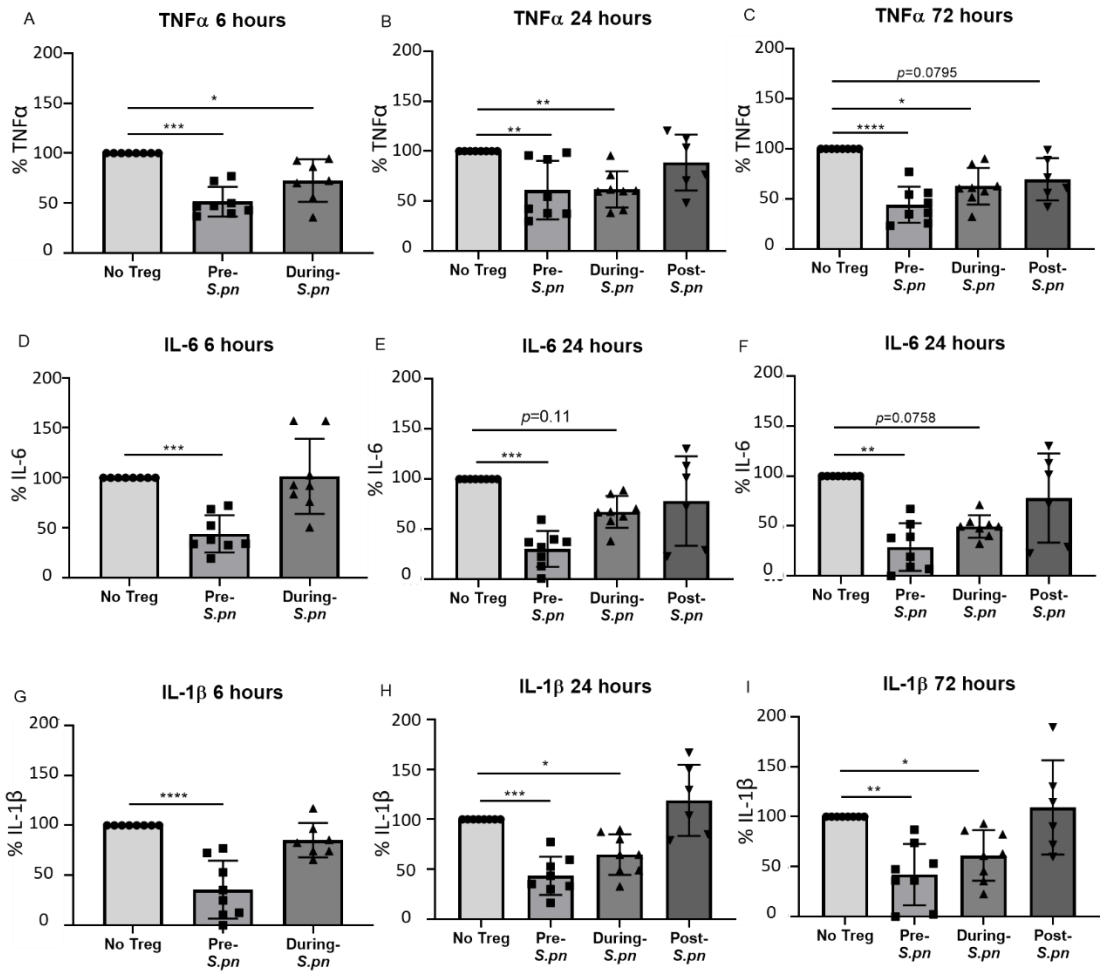

Figure 4.5 Pro-inflammatory cytokine production of *S. pneumoniae*-infected MDMs with addition of Treg cells either pre-infection, during infection or post-infection

Treg cells were added to MOI 2 TIGR4 *S. pneumoniae*-infected MDMs either pre-infection, during infection, or post-infection as described in 4.2, 4.3 and 4.4, respectively. Supernatant samples were taken at 6, 24 and 72 hours post-infection for detection of TNFα (A-C), IL-6 (D-F), and IL-1β (G-I) measured by ELISA. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Graphs show means of biological replicates of cells taken from 8 donors for pre-infection and during-infection groups and 6 donors for post-infection groups. Error bars show +/- SD of the mean. Statistical analysis was by Kruskal-Wallis (p<0.01 in B, F; p<0.001 in A, C, D, E, G, I; p<0.0001 in H) with Dunn's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

4.6 Comparison of effects on MDM inflammatory responses to *S. pneumoniae* with Treg or Teff cells added pre-infection or during infection

While Treg cells had anti-inflammatory effects on MDMs when added either pre-infection or during infection as shown in part 4.2 and 4.4, the effects of Teff cells were more variable and more difficult to define. Teff cells were observed to either promote or reduce pro-inflammatory cytokine concentration by MDMs, although the effects of Teff cells when comparing absolute cytokine values across all time points was not statistically significant. While many of these data show statistically non-significant increases or decreases in cytokine concentrations, these effects may be significant *in vivo*. For example, very steep decreases or increases in cytokine concentration may be detrimental to the host's survival, whereas less pronounced effects may be required to maintain balance between bacterial clearance and preservation of host tissue. Therefore, the relative concentrations of TNFα, IL-6 and IL-1β between MDMs incubated with Treg cells and MDMs incubated with Teff cells was compared for cell addition pre-infection and during infection. Absolute cytokine concentrations measured in part 4.2 and 4.4 were converted into a percentage of the MDM + *S. pneumoniae* group in order to reduce donor variability from the overall effects of Treg/Teff addition and individual time points were examined. The post-infection data were omitted due to the overall lack of differences observed compared to MDMs infected alone.

The results suggest that examining the converted cytokine concentrations at individual time points showed that in most cases the MDM cytokine responses to *S. pneumoniae* upon Teff co-culture did not differ in a statistically significant manner compared to MDM cytokine responses to *S. pneumoniae* upon Treg co-culture. The biggest differences between Treg and Teff effects can be seen in the TNFα response. In the pre-infection experiments at 6 hours, Treg cell co-culture reduced TNFα concentration to a mean of 52% of that seen in the MDM + *S. pneumoniae* group

without Treg/Teff cells, while Teff cell co-culture increased the TNFα concentration to a mean of 150% (figure 4.6 A). This trend remains similar over all 3 time points, with 24 hour and 72 hour Treg co-cultured MDMs producing means of 61% and 44% of the total TNFα of *S. pneumoniae* infected MDMs without Treg/Teff co-culture, respectively, and with 24 hour and 72 hour Teff co-cultured MDMs producing means of 147% and 151% of the total TNFα of *S. pneumoniae* infected MDMs without Treg/Teff co-culture, respectively (figure 4.6 B and C). The results between Treg and Teff addition pre-infection were statistically significant as per the Tukey's post-test. However, when added during infection, Teff cell addition did not increase TNFα concentrations, in fact there was a trend towards decreased TNFα concentration (figure 4.6 A-C). Treg addition to *S. pneumoniae*-infected MDMs resulted in means of 73%, 62% and 63% of the TNFα produced by MDMs infected without Treg/Teff cell addition at 6 hours, 24 hours and 72 hours post-infection, respectively, whereas Teff addition resulted in means of 92%, 86% and 82% of the TNFα at 6 hours, 24 hours and 72 hours post-infection, respectively (figure 4.6 A-C). Overall, the two-way ANOVA analysis of the TNFα data indicate that there are statistically significant differences in the Treg results compared to the Teff results at all time points, that there are only statistically significant differences between timing of cell addition (pre vs during) in the 24 hours data where $p<0.05$, and that there is statistically significant interaction between these two factors, i.e. differences between Treg and Teff results are different for pre-infection and during infection data at all 3 time points.

The effects of Treg or Teff cell addition on IL-6 concentration by *S. pneumoniae*-infected MDMs is quite different than those on TNFα concentration. For pre-infection co-culture with Treg or Teff cells, trends towards initial reductions in IL-6 concentration are observed, with a trend of more pronounced reduction in Treg cell co-culture at a mean of 46% IL-6 concentration, whereas Teff co-cultured MDM IL-6 concentration was at a mean of 66% (figure 4.6 D). At 24 hours, IL-6 concentration

by Treg-co-cultured MDMs is even lower at a mean of 30%, while Teff-co-cultured MDMs remain similar to the 6 hour concentrations with a mean of 68% (figure 4.6 E). At 72 hours, Treg-co-cultured MDMs were making a mean of 29% of the IL-6 made by MDMs infected without Treg/Teff co-culture, whereas Teff co-cultured MDMs are producing similar concentrations as the no Treg/Teff co-culture control with a mean of 107%, and these differences are statistically significant (figure 4.6 F). These data suggest that while Treg co-culture prior to *S. pneumoniae* infection keeps MDM IL-6 concentration at low levels (~30-50%) throughout 72 hours, Teff co-culture results in trends towards initial, less pronounced decreases in IL-6 concentration, which gradually rises to similar levels as the no Treg/Teff co-culture control by 72 hours. When added during infection, both Treg and Teff cell addition results in a trend towards gradual reductions in IL-6 concentration over 24-72 hours, although Treg cells cause greater reductions at the later time points. At 6 hours, Treg addition results in a mean of 102% IL-6 concentration and Teff addition results in a mean of 96% IL-6 concentration. At 24 hours, Treg addition has resulted in a trend towards reduced IL-6 concentration to a mean of 67%, while Teff addition has resulted in a trend towards reduced IL-6 concentration to a mean of 82% (figure 4.6 E). At 72 hours, Treg addition during infection resulted in a mean IL-6 concentration of 50%, while the mean IL-6 concentration remains at 82% with Teff addition (figure 4.6 F). Overall, the two-way ANOVA analysis of the IL-6 data indicate that there are statistically significant differences in the Treg results compared to the Teff results at 24 hours and 72 hours, that there are statistically significant differences between timing of cell addition (pre vs during) at 6 hours and 24 hours, and that there is significant interaction between these two factors, i.e. differences between Treg and Teff results are different for pre-infection and during infection data at 72 hours.

Co-culture of MDMs with Treg or Teff cells prior to *S. pneumoniae* infection both caused trends towards reduced concentration of IL-1β by the MDMs upon infection

from the early time point of 6 hours. With Treg addition, these concentrations remained fairly stable over time, with mean IL-1β concentrations at 36%, 44% and 44% at 6 hours, 24 hours, and 72 hours, respectively (figure 4.6 G-I). With Teff addition, the trend towards decrease in IL-1β concentration was less stable over time and tended towards increase, with mean IL-1β concentrations at 37%, 72% and 69% at 6 hours, 24 hours, and 72 hours, respectively (figure 4.6 G-I). When added during infection, Treg cells caused a trend towards gradual reduction in IL-1β concentration, with means of 85%, 65% and 65% of the total IL-1β concentrations by MDMs infected without Treg/Teff addition at 6 hours, 24 hours and 72 hours, respectively (figure 4.6 G-I). Teff addition resulted in 118%, 66%, and 54% of the total IL-1β concentrations by MDMs infected without Treg/Teff addition at 6 hours, 24 hours and 72 hours, respectively (figure 4.6 G-I). Surprisingly, Teff addition resulted in the lowest IL-1β concentrations at 72 hours (figure 4.6 I). Despite these observed trends, none of these differences were statistically significant as per the Tukey's post-test. Overall, the two-way ANOVA analysis of the IL-1β data indicate that there are no statistically significant differences in the Treg results compared to the Teff results at any time point, that there are only statistically significant differences between timing of cell addition (pre vs during) in the 6 hours data where $p<0.0001$, and that there is no statistically significant interaction between these two factors, i.e. differences between Treg and Teff results are different for pre-infection and during infection data at all 3 time points.

Overall these data suggest that Teff co-culture with MDMs prior to infection promotes increased TNFα concentration by the macrophages upon infection, while a trend towards moderate decreases are observed when Teff cells are added during infection. For the Treg cells, addition of Treg cells prior to infection or during infection decreases TNFα concentration in both scenarios, although co-culture prior to infection caused the greater decreases. Treg co-culture pre-infection caused trends

towards large reductions in IL-6 concentration by MDMs upon *S. pneumoniae* infection, with the decreases becoming more pronounced over 72 hours, while Teff co-culture pre-infection caused a trend towards initial decreases in IL-6, which were raised back to 100% at 72 hours, reaching statistical significance compared to Treg co-culture IL-6 concentrations. Addition of Treg or Teff cells during *S. pneumoniae* infection both caused trends towards reduction in IL-6 concentration by MDMs at 24 and 72 hours, however the mean concentrations were lower with Treg addition. Pre-infection co-culture of MDMs with Treg or Teff cells both caused trends towards large decreases in IL-1β concentrations at the early time point of 6 hours, remaining fairly stable for Treg-co-cultured MDMs and rising to around 70% for Teff-co-cultured MDMs. When added during infection, Treg cells caused a trend towards moderate decrease in IL-1β concentration to around 65%, whereas Teff cell addition caused the lowest IL-1β concentrations of around 54%. The data showing different trends for Treg and Teff cells and for addition pre- or during infection suggest that these cell types and timings of MDM-T cell interactions have different effects per cytokine. Overall mean cytokine concentrations are summarised in table 4.6.

		Treg		**Teff**	
		Pre	During	Pre	During
TNFα	**6h**	52	73	150	92
	24h	61	62	147	86
	72h	44	63	151	82
IL-6	**6h**	44	102	66	96
	24h	30	67	68	82
	72h	29	50	107	63
IL-1β	**6h**	36	85	37	118
	24h	44	65	72	66
	72h	44	65	69	54

Table 4.6 Cytokine concentrations as a percentage of the concentrations measured in *S. pneumoniae*-infected MDMs without Treg/Teff co-culture pre-infection or during infection

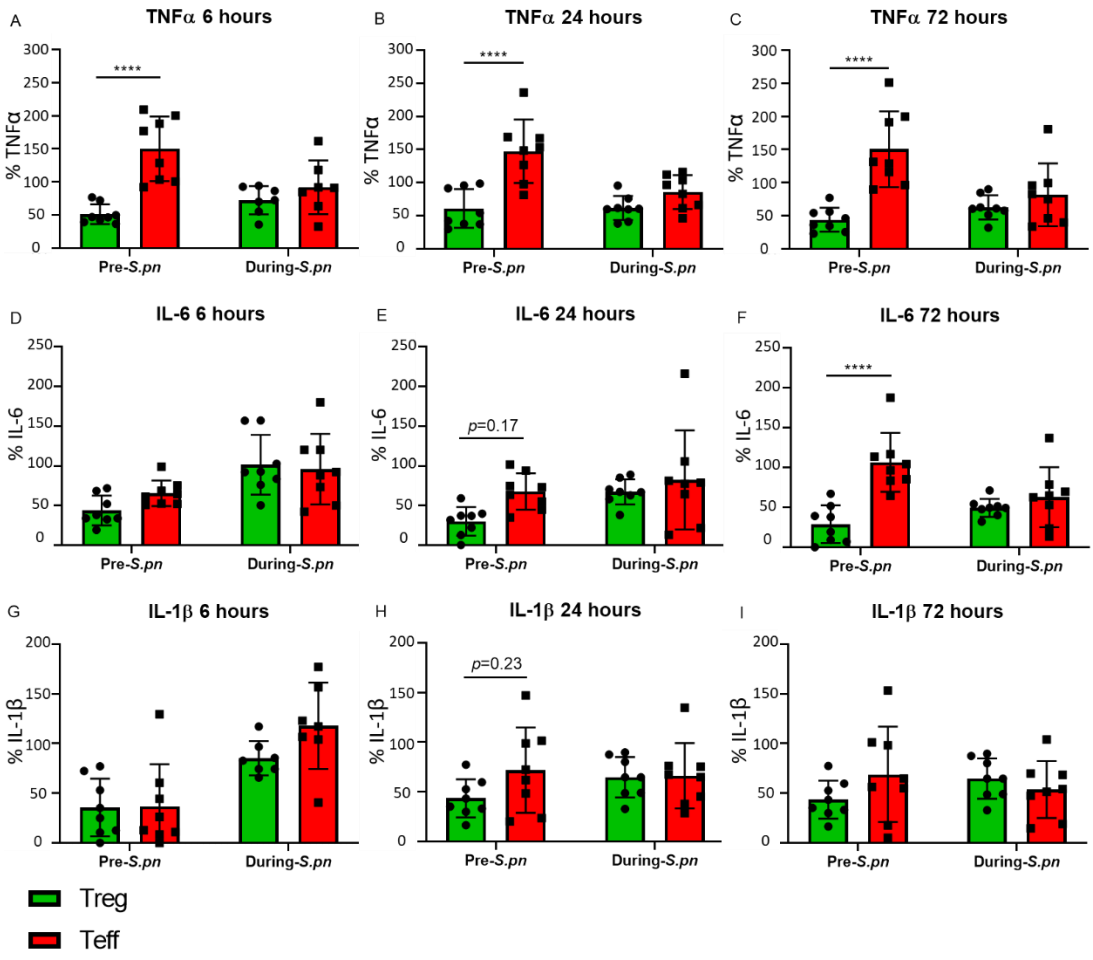

Figure 4.6 Pro-inflammatory cytokine production by MDMs incubated with Treg cells compared to MDMs incubated with Teff cells pre-infection or during infection

MDMs were cultured in the presence of Treg or Teff cells pre-infection or during infection with MOI 2 TIGR4 *S. pneumoniae* and supernatant samples were analysed for TNFα (A-C), IL-6 (D-F) or IL-1β (G-I) measured by ELISA. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Graphs show means of biological replicates of cells taken from 8 donors with error bars showing +/- SD of the mean. Statistical analysis was by two-way ANOVA (interaction factor $p<0.05$ in B, $p<0.01$ in A, C, and F, not significant in D, E, G, H and I; row factor "pre vs during" $p<0.05$ in B and E, $p<0.001$ in D, not significant in A, C, F, H and I; column factor "Treg vs Teff" $p<0.05$ in E, $p<0.001$ in C and F, $p<0.0001$ in A and B, not significant in D, H and I) with Tukey's multiple comparisons test (****$p<0.0001$).

4.7 Effect of decreasing the number of Treg and Teff cells incubated with MDMs prior to infection on the inflammatory response to *S. pneumoniae*

To identify the ratio of Treg cells to MDMs required for suppression of pro-inflammatory cytokine responses to *S. pneumoniae*, co-culture experiments were repeated using Treg or Teff cells to MDMs ratios of 1:15, 1:10 and 1:5. MDMs were incubated with these Treg/Teff:MDM ratios for 14 hours before the Treg/Teff cells were removed, and the MDMs infected with *S. pneumoniae* at an MOI of 2. After 6 hours infection, the *S. pneumoniae* were killed by addition of penicillin-streptomycin, and supernatant samples were taken at 6 hours, 24 hours, and 72 hours post-infection for measurement of TNFα, IL-6 and IL-1β by ELISA. For comparions with a Treg: MDM ratio of 1:3, results from the co-culture experiments shown in section 4.4 were included in this section. Absolute cytokine concentrations measured were converted into a percentage of the MDM + *S. pneumoniae* group in order to reduce donor variability from the overall effects of Treg/Teff addition and individual time points were examined.

Increasing the number of Treg cells to MDMs resulted in a trend to decreasing the concentration of TNFα produced by the MDMs at 6 hours, 24 hours and 72 hours post-infection, with statistically significant difference at all time points with a ratio of 1 Treg per MDM (figure 4.7.1 A, B and C). Statistically significant decreases in IL-6 at 6 hours were seen at ratios of 1 Treg per 5 MDMs and at 1 Treg per 3 MDMs, with the latter having larger statistical significance (figure 4.7.1 C). At 24 hours, statistically significant reduction in IL-6 production was seen at ratios of 1 Treg per 10 MDMs and at 1 Treg per 5 MDMs, and ratios of 1 Treg per 15 MDMs and 1 Treg per 3 MDMs close to statistical significance at p=0.089 and p=0.099, respectively (figure 4.7.1 D). At 72 hours, IL-6 production was decreased in MDMs incubated with 1 Treg per 5 MDMs and 1 Treg per 3 MDMs (figure 4.7.1 F). In this experiment, IL-1β production at 6 hours was very low, so these data are not shown. At 24 hours, a clearer trend

can be seen between increasing the number of Treg cells per MDM and the decrease in IL-1β production by the MDMs, with a statistically significant decrease seen when MDMs were incubated with 1 Treg per 3 MDMs (figure 4.7.1 G). At 72 hours post-infection, statistically significant decreases in IL-1β were seen when MDMs were incubated with Treg cells at ratios of 1 Treg per 15 MDMs, 1 Treg per 10 MDMs and 1 Treg per 3 MDMs, with the difference close to statistically significant for the ratio of 1 Treg per 5 MDMs at p=0.063 (figure 4.7.1 H).

Overall, there are general trends between increasing the number of Treg cells in MDM culture and decreased production of pro-inflammatory cytokine production. statistically significant decrease in all cytokines at all time points resulted with addition of 1 Treg per 3 MDMs, except IL-6 at 24 hours which was close to statistically significant at p=0.099. With addition of fewer Treg cells than 1 Treg per MDM, results were less consistently statistically significant, indicating that the optimal ratio for suppression of MDM responses is at least 1:3.

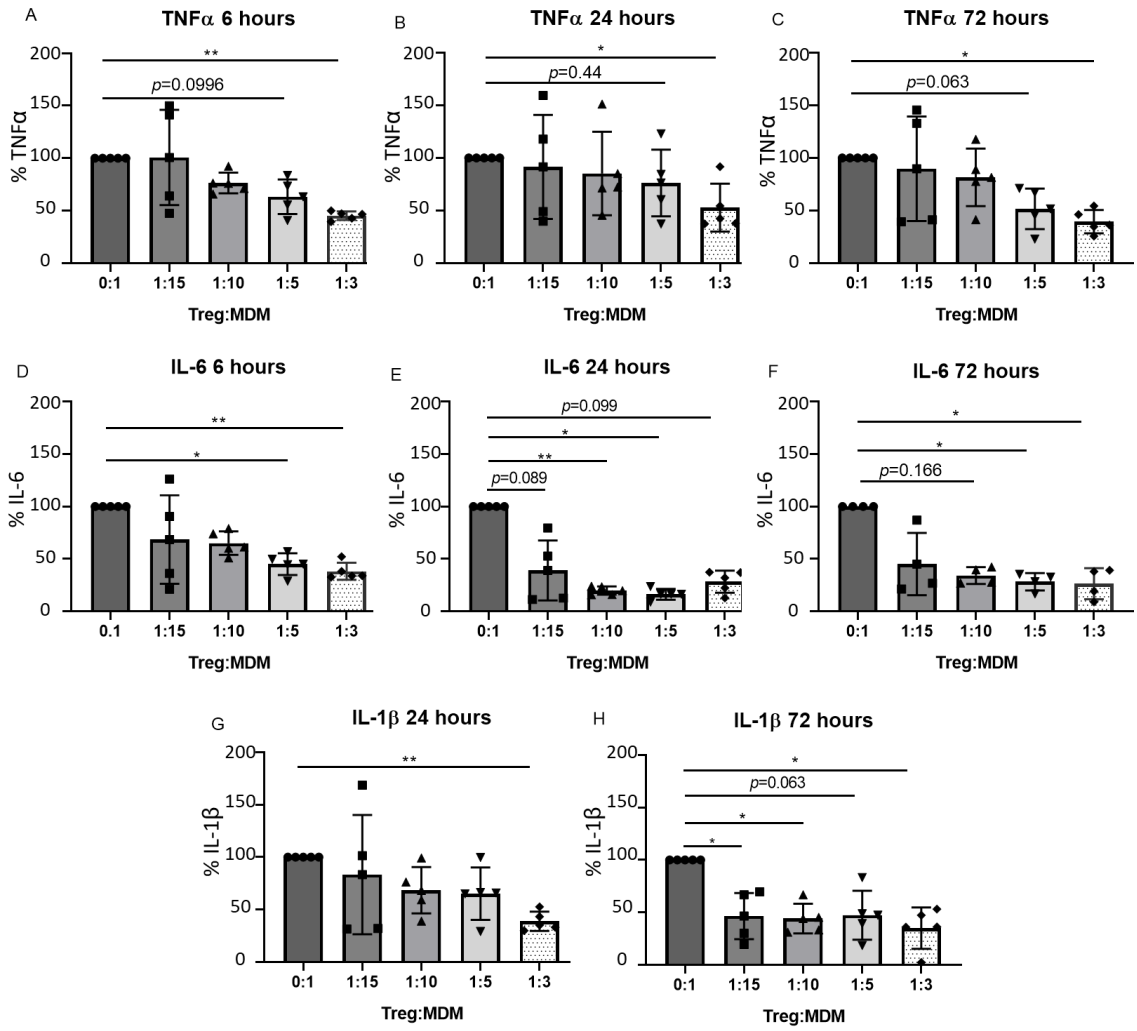

Figure 4.7.1 Pro-inflammatory cytokine production by MDMs co-cultured with increasing numbers of Treg cells prior to *S. pneumoniae* infection

MDMs cultured with an increasing number of Treg cells for 14 hours were then infected with MOI 2 TIGR4 *S. pneumoniae* for 6 hours. Supernatant samples taken at 6 hours, 24 hours and 72 hours were analysed by ELISA for concentration of TNFα (A, B and C), IL-6 (D, E and F) and IL-1β (G and H). 6 hour IL-1β data are omitted due to undetectable cytokine levels. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Groups 0:1, 1:15, 1:10 and 1:5 bars show the means of 5 technical replicates from 1 donor (5 wells per condition containing cells from 1 donor). Group 1:3 bars show the means from 5 donors (data from 4.4). Error bars represent +/- SD from the mean. Statistical analysis was by Kruskal-Wallis (not statistically significant in B; $p<0.05$ in C, E, F, G, H; $p<0.01$ in A, D) with Dunn's multiple comparisons test (*$p<0.05$; **$p<0.01$).

Increasing the numbers of Teff cells in the MDM co-culture resulted in increased TNFα production. TNFα was increased at 6 hours when MDMs had been cultured with 1 Teff per 5 MDMs and with 1 Teff per 3 MDMs (figure 4.7.2 A). At 1 Teff per 15 MDMs, TNFα was close to statistically significant increase at p=0.056. At 24 hours, statistically significant increase in TNFα was observed at the 1:3 ratio of Teff to MDMs (figure 4.7.2 B). At 72 hours statistically significant increase in TNFα was observed at 1:5 and 1:3 ratios of Teff cells to MDMs, with the latter having greater statistical significance (figure 4.7.2 B). Overall, a trend towards increasing TNFα with increasing numbers of Teff cells in the culture with MDMs was observed.

Trends in the data for IL-6 production with increasing number of Teff cells was less apparent. At 6 hours, there were no statistically significant differences in IL-6 production at any ratio of Teff cells to MDMs compared to MDMs cultured without Teff cells (figure 4.7.2 D). At 24 hours, statistically significant decrease in IL-6 production was observed at ratios of 1 Teff per 15 MDMs, 1 Teff per 10 MDMs and 1 Teff per 5 MDMs, and but not at 1 Teff per 3 MDMs at p=0.19 (figure 4.7.2 E). This suggests that the Teff cells promoted suppression of IL-6 production at 24 hours. Interestingly, this suppression was most statistically significant with the fewest Teff cells (1 Teff per 15 MDMs), less so but still statistically significant with 1 Teff per 10 and 1 Teff per 5 MDMs, and then losing statistical significance at a ratio of 1 Teff per 3 MDMs. At 72 hours, no statistically significant differences in IL-6 production could be seen at any ratio (figure 4.7.2 F). No statistically significant differences in IL-1β production could be seen at any ratio (figure 4.7.2 G and H).

Overall, the increasing the number of Teff cells in culture with MDMs caused a trend towards increasing TNFα production at all time points and a temporary decrease in IL-6 production at 24 hours post-infection. Trends for effects of increasing Teff cell numbers in culture were not seen for IL-6 at 6 hours and 72 hours post-infection, or in IL-1β production at any time points (figure 4.7.2).

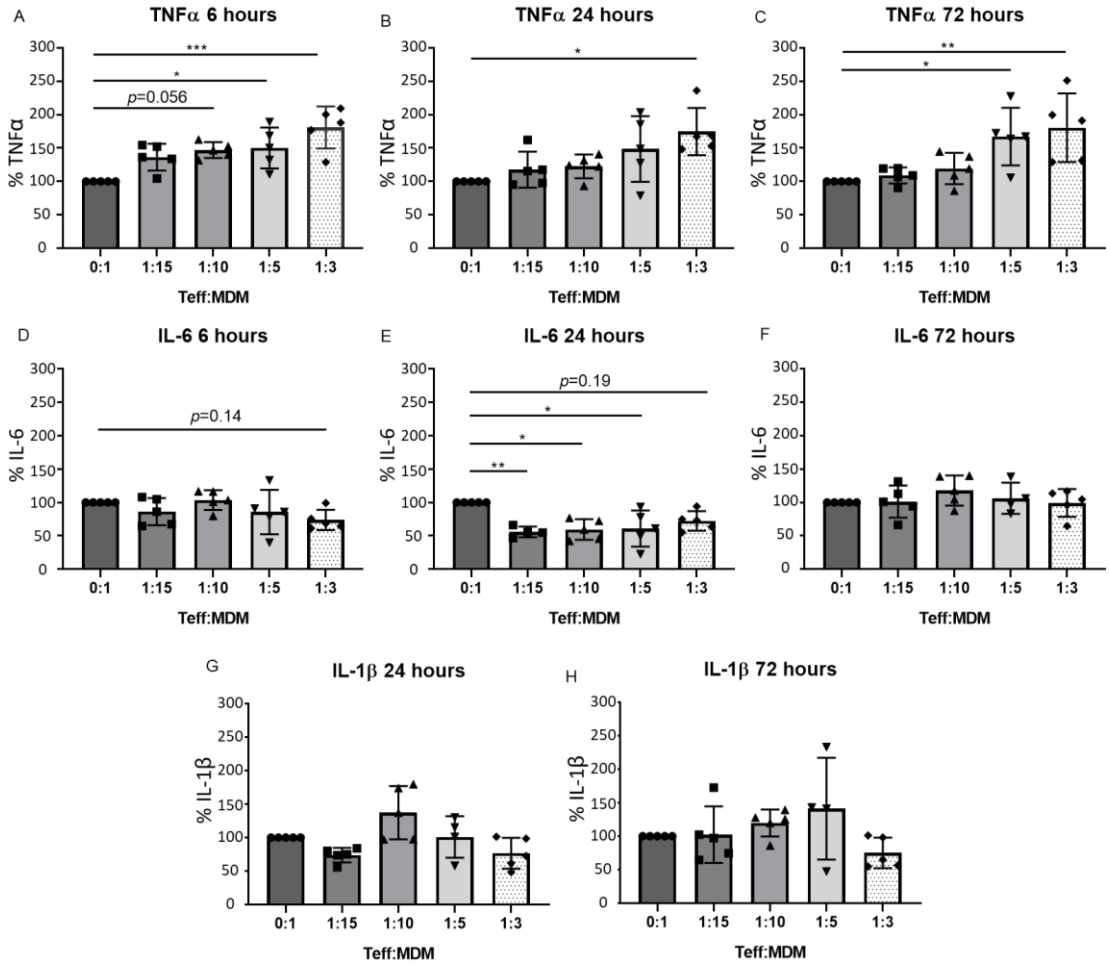

Figure 4.7.2 Pro-inflammatory cytokine production by MDMs co-cultured with increasing numbers of Teff cells prior to *S. pneumoniae* infection

MDMs cultured with an increasing number of Teff cells for 14 hours were then infected with MOI 2 TIGR4 *S. pneumoniae* for 6 hours. Supernatant samples taken at 6 hours, 24 hours and 72 hours were analysed by ELISA for concentration of TNFα (A, B and C), IL-6 (D, E and F) and IL-1β (G and H). 6 hour IL-1β data are omitted due to undetectable cytokine levels. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Groups 0:1, 1:15, 1:10 and 1:5 bars show the means of 5 technical replicates from 1 donor (5 wells per condition containing cells from 1 donor). Group 1:3 bars show the means from 5 donors (data from 4.4). Error bars represent +/- SD of the mean. Statistical analysis was by Kruskal-Wallis (not statistically significant in D, F, H; $p<0.05$ in B, E, G; $p<0.01$ in A, C) with Dunn's multiple comparisons test (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

4.8 *S. pneumoniae* supernatant CFU in the presence of MDMs co-cultured in the presence or absence of Treg or Teff cells

To assess whether Tregs affected control of *S. pneumonaie* by MDMs, co-culture experiment supernatant samples were plated to count the number of bacterial CFUs. The results showed a strong trend to increasing CFU with an increasing ratio of Treg cells to MDMs, with the differences reaching statistical significance for MDMs co-cultured in a ratio of 1 Treg cell per 5 MDMs compared to MDMs cultured alone (figure 4.8 A). MDMs co-cultured in the presence of Teff cells showed little difference compared to MDMs cultured alone, regardless of the number of Teff cells incubated on the Teff cells. This result was taken from 4 technical replicates per group from the same donor. To see whether the *S. pneumoniae* number was consistently higher when infecting MDMs cultured in the presence of Treg cells compared to MDMs cultured alone, the experiment was repeated with cells from 6 individual donors, using the higher ratio of 1 Treg cell to 3 MDMs. This result showed that although the mean CFU number was higher in Treg-MDM co-culture compared to MDMs cultured alone in 3 out of 6 donors, there was no statistically significant difference overall (figure 4.8 B).

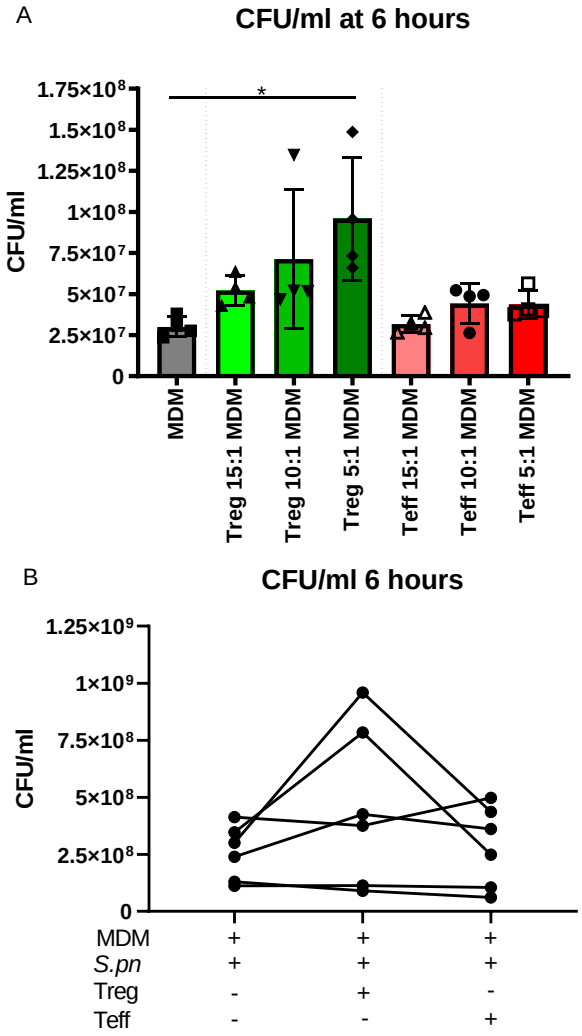

Figure 4.8 CFUs after a 6 hour S*. pneumoniae* infection of MDMs co-cultured in the presence or absence of Treg or Teff cells

MDMs cultured for 14 hours in the presence or absence of increasing numbers of Treg or Teff cells (A), or in the presence or absence of 1 Treg/Teff per 3 MDMs (B), were infected with an MOI of 2 TIGR4 *S. pneumoniae* per MDM for 6 hours. Bacterial numbers were determined by plating the supernatant and counting the CFUs. Graph A shows the mean CFU/ml of technical replicates from 4 wells of the same condition containing cells from 1 donor, with error bars showing +/- SD of the mean and analysed by Kruskal-Wallis (p<0.01) with Dunn's multiple comparisons test (*p<0.05). Data points in B show biological replicates from 6 donors, are paired across conditions per donor, and analysed by Friedman test (not statistically significant) with Dunn's multiple comparisons test (not statistically significant).

4.9 Effect of Treg/Teff cell co-culture on phagocytosis of *S. pneumoniae* by MDMs

An antibiotic protection assay was used to assess whether co-culture with Treg cells affected MDM phagocytosis of *S. pneumoniae*. MDMs co-cultured in the presence or absence of Treg or Teff cells at a ratio of 1 Treg/Teff cell per 3 MDMs were infected with an MOI of 10 *S. pneumoniae* per MDM. After 4 hours infection, the external bacteria were removed and plated to count CFUs. The remaining external bacteria were killed by addition of gentamicin for 45 minutes before washing off the antibiotic and lysing the MDMs by addition of distilled water to release intracellular bacteria, which were plated for CFU growth.

No statistically significant difference was found between the number of intracellular CFUs in MDMs cultured alone, or with Treg or Teff cells (figure 4.9 A). A similar result was obtained upon repeating the experiment. However, in the representative shown for 1 donor, the extracellular bacteria were increased in the presence of the MDMs co-cultured with either Treg or Teff cells compared to MDMs cultured alone (figure 4.9 B), however this was not a consistent finding when repeated across further donors.

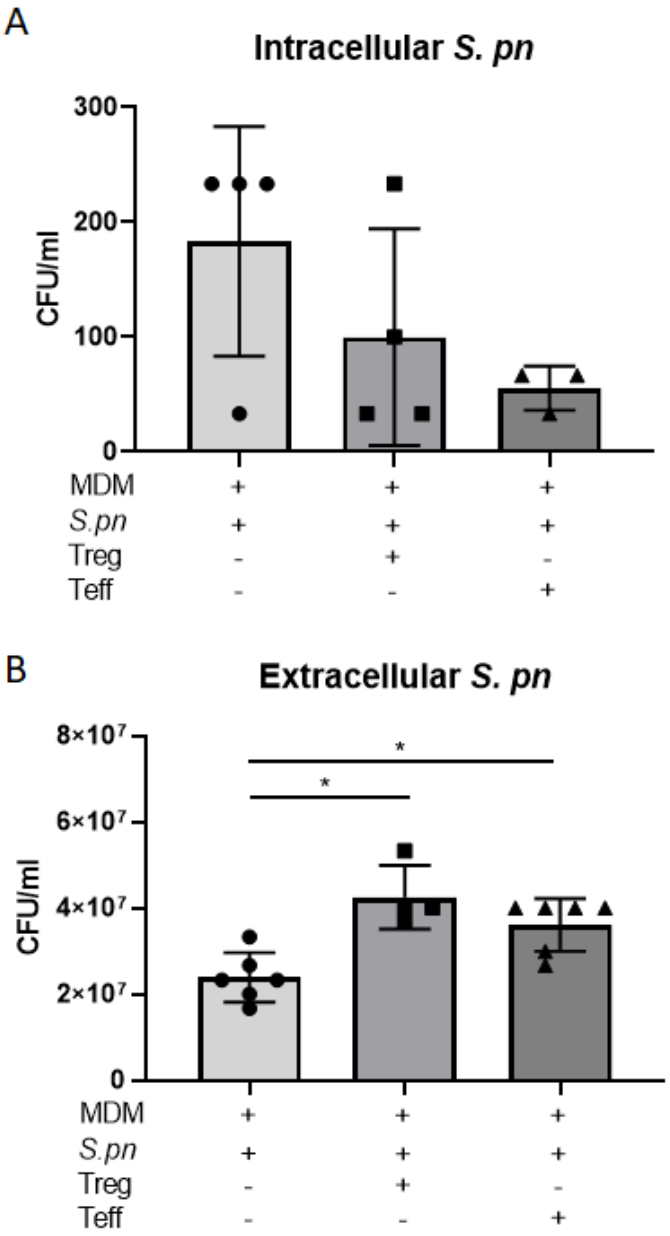

Figure 4.9 Intracellular and extracellular CFUs after *S. pneumoniae* infection of MDMs cultured in the presence or absence of Treg or Teff cells

MDMs cultured in the presence or absence of Treg or Teff cells were infected with MOI 10 TIGR4 *S. pneumoniae* for 4 hours. Intracellular (A) and extracellular (B) bacteria were plated and CFUs counted. Graphs show means from technical replicates taken from 6 wells of the same condition with cells from 1 donor, with error bars showing +/- SD of the means. Statistical analysis was by Kruskal-Wallis (A is not statistically significant, $p<0.01$ in B) with Dunn's multiple comparisons test (*$p<0.05$).

4.10 Effect of delaying infection of MDMs co-cultured in the presence or absence of Treg or Teff cells on the inflammatory response to *S. pneumoniae*

These experiments investigated whether delaying the infection after co-culture of MDMs with Treg cells retained the suppressive effect on MDMs pro-inflammatory cyokne responses to *S. pneumoniae*. MDMs were cultured in the presence or absence of Treg or Teff cells at a ratio of 1 Treg/Teff cell per 3 MDMs for 14 hours, the Treg/Teff cells were then removed from the MDMs, which were then incubated for a further 24 hours in the absence of Treg/Teff cells. The MDMs were then infected with an MOI of 2 *S. pneumoniae* per MDM for 6 hours. The bacteria were killed by addition of penicillin-streptomycin, and supernatant samples taken at 6 hours, 24 hours and 72 hours post-infection. Absolute cytokine concentrations measured were converted into a percentage of the MDM + *S. pneumoniae* group in order to reduce donor variability from the overall effects of Treg/Teff addition and individual time points were examined.

TNFα and IL6 production was suppressed at all time points in MDMs that had been co-cultured with Treg cells (figure 4.10 A to F). At 6 hours and 72 hours post-infection statistically significant reduction in IL-1β production by MDMs that had been co-cultured with Treg cells compared to MDMs cultured alone was observed (figure 4.10 G and I), with the results at 24 hours falling short of of statistical significance *p* value of 0.092 (figure 4.10 H). In contrast, in general TNFα and IL6 production by MDMs co-cultured with Teff cells was higher compared to MDMs co-cultured with Treg cells (figure 4.10 A, B and C). Production of IL-1β at 6 hours, 24 hours and 72 hours by the MDMs co-cultured with Teff cells did not significantly differ statistically from MDMs cultured alone (figure 4.10 G, H and I).

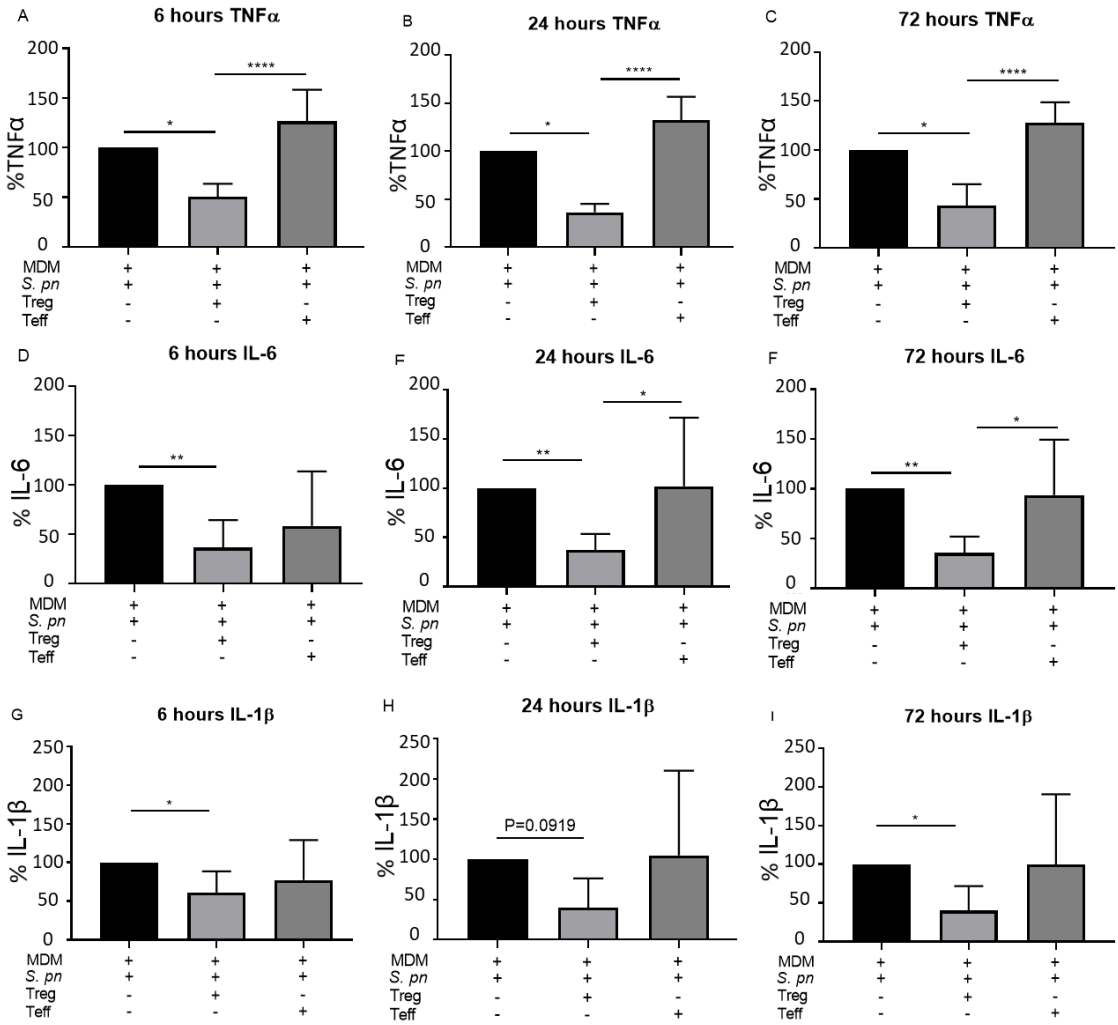

Figure 4.10 Pro-inflammatory cytokine production by MDMs co-cultured in the presence or absence of Treg or Teff cells upon a delayed infection with *S. pneumoniae*

MDMs cultured in the presence or absence of Treg or Teff cells for 14 hours were then incubated in the absence of the Treg/Teff cells for a further 24 hours, followed by infection of the MDMs with MOI 2 TIGR4 *S. pneumoniae* for 6 hours. Supernatant samples taken at 6 hours, 24 hours and 72 hours post-infection were analysed for concentration of TNFα (A, B, and C), IL-6 (D, E and F), and IL-1β (G, H and I) by ELISA. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Graphs show means of technical replicates from 4 wells of each condition with cells from 1 donor and error bars showing +/- SD of the mean. Statistical analysis was by Kruskal-Wallis (A, B and C: $p<0.0001$. D: $p<0.001$. E, F: $P<0.01$. G, I: $P<0.05$. H: not statistically significant) with Dunn's multiple comparisons test (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

4.11 Effect of co-culturing MDMs with Treg cells on the inflammatory response to *A. baumannii*

These experiments aimed to see whether similar suppressive effects exerted by Treg cells on MDM inflammatory responses to *S. pneumoniae* could be seen when the MDMs were instead infected with another pneumonia pathogen, *A. baumannii*, which are Gram-positive bacteria. Like *S. pneumoniae*, they have a polysaccharide capsule, but also produce LPS and have abundant outer membrane proteins that are important virulence factors.

Replicating the same conditions optimised for the *S. pneumoniae* experiments in 4.4, MDMs were cultured in the presence or absence of Treg or Teff cells in a ratio of 1 Treg/Teff cell per 3 MDMs for 14 hours, before removing the Treg/Teff cells and infecting the MDMs with an MOI of 2 *A. baumannii* per MDM. After 6 hours infection, penicillin-streptomycin was added to the infected MDMs and supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for analysis of cytokine concentrations by ELISA. Absolute cytokine concentrations measured were converted into a percentage of the MDM + *S. pneumoniae* group in order to reduce donor variability from the overall effects of Treg/Teff addition and individual time points were examined.

The results showed that there was no decrease at any timepoints in TNFα (figure 4.11.1 A, B and C), IL-6 (figure 4.11.1 D, E and F), or IL-1β (figure 4.11.1 G and H) production by MDMs that had been co-cultured in the presence of Treg cells compared to MDMs cultured alone. MDMs co-cultured in the presence of Teff cells showed lower IL-6 at 24 hours (figure 4.11.1 E) compared to MDMs cultured alone. MDM inflammatory responses to *A. baumannii* was approximately 10-fold higher than that to *S. pneumoniae*, with *S. pneumoniae* generally producing around 2000 pg/ml TNFα by 6 hours and *A. baumannii* approximately 20000 pg/ml under the same conditions. Plating the inoculum for this experiment ensured that the correct number

of bacteria had been added, and plating 6 hour post-infection supernatants showed comparable CFU numbers to *S. pneumoniae* when added at the same MOI (figure 4.10.2). Hence, in case the excessive inflammatory response had hindered Treg suppression of the MDMs this experiment was repeated with a 10-fold and 100-fold fewer *A. baumannii* added.

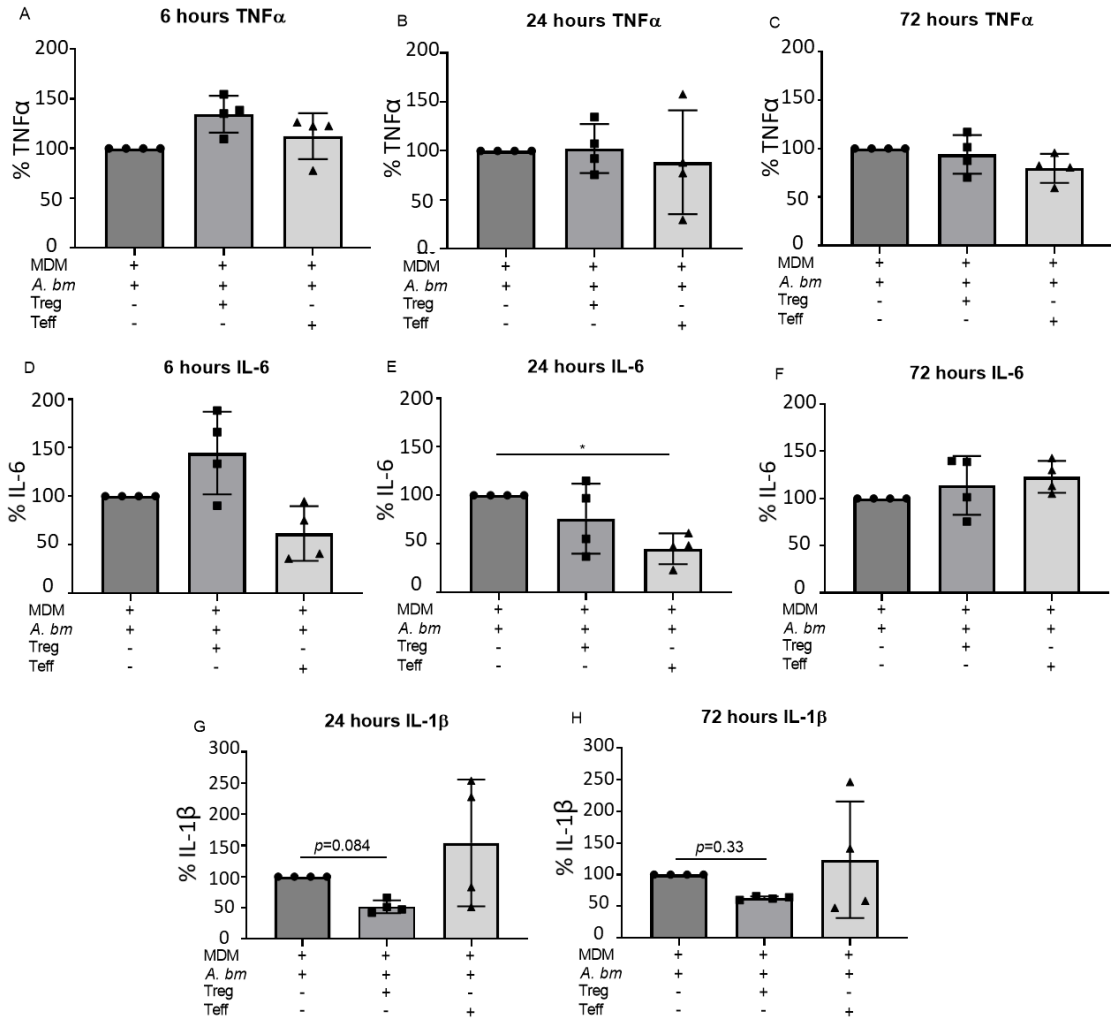

Figure 4.11.1 Pro-inflammatory cytokine production by MDMs cultured in the presence or absence of Treg or Teff cells upon infection with an MOI of 2 *A. baumannii* per MDM

MDMs were cultured in the presence or absence of Treg or Teff cells for 14 hours before removing the Treg/Teff cells and infecting the MDMs with MOI 2 ST2 *A. baumannii*. Supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for detection of TNFα (A, B, C), IL-6 (D, E, F) and IL-1β (G, H, I) by ELISA. 6 hour IL-1β data are omitted due to undetectable cytokine levels. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Graphs show means of technical replicates taken from 4 wells per condition containing cells from 1 donor, with error bars representing +/- SD of the mean. Statistical analysis was by Kruskal-Wallis (not significant in A, B, C, F; $p<0.05$ in D, E; $p<0.01$ in G; $p<0.001$ in H, I) with Dunn's multiple comparisons test (*$p<0.05$, **$p<0.01$).

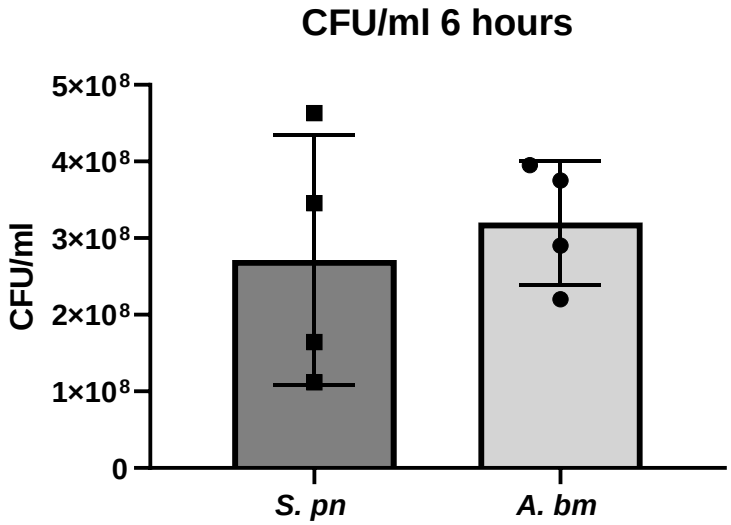

Figure 4.11.2 Comparison of CFUs of *A. baumannii* and *S. pneumoniae* after 6 hour infection of MDMs at an MOI of 2 bacteria per MDM

MDMs were infected with TIGR4 *S. pneumoniae* or ST2 *A. baumannii* at an MOI of 2 bacteria per MDM for 6 hours, the supernatant was plated and incubated for 14 hours and the resulting CFUs counted. *S. pneumoniae* data shown are the mean CFU/ml from biological replicates from 4 MDM donors. *A. baumannii* data show the mean CFU/ml of technical replicates of a supernatant sample each from 4 wells of cells from 1 donor. Graphs show mean values with error bars showing +/- SD of the means. Statisitical analysis by unpaired t test was not statistically significant.

Similar to the results for an MOI of 2, Treg cells failed to have statistically significant suppressive effects on pro-inlammatory cytokine levels at almost all timepoints for MDMs infected with an MOI of 0.2 or 0.02 of *A. baumannii* (figures 4.11.3 and 4.11.4), with the exception of IL-6 at 6 hours for MOI 0.2 (figure 4.11.3 D). Addition of Teffs cells also had limited effects on the pro-inflammatory cytokine response by MDMs to *A. baumannii* (figures 4.11.3 and 4.11.4).

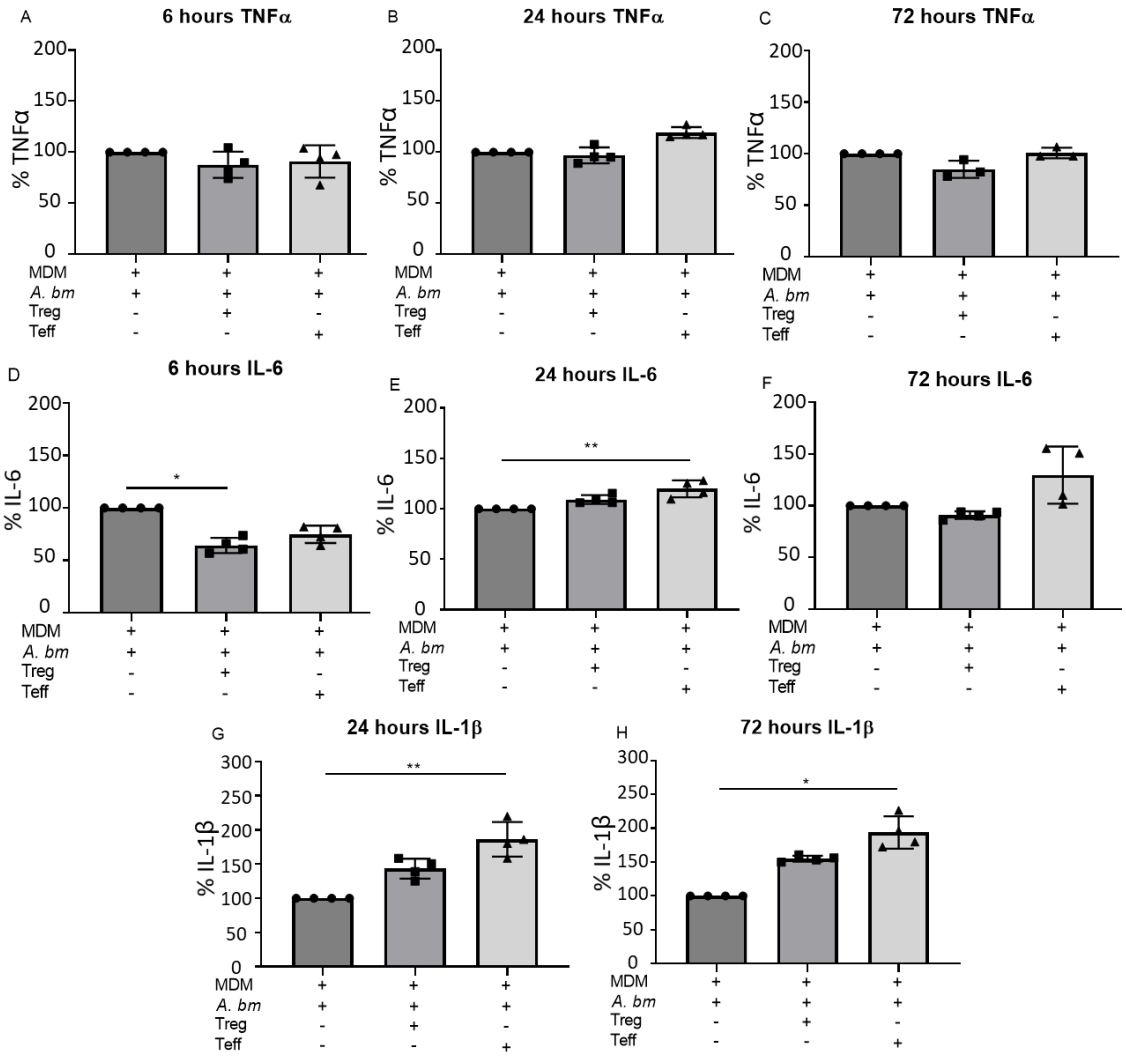

Figure 4.11.3 Pro-inflammatory cytokine production of MDMs cultured in the presence or absence of Treg or Teff cells upon infection with an MOI of 0.2 *A. baumannii* per MDM

MDMs were cultured in the presence or absence of Treg or Teff cells for 14 hours before removing the Treg/Teff cells and infecting the MDMs with an MOI of 0.2 ST2 *A. baumannii* per MDM. Supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for detection of TNFα (A, B, C), IL-6 (D, E, F) and IL-1β (G, H, I) by ELISA. IL-1β data are omitted due to undetectable cytokine levels. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Graphs show the mean ratio of technical replicates from 4 wells of each condition containing cells from 1 donor with error bars showing +/- SD from the mean. Statistical analysis was by Kruskal-Wallis (not statistically significant in A; $p<0.05$ in C, $p<0.01$ in B, D, F, H; $p<0.001$ in E and G) with Dunn's multiple comparisons test (*$p<0.05$, **$p<0.01$).

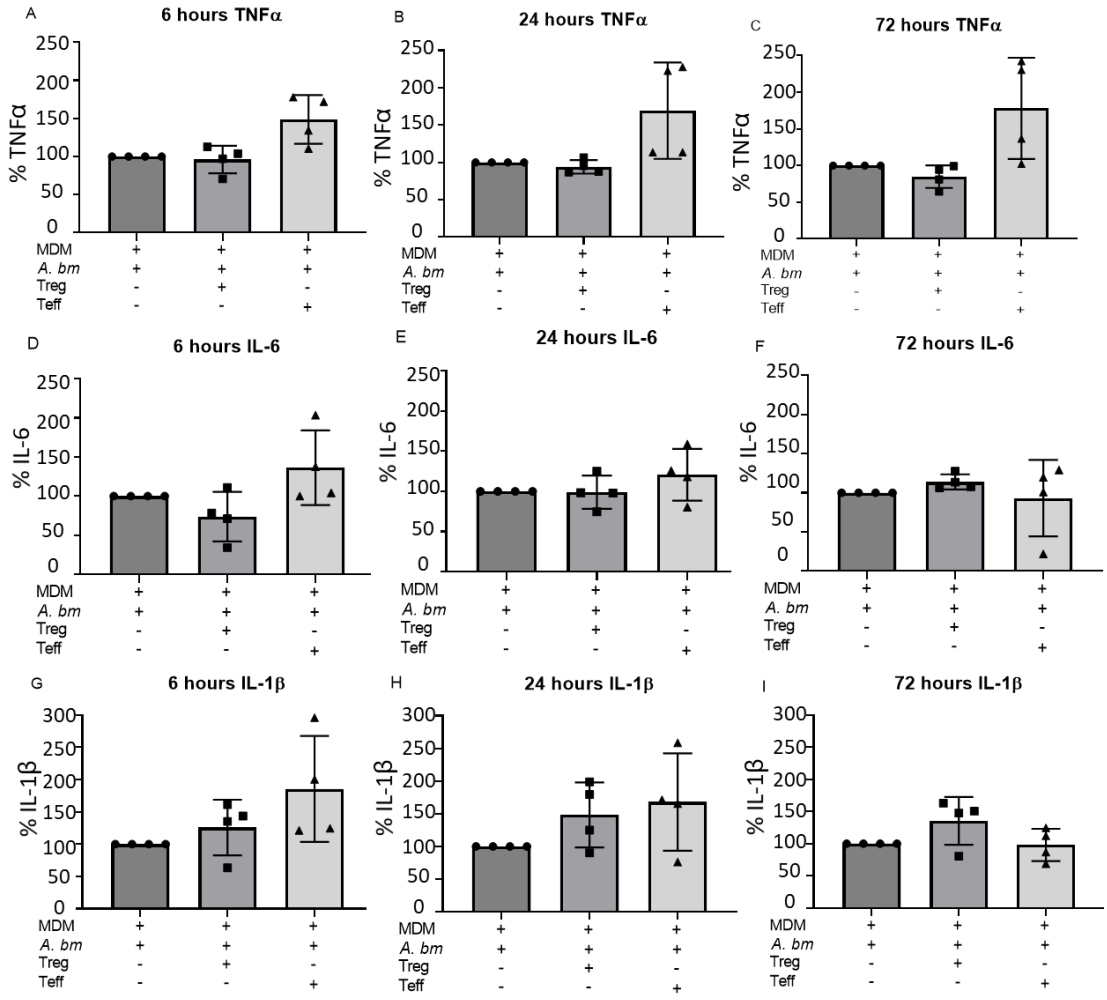

Figure 4.11.4 Pro-inflammatory cytokine production of MDMs cultured in the presence or absence of Treg or Teff cells upon infection with an MOI of 0.02 *A. baumannii* per MDM

MDMs were cultured in the presence or absence of Treg or Teff cells for 14 hours before removing the Treg/Teff cells and infecting the MDMs with an MOI of 0.02 ST2 *A. baumannii* per MDM. Supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for detection of TNFα (A, B, C), IL-6 (D, E, F) and IL-1β (G, H, I) by ELISA. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Graphs show the mean ratio of technical replicates from 4 wells per condition containing the cells from 1 donor with error bars showing +/- SD from the mean. Statistical analysis was by Kruskal-Wallis (not statistically significant for all) with Dunn's multiple comparisons test (not statistically significant for all).

Of note, with an MOI of 0.02 *A. baumannii* per MDM comparable concentrations of cytokines were seen to MDM infection with MOI of 2 for *S. pneumoniae* (figure 4.11.5). There was no statistically significant difference at 6 hours post-infection in the concentrations of TNFα, IL-6 or IL-1β when comparing MOI 2 *S. pneumoniae* with MOI 0.02 *A. baumannii*, although IL-6 concentration was approximately double that of *S. pneumoniae* and was close to statistically significant difference with a *p* value of 0.0767 (figure 4.11.5 A and B).

To confirm that the decreasing inoculi of *A. baumannii* resulted in correspondingly lowered CFU numbers after the 6 hour infection of the MDMs, supernatant samples were taken and plated for CFU growth at this time. These data were compared against CFU number of *S. pneumoniae* after a 6 hour infection of MDMs at an MOI of 2 bacteria per MDM. No statistically significant difference was found between CFU numbers of *S. pneumoniae* and *A. baumannii* infecting the MDMs at MOI 2, however statistically significant differences were found when comparing the CFU numbers of *A. baumannii* or *S. pneumoniae* at MOI 2 to *A. baumannii* MOI 0.2 and MOI 0.02 (figure 4.11.6).

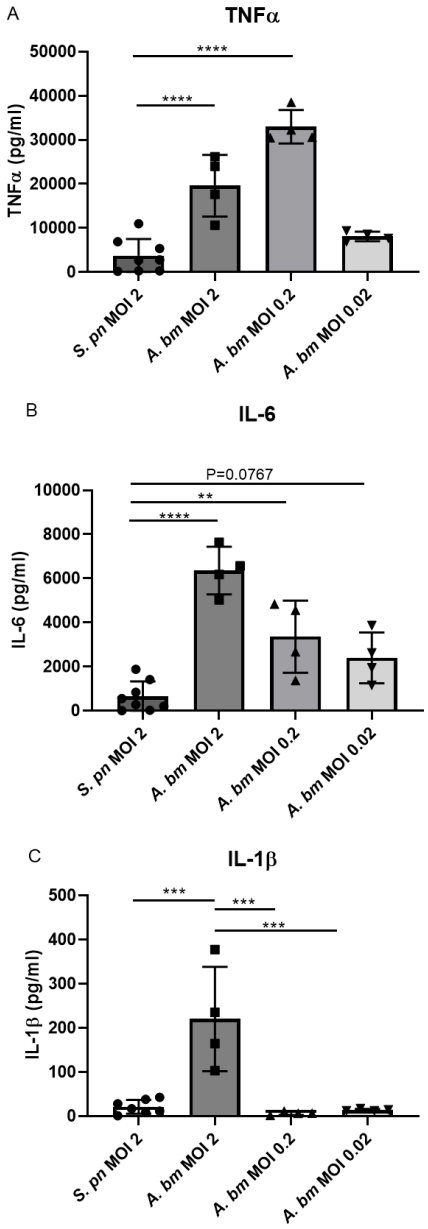

Figure 4.11.5 Pro-inflammatory cytokine production by MDMs infected with an MOI of 2 *S. pneumoniae* per MDM or a decreasing MOI of *A. baumannii* per MDM

MDMs were infected with either an MOI 2 of TIGR4 *S. pneumoniae* per MDM, or an MOI of 2, 0.2 or 0.02 of ST2 *A. baumannii* per MDM, for 6 hours. Supernatants were analysed for concentration of TNFα (A), IL-6 (B) and IL-1β (C) by ELISA. *S. pneumoniae* data shown are the mean concentrations of biological replicates on cells taken from 8 donors. *A. baumannii* data shown are the mean concentrations of 4 technical replicates with a sample from each of 4 wells per condition with cells from 1 donor. Error bars show +/- SD of the mean. Statistical analysis was by one-way ANOVA (A, B $p<0.0001$; C $p<0.001$) with Tukey's multiple comparisons test (**$p<0.01$, ****$p<0.0001$).

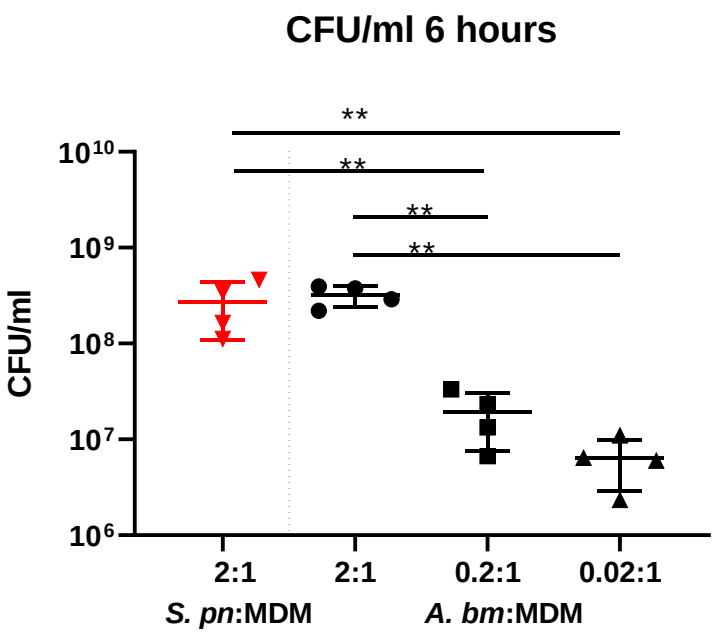

Figure 4.11.6 CFUs after a 6 hour infection of MDMs with an MOI of 2 *S. pneumoniae* per MDM or a decreasing MOI of *A. baumannii* per MDM

MDMs were infected with either an MOI 2 TIGR4 *S. pneumoniae* per MDM, or an MOI of 2, 0.2 or 0.02 ST2 *A. baumannii* per MDM, for 6 hours. Supernatants were plated at 6 hours post-infection and incubated for 14 hours to count the resulting CFU numbers. *S. pneumoniae* data points represent CFU/ml of 4 biological replicates with cells taken from 4 donors. *A. baumannii* data points show CFU/ml of technical replicates with a sample taken from each of 4 wells containing cells from 1 donor. Error bars show +/- SD of the means. Statistical analysis was by one-way ANOVA (p<0.001) with Tukey's multiple comparisons test (**p<0.01).

4.12 Effect of culturing MDMs in the presence or absence of Treg or Teff cells on CFU number after a 6 hour infection with a decreasing MOI of *A. baumannii* per MDM

MDMs were cultured in the presence or absence of Treg or Teff cells in a ratio of 1 Treg/Teff cell per 3 MDMs for 14 hours, before removing the Treg/Teff cells and infecting the MDMs with either an MOI of 2, 0.2, or 0.02 of *A. baumannii* per MDM. After a 6 hour infection, supernatant samples were plated and incubated for 14 hours and the resulting CFU numbers counted. There were no statistically significant differences between CFU numbers counted from *A. baumannii*-infected MDMs that had been cultured alone, MDMs cultured in the presence of Treg cells, or MDMs cultured in the presence of Teff cells, at all MOIs of bacteria per MDM (figure 4.12). Despite lack of statistical significance, a trend towards decreasing CFU numbers counted in the supernatant of MDMs with Treg cell co-culture was observed at an MOI of 0.2 and an MOI of 0.02 *A. baumannii* per MDM, which is contradictory to the trend seen in 4.8, where a trend of increased *S. pneumoniae* CFUs in the presence of MDMs co-cultured with Treg cells in 3 out of 6 experiments could be seen (figure 4.8).

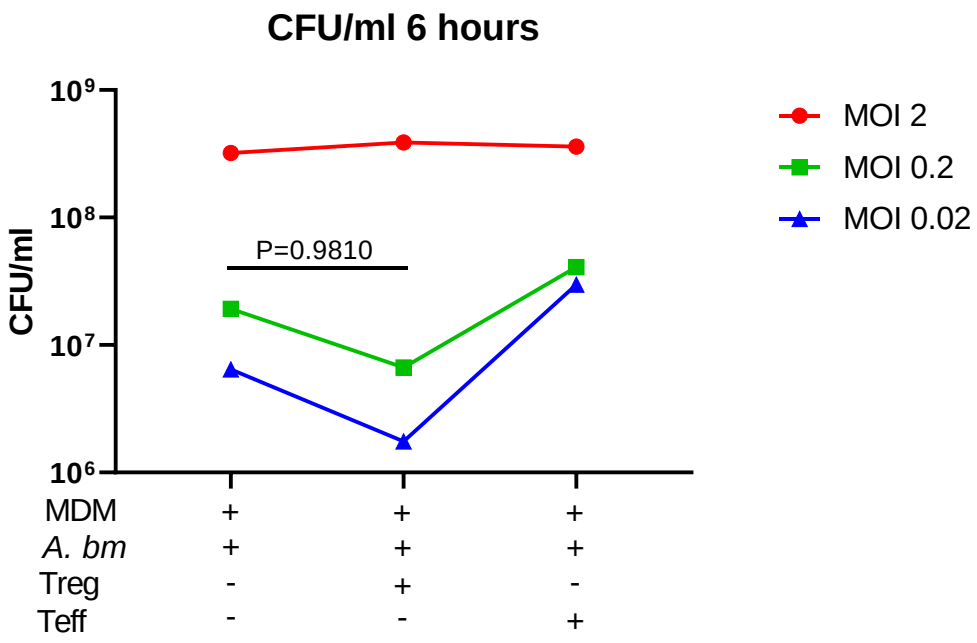

Figure 4.12 CFUs from *A. baumannii*-infected MDMs co-cultured in the presence or absence of Treg or Teff cells

MDMs were cultured in the presence or absence of Treg or Teff cells and infected with a decreasing MOI of ST2 *A. baumannii* per MDM for 6 hours. Post-infection supernatant samples were plated and incubated for 14 hours to count the resulting CFUs grown. Data points shown are the means of 4 technical replicates with a sample from each of 4 wells containing cells taken from 1 donor per MOI. Statistical analysis was by two-way ANOVA (not statistically significant) with Tukey's multiple comparisons test (not statistically significant). *p*=0.9810 for both MOI 0.2 and MOI 0.02 comparing MDMs +/- Treg co-culture.

4.13 Chapter 4 Summary

The experiments in this chapter aimed to investigate whether Treg cells could suppress MDM inflammatory responses when co-cultured before, during or after *S. pneumoniae* infection, additionally comparing the effect that Teff-MDM co-culture has on this inflammatory response.

Adding Treg cells to MDMs simultaneously with *S. pneumoniae* resulted in decreased TNFα, IL-6 and IL-1β production by these MDMs compared to MDMs that had been infected alone. This may occur physiologically *in vivo* as non-local macrophages and lymphocytes are reportedly recruited at similar timings during *S. pneumoniae* pneumonia, and therefore this could be a potential mechanism of inflammatory control (Kadioglu et al., 2000; Bergeron et al., 1998). Conversely, addition of Treg cells to *S. pneumoniae*-infected MDMs post-infection had little effect on subsequent MDM cytokine production, with the exception of TNFα being lowered with Treg cells at 72 hours. Other reports suggest that macrophages are capable of modifying their phenotype and cytokine production when stimuli change (Edwards et al., 2006), therefore theoretically they should be capable of adopting an anti-inflammatory role when infection is clearing. Shift to an anti-inflammatory role may have been hindered in the post-infection Treg addition experiments as penicillin-streptomycin addition may have resulted in a large output of inflammatory bacterial components as a result of bacterial cell lysis, causing resolution of the inflammatory response of the macrophages to be delayed and therefore not clearly observed in these experiments.

The most statistically significant effect on MDM inflammatory responses was found when co-culturing Treg cells with MDMs prior to infection, in which Treg co-culture resulted in decreased TNFα, IL-6 and IL-1β production by MDMs upon infection. Therefore, the effects of this co-culture were then examined in more detail. In the pre-infection co-culture experiments, Treg/Teff cells were not present during the actual infection, suggesting that the Treg cells were able to condition the MDMs

towards a lessened inflammatory response which was maintained without the need for Treg cells to remain present upon infection. This was further confirmed by finding that MDM inflammatory responses were still decreased when repeating the experiment but delaying the addition of *S. pneumoniae* by 24 hours after the removal of Treg cells. This finding could be potentially important when considering that prior respiratory infections such as influenza can predispose to a worsened second infection, as macrophages exposed to Treg cells during resolution of a prior infection could maintain a suppressed inflammatory ability into the second infection (Roquilly et al., 2015). Alternatively, macrophages encountering Treg cells prior to infection could promote this suppressed inflammatory ability upon subsequent bacterial challenge, which may perhaps be brought about by resident Treg populations at the infection site.

Comparison of the pre-infection and during infection Treg addition experiments does not take into account the fact that in the pre-infection experiments the MDMs were exposed to the Treg cells for longer. The pre-infection co-culture of MDMs with Treg cells was 14 hours, therefore Treg cells have had this amount of time to exert a suppressive effect on the macrophage. By the 6 hour post-infection sample collection, the Treg cells and MDMs had spent a total of 20 hours in co-culture, only 6 of which was in an inflammatory environment. Conversely, addition of the Treg cells simultaneously with infection meant that the Treg cells only had 6 hours to work on the MDMs, the duration of which was an inflammatory environment. It would be important to deduce how long the Treg cells need to remain in the culture without infection in order to produce a significant suppressive effect on the MDMs and the time at which maximum suppression occurs. Following this, it would be important to determine if the inflammatory environment of the infection affects the suppressive capacity and mechanisms of suppression used by the Treg compared to those in the absence of infection.

The direct effects of *S. pneumoniae* on Treg/Teff cells alone was not investigated, however it is likely that the presence of the bacteria affected the functions of the T cells. For example, *S. pneumoniae* NanA can activate latent TGFβ by removing LAP (Gratz et al., 2017), which may have aided Treg-mediated suppression of the macrophage responses if TGFβ is involved in the suppressive mechanism used by Treg. Conversely, Treg cells express TLR2 receptors and can shift to a more Th17-like phenotype by stimulation with TLR2 agonists (Nyirenda et al., 2015), therefore *S. pneumoniae* lipoproteins may have caused Treg cells to lose some suppressive ability by this mechanism. Bacterial properties likely affected the experiments in which the T cells were added to the MDMs simultaneouslty with *S. pneumoniae* and may partly explain differences in the suppressive capacity compared with pre-infection T cell-MDM co-culture.

Results of pro-inflammatory cytokine production by MDMs co-cultured with Teff cells prior to infection or infected in the presence of Teff cells was more variable than those of Treg-MDMs. For MDMs infected simultaneously with Teff addition, some donors showed a trend of increased cytokine production, some decreased, and some remained similar to MDMs infected alone. Results when comparing absolute cytokine concentrations over all 3 time points did not produce any statistically significant increases or decreases in cytokine production upon infection with simultaneous Teff addition, although for most donors a trend towards decreased IL-6 and IL-1β, especially at the later time points of 24 hours and 72 hours. Conversely, Teff addition pre-infection caused trends towards increased TNFα, early decreases in IL-6 which recovered by 72 hours to be similar to the MDM + *S. pneumoniae* group, and a trend towards decrease in IL-1β for most donors. Post-infection addition of Teff cells to MDMs did not show any clear trends on TNFα, IL-6 or IL-1β production. The differences in responses of MDMs with Treg co-culture compared to Teff co-culture suggest that suppressive effects are not just simply a result of T cell co-culture, and

these T cell subsets cause different effects on the MDM inflammatory responses. In dose response experiments a general trend towards decreasing pro-inflammatory cytokine production with an increasing number of Treg cells added to the MDMs was seen. Although for some donors an increase in bacterial number could be seen when Treg- co-cultured MDMs were infected with *S. pneumoniae* compared to infected MDMs that had been cultured alone, this was not found to be statistically significant across all donors tested. For MDMs with pre-infection co-culture with Teff cells, TNFα production was increased with increasing number of Teff cells, whereas IL-6 and IL-1β production showed no trend between cytokine production and increasing Teff cell numbers, except for overall decreases in IL-6 at 24 hours. Lack of statistical significance does not mean that these differences do not have a significant biological effect *in vivo*, in fact extremes of reduction or increase in inflammatory responses are likely to be detrimental.

The low MOI of 2 bacteria per MDM used in these experiments was optimised in chapter 3 in order to produce measurable, statistically significant reductions in MDM cytokine production, while higher MOIs resulted in less suppression of MDM responses by Treg cells. However, *in vivo*, the numbers of bacteria present will be hugely variable and will affect the inflammatory response, success of carriage and severity of disease. Low density of *S. pneumoniae* in the nasopharynx promotes longer carriage, associated with increased Treg numbers and anti-inflammatory macrophages (Neill et al., 2014). The data in this chapter may support the *in vivo* findings of Neill and colleagues as the increased Treg numbers may be promoting suppression of the macrophage responses and thereby prolonging carriage. The TIGR4 strain used in this chapter is not common in pneumococcal carriage, therefore it would be interesting to repeat the experiments with increasing concentrations of a strain more commonly seen in carriage, such as 23F.

No statistically significant difference in phagocytosis of *S. pneumoniae* by MDMs cultured alone compared to MDMs cultured prior to infection in the presence of Treg or Teff cells could be seen. A limitation of the protocol used is that very low numbers of bacteria are released from the MDMs upon lysing the cells, making the counting of CFUs prone to error. These experiments could be repeated using fluorescently labelled *S. pneumoniae* to visualise the number of internalised bacteria by microscopy as a more accurate measure of phagocytosis.

In the literature, mouse data on Treg modulation of APCs in pneumonia vary by pathogen (Delano et al., 2013). Repeating the pre-infection MDM- Treg co-culture experiments infecting MDMs with *A. baumannii* did not show any ability of Treg cells in decreasing pro-inflammatory cytokine production by MDMs. Therefore, *A. baumannii* possess factors that overcome the suppressive effects exerted by Treg cells on MDMs more successfully that those of *S. pneumoniae*. Such bacterial factors of *A. baumannii* may be LPS or outer membrane proteins. *A. baumannii* infection resulted in an approximate 10-fold increase in pro-inflammatory cytokine production by the MDMs when added at the same MOI as *S. pneumoniae*, but Treg cells still failed to supporess MDM responses even when the MOI was lowered by 100-fold to result in pro-inflammatory cytokine production to similar levels seen in the *S. pneumoniae* experiments. The ability of *S. pneumoniae* to evade the immune system and colonise the nasopharynx while *A. baumannii* provokes a highly pro-inflammatory response normally resulting in rapid clearance may explain these differences, and perhaps *S. pneumoniae* utilise Treg manipulation of macrophages to achieve prolonged colonisation *in vivo*. In the literature, clearance of *S. pneumoniae* infection is reported as being highly dependent on macrophage responses, whereas clearace of *A. baumannii* seems less dependent on macrophages and more dependent on neutrophils (reviewed by García-Patiño et al., 2017). This may indicate that modulation of macrophage responses is not beneficial to *A. baumannii*. The *A.*

baumannii experiments in this thesis were only carried out with cells from one donor each for the three MOIs of *A. baumannii* tested, and to strengthen the data need repeating using cells from mulitple donors at a consistent MOI. In addition, repeating these experiments with another Gram positive bacterium may be useful in order to see if these bacteria show more similar results to those seen with *S. pneumoniae* compared with the Gram negative *A. baumannii*.

In conclusion:

- Addition of Treg cells to MDMs simultaneously with *S. pneumoniae* infection resulted in suppressed TNFα, IL-6 and IL-1β production by the MDMs
- Post-infection Treg addition reduced MDM TNFα production by 72 hours, but not IL-6 or IL-1β levels at any timepoint
- Co-culturing MDMs with Treg cells prior to infection lowered TNFα, IL-6 and IL-1β MDM responses upon infection with *S. pneumoniae*, and this suppression was more statistically significant than that seen when Treg cells were added to MDMs simultaneously with the bacteria
- Addition of Teff cells to MDMs pre-infection, during infection and post infection had variable effects on TNFα, IL-6 and IL-1β production
- Suppression of TNFα, IL-6 and IL-1β production by MDMs co-cultured with Treg cells prior to infection did not require the Treg cells to remain present and the suppressive effect was retained upon delaying infection for a further 24 hours after Treg removal
- No consistent effect of Treg-MDM co-culture on phagocytosis and bacterial replication of *S. pneumoniae* were found in these data, although the data suggest there maybe a degree of impaired bacterial clearance for MDMs incubated with Treg cells.
- There was no suppression of the production of TNFα, IL-6 or IL-1β by Treg-co-cultured MDMs upon subsequent infection with *A. baumannii*.

Chapter 5: Mechanisms of Treg cell suppression of MDM inflammatory responses to *S. pneumoniae*

5.1 Chapter 5 Introduction

Chapter 4 demonstrated that Treg cells could suppress the inflammatory response of MDMs upon infection with *S. pneumoniae*. This suppression was most statistically significant when MDMs were cultured in the presence of Treg cells prior to infection, as seen in part 4.4. The present chapter aimed to investigate the mechanisms by which this suppression may occur.

Aims:

- Can IL-10 or TGFβ suppress MDM inflammatory responses to *S. pneumoniae* and are these cytokines produced during MDM-Treg or MDM-Teff co-culture?
- Is contact between the Treg cells and MDMs required for suppression or can suppression be mediated by soluble factors (e.g. cytokines)?
- What are the effects of blocking TGFβ signalling in Treg-MDM co-culture on the inflammatory responses of MDMs to *S. pneumoniae*?
- Do the MDM co-culture conditions encourage Treg differentiation from $CD4^+$ T cells?

5.2 Effect of exogenous addition of IL-10 or TGFβ on MDM inflammatory responses to *S. pneumoniae*

IL-10 and TGFβ are two anti-inflammatory cytokines reported to be produced by Treg cells (Groux *et al*., 1997, Miller *et al*., 1992). Therefore, the effect of the exogenous addition of these cytokines to MDMs on their inflammatory response to infection with *S. pneumoniae* was investigated. MDMs were incubated with either IL-10 at 1ng/ml or 100 ng/ml or with TGFβ at 1ng/ml or 50ng/ml for 1 hour before infection of the MDMs with *S. pneumoniae* for 6 hours. After the 6 hour infection, the supernatants were collected and analysed for the concentration of TNFα, IL-6 and IL-1β by ELISA. Addition of IL-10 at 100ng/ml reduced TNFα, IL-6 and IL-1β production by MDMs during *S. pneumoniae* infection, with 1ng/ml IL-10 causing a trend towards reduced production of these cytokines (figure 5.2 A, B and C). Addition of TGFβ at 50ng/ml also decreased TNFα and IL-6 production by the *S. pneumoniae*-infected MDMs; however, the reduction in IL-1β production was not statistically significant (p=0.14, figure 5.2 D, E and F). Addition of TGFβ at 1 ng/ml resulted in a trend towards decrease in the production of the measured cytokines (figure 5.2 D, E and F).

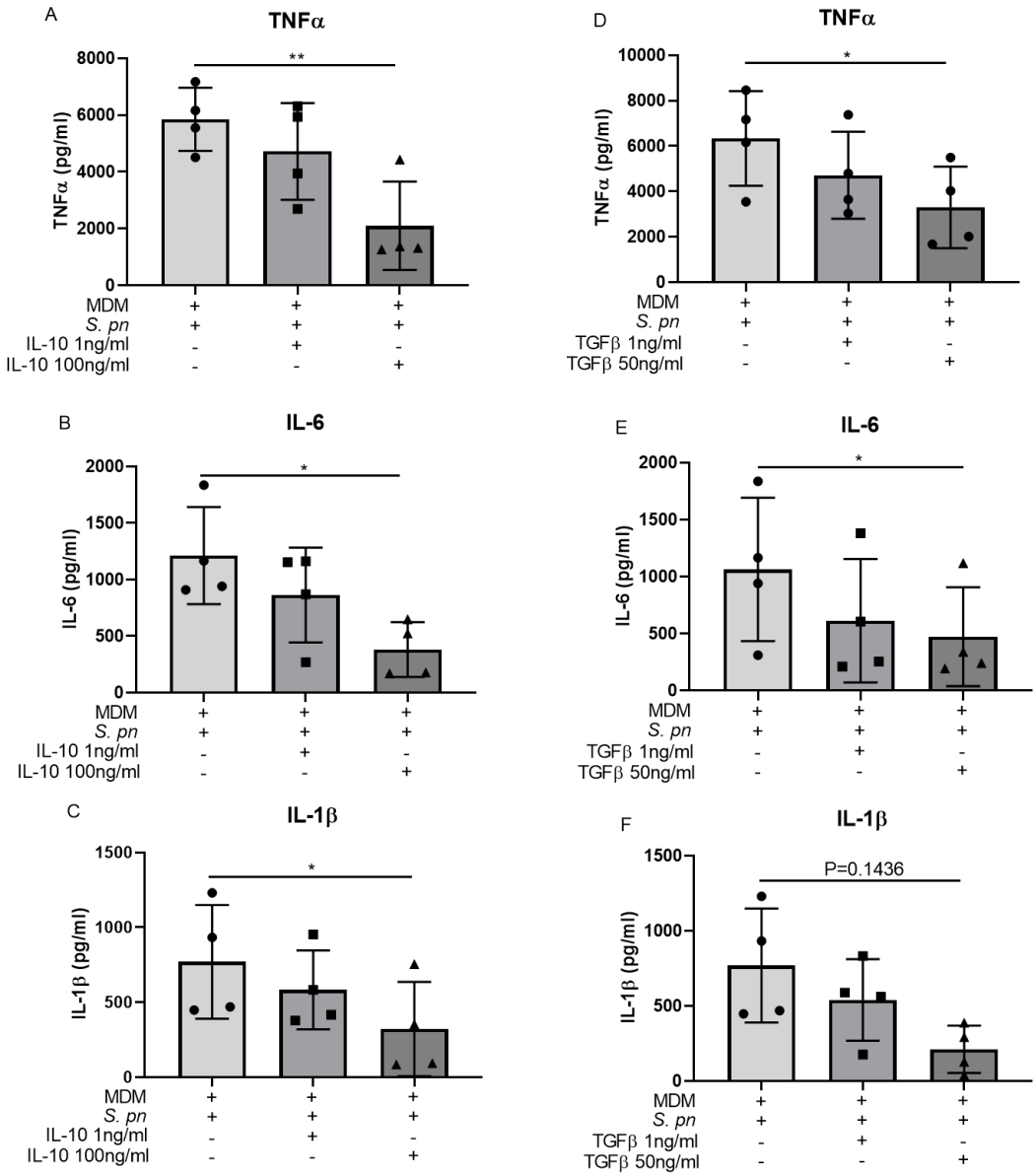

Figure 5.2 TNFα, IL-6 and IL-1β production by MDMs incubated with exogenous IL-10 or TGFβ and infection with *S. pneumoniae*

MDMs were incubated for 1 hour with either IL-10 at 1 ng/ml or 100 ng/ml IL-10 (A, B, and C) or with TGFβ at 1 ng/ml or 50ng/ml (D, E, and F) before infection of the MDMs with MOI 10 TIGR4 *S. pneumoniae* for 6 hours, then analysis of the supernatants for the concentration of TNFα (A and D), IL-6 (B and E) and IL-1β (C and F) by ELISA. Graphs show the mean concentration of cytokine measured from 4 biological replicates (cells taken from 4 donors) and error bars showing +/- SD of the mean. Data are paired across conditions for each donor. Statistical analysis was by repeated measures ANOVA (P<0.05 B, C, E; P<0.01 A, D; not statistically significant F) with Dunnett's multiple comparisons test (*P<0.05, **P<0.01).

5.3 IL-10 production during MDM-Treg/Teff cell co-culture

Due to the finding that IL-10 can suppress MDM inflammatory responses to *S. pneumoniae*, the production of IL-10 during MDM-Treg cell and MDM-Teff cell co-culture was investigated. Treg cells or Teff cells were either cultured with MDMs prior to infection then removed from the culture before infection of the MDMs with *S. pneumoniae*, or the Treg/Teff cells were added to the MDMs simultaneously with and remained present during infection with *S. pneumoniae*. Additional groups included uninfected MDMs cultured alone, MDMs infected in the absence of Treg/Teff cells, and MDMs cultured with Treg/Teff cells without infection. IL-10 in the supernatants was detected by ELISA at 24 hours and 48 hours post-infection.

By 24 hours, there was no statistically significant production of IL-10 in any conditions (figure 5.3 A). At 48 hours, IL-10 production was not increased by MDMs that had been previously co-cultured with Treg cells with or without *S. pneumoniae* infection compared to uninfected MDMs cultured alone (figure 5.3 B). In addition, IL-10 was not produced when Treg cells were not removed from the co-culture and remained present during infection (figure 5.3 B). The lack of IL-10 production during MDM-Treg cell co-culture suggests that secretion of IL-10 is not the mechanism by which Treg cells reduce MDM inflammatory responses upon infection.

In contrast, the supernatants from MDMs infected in the presence of Teff cells did contain a statistically significant concentration of IL-10 compared to unstimulated MDMs (figure 5.3 B). MDMs that had been cultured with Teff cells prior to infection but had no Teff cells present during the infection showed a slightly increased concentration of IL-10 compared to MDMs cultured alone, however this was short of statisitical significance at p=0.0706. Similarly, MDMs that had been previously cultured with Teff cells before removal of the Teff cells and continued incubation of the MDMs in the absence of Teff cells and *S. pneumoniae* also showed a slight but statistically not significant increase in IL-10 compared to MDMs cultured alone

(*p*=0.074, figure 5.3 B). These slight increases may be explained by either residual Teff cells from the prior co-culture remaining on the MDMs and producing IL-10, or that the MDMs had been stimulated to produce IL-10 by the presence of Teff cells, or a combination of both. IL-10 produced by either the MDMs or the Teff cells are also likely to promote a feedback loop in which further IL-10 is then produced by the MDMs, further suppressing the production of pro-inflammatory cytokine by the MDMs (Couper et al., 2008).

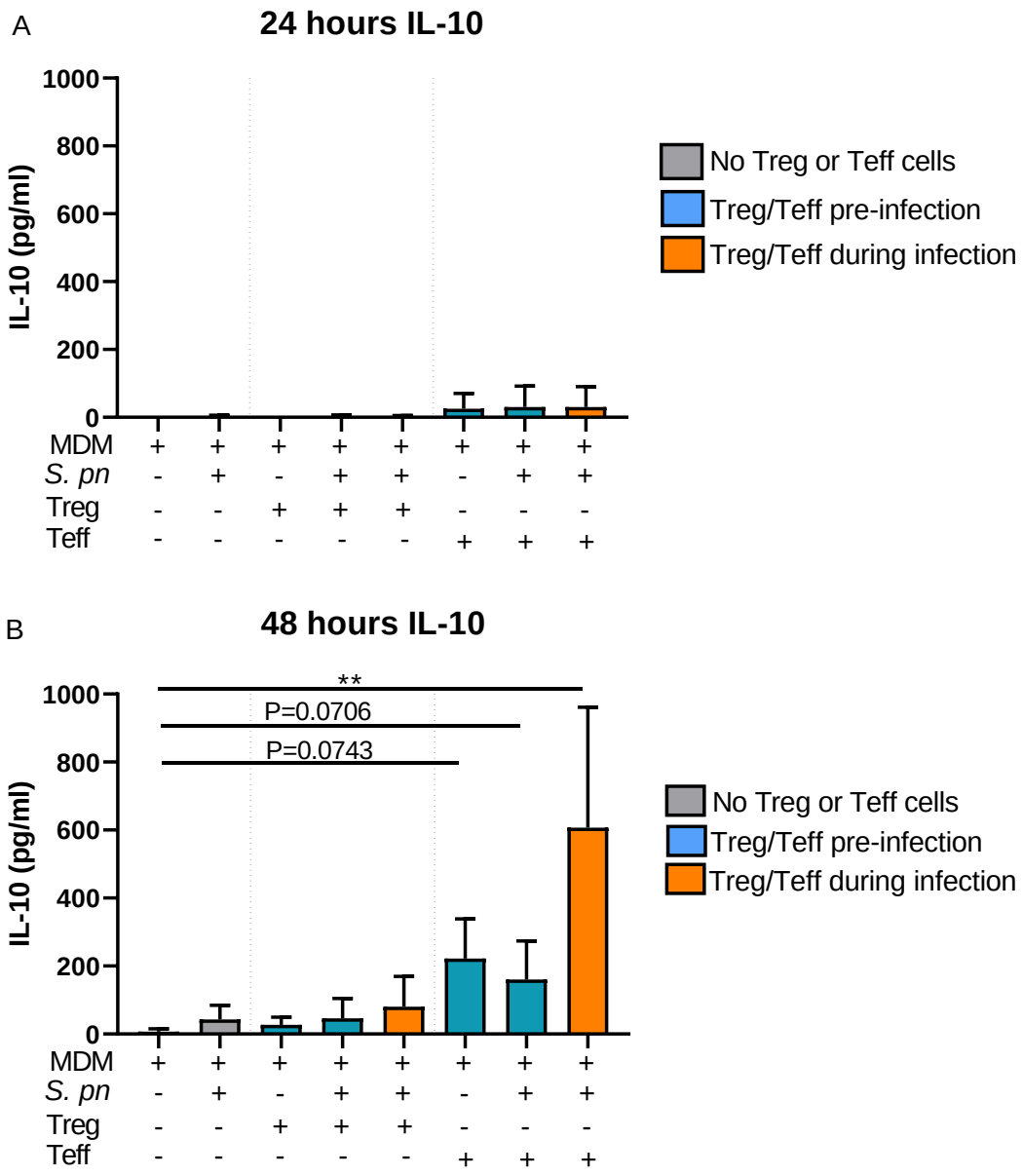

Figure 5.3 IL-10 production by MDM-Treg/Teff cell co-culture in the presence or absence of *S. pneumoniae* infection

MDMs were cultured with Treg or Teff cells before removal of the Treg/Teff cells and either infection of the MDMs with MOI 2 TIGR4 *S. pneumoniae* or continued culture of the MDMs for 48 hours without infection ("pre-infection"), or Treg/Teff cells were added to the MDMs simultaneously with *S. pneumoniae* infection ("during infection"). IL-10 concentration in the supernatants was measured at 24 hours (A) and 48 hours (B) post-infection/continued incubation. Graph shows the mean IL-10 concentration from 6 biological replicates (cells taken from 6 donors) with error bars showing +/- SD of the mean. Statistical analysis was by Kruskal-Wallis test (not statistically significant in A, P<0.01 in B) with Dunn's multiple comparisons test (**P<0.01).

5.4 TGFβ concentration in MDM-Treg/Teff co-culture

As exogenous addition of TGFβ decreased production of inflammatory cytokines by MDMs after infection with *S. pneumoniae* (figure 5.2), the production of TGFβ in the MDM-Treg/Teff cell co-culture was investigated as a possible mechanism for suppression of MDM inflammatory responses to *S. pneumoniae* by Treg cell co-culture. MDMs were cultured in the presence of Treg or Teff cells for 14 hours before analysis of the supernatants for TGFβ concentration by ELISA. However, a large concentration of TGFβ was present in the supernatant of unstimulated MDMs, perhaps due to either pre-existing TGFβ in the serum supplementing the medium or by MDM production of TGFβ, and these concentrations did not statistically differ significantly from the supernatants taken from MDMs cultured in the presence of Treg or Teff cells (figure 5.4).

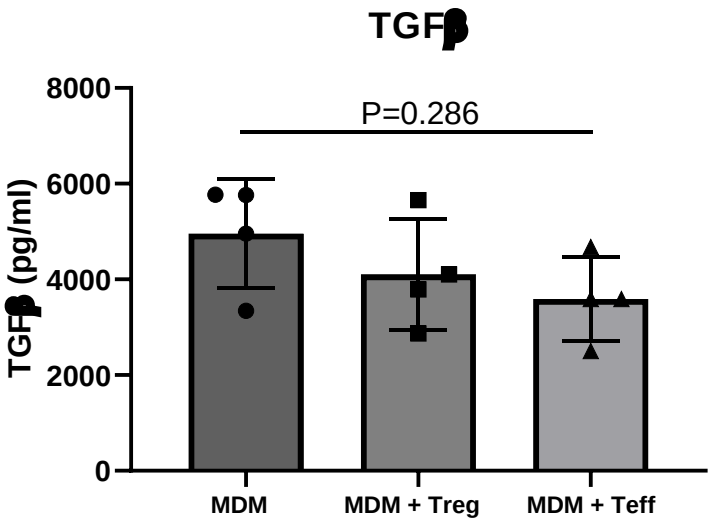

Figure 5.4 TGFβ in supernatants from MDMs cultured in the presence or absence of Treg or Teff cells

MDMs were cultured in the presence of Treg or Teff cells for 14 hours before analysis of the supernatants for TGFβ concentration by ELISA. Graph represents the mean of technical replicates taken from 4 wells per condition with cells from 1 donor. Error bars showing +/- SD of the mean. The experiment was repeated twice with similar results. Statistical analysis was by Kruskal-Wallis (not statistically significant) with Dunnett's multiple comparisons test (not statistically significant between any conditions).

5.5 FACS-sorted Treg cell and Teff cell cytokine production

Flow cytometry was used to investigate the potential cytokine production of FACS-sorted Treg and Teff cells during MDM co-culture. Treg or Teff cells were incubated on MDMs for 14 hours then stimulated with PMA, ionomycin and Golgistop and stained for IL-2, IL-10, IL-17A and IFNγ.

IL-2 expression was statistically significantly increased by Teff cells but not by Treg cells compared to unstimulated $CD4^+$ T cells (figure 5.5 A). Similarly, IL-10 and IL-17A expression was increased by Teff cells but not by Treg cells compared to unstimulated $CD4^+$ T cells (figure 5.5 B and C). Expression of IFNγ by Teff cells was close to statistical significance compared to unstimulated $CD4^+$ T cells at p=0.08, with large variation in expression between donors (figure 5.5 D). Overall, sorted Treg cells do not make express statistically significant levels of these cytokines, whereas Teff cells express high levels of IL-10 and IL-17A, a modest but statistically significant level of IL-2, and variable levels of IFNγ.

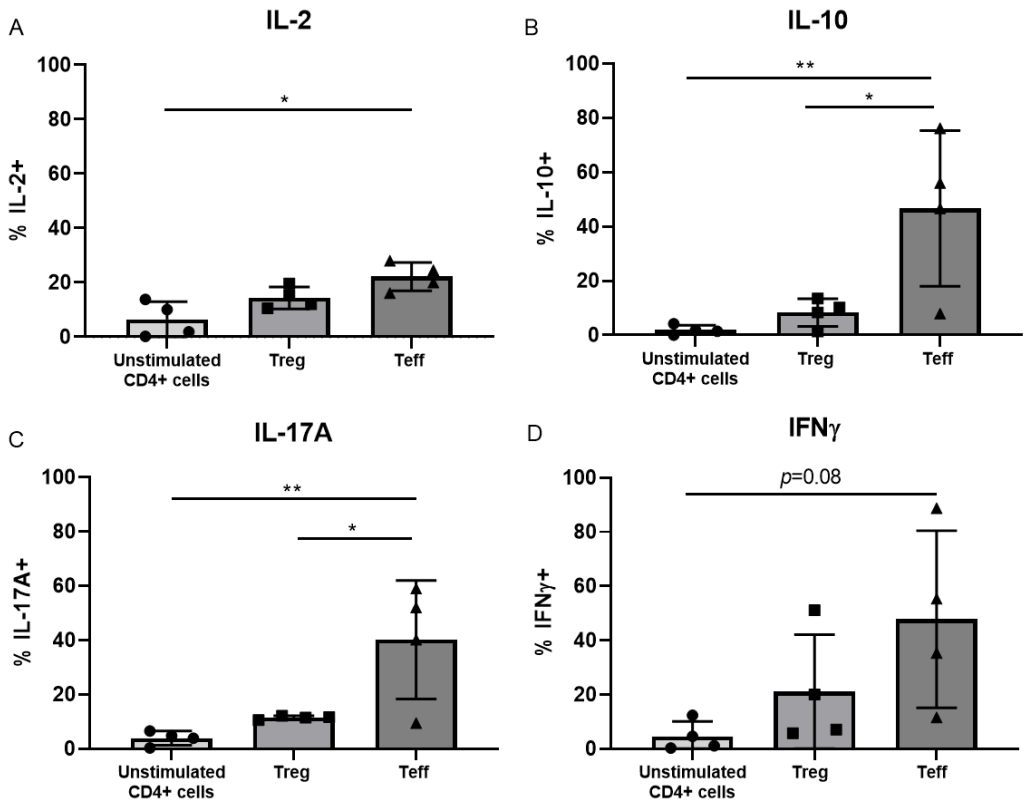

Figure 5.5 IL-2, IL-10, IL-17A and IFNγ production by FACS-sorted Treg and Teff cells cultured on MDMs and stimulated with PMA, ionomycin and Golgistop

FACS-sorted Treg and Teff cells were incubated on MDMs for 14 hours followed by addition of with PMA, ionomycin and Golgistop for 6 hours and staining for IL-2, IL-10, IL-17A and IFNγ for analysis by flow cytometry. Expression of these cytokines by Treg and Teff cells were compared against unstimulated $CD4^+$ cells with gating determined by fluorescent minus one (FMO) controls. Graphs represent mean percent expression of cytokine from 4 biological replicates (cells taken from 4 donors) with error bars showing +/- SD of the mean. Statistical analysis was by one-way ANOVA (not statistically significant in D; $P<0.05$ A; $P<0.01$ B and C) with Tukey's multiple comparisons test (*$P<0.05$; **$P<0.01$).

5.6 Effect of MDM-Treg/Teff cell co-culture supernatant on MDM inflammatory responses to *S. pneumoniae*

To investigate the possibility that any secreted cytokines produced through MDM-Treg cell co-culture caused suppression of MDM inflammatory responses after *S. pneumoniae* infection, MDMs were cultured in the supernatants from previous MDM-Treg/Teff cell co-culture then infected with *S. pneumoniae*. MDMs were co-cultured with Treg or Teff cells for 14 hours, the resulting supernatant removed and added to fresh MDMs for a further 14 hours, followed by removal of the supernatant and infection of these MDMs with *S. pneumoniae* in fresh medium. After 6 hours infection followed by addition of penicillin-streptomycin and further incubation up to 72 hours, the supernatants were collected and analysed for TNFα, IL-6 and IL-1β concentration by ELISA. The results showed no difference in concentration of TNFα (figure 5.6 A, B and C), IL-6 (figure 5.6 D, E and F) or IL-1β (figure 5.6 G, H and I) at any time point between MDMs cultured alone and MDMs cultured in the presence of MDM-Treg/Teff cell co-culture supernatant. This suggests that cytokines produced as a result of MDM-Treg cell co-culture are not required for the suppression of MDM inflammatory responses to *S. pneumoniae* seen in part 4.4.

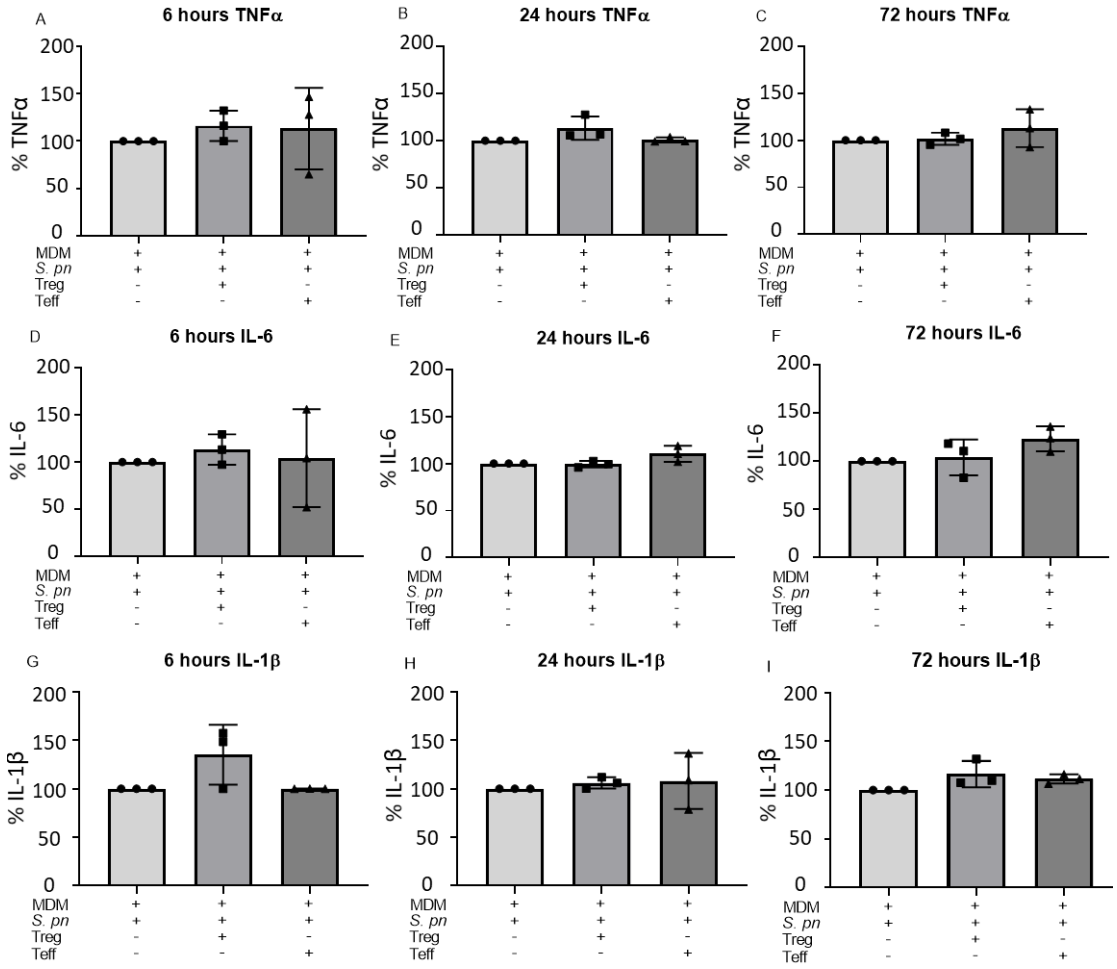

Figure 5.6 TNFα, IL-6 and IL-1β production by MDMs incubated in supernatants from MDM-Treg/Teff cell co-culture upon infection with *S. pneumoniae*

MDMs were incubated for 14 hours in the supernatant from a previous MDM-Treg/Teff co-culture from the same donor then infected with MOI 2 TIGR4 *S. pneumoniae* in fresh medium for 6 hours followed by addition of penicillin-streptomycin and further incubation up to 72 hours. The supernatants were collected and analysed for TNFα (A, B and C), IL-6 (D, E and F) and IL-1β (G, H and I) concentration by ELISA at 6 hours, 24 hours and 72 hours post-infection. Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Graphs show mean concentrations detected from 3 biological replicates (cells taken from 3 donors) with error bars showing +/- SD of the mean. Statistical analysis was by one-way ANOVA (not statistically significant) with Tukey's multiple comparisons test (not statistically significant between any conditions).

5.7 Effect of co-culturing MDMs with Treg/Teff cells separated by a transwell insert on MDM inflammatory responses to *S. pneumoniae*

To confirm whether direct contact between the MDMs and the Treg cells was necessary for suppression of MDM inflammatory responses to *S. pneumoniae*, the experiment in part 4.4 was repeated with MDMs cultured with Treg or Teff cells separated from the MDMs by transwell inserts with 3μm pores, allowing passage of cytokines but not cells. After 14 hours, the MDMs were then infected with *S. pneumoniae*. Supernatants were taken at 6 hours, 24 hours and 72 hours post-infection for measurement of TNFα, IL-6 and IL-1β concentrations by ELISA.

The results showed that there was no suppression of TNFα (figure 5.7 A, B and C), IL-6 (figure 5.7 D, E and F), or IL-1β (figure 5.7 G, H and I) at any of the measured time points when comparing infected MDMs cultured alone and MDMs co-cultured with Treg cells separated by a transwell insert. Additionally, no statistically significant differences were seen in the concentrations of TNFα, IL-6 or IL-1β when the MDMs had been co-cultured with Teff cells prior to infection compared to MDMs cultured alone or MDMs co-cultured with Treg cells. The experiments were repeated with the additional step of pre-incubating the Treg and Teff cells in contact with MDMs prior to the 14 hour transwell incubation, in order to ensure that the lack of effect seen with transwell-separated Treg cells was not due to lack of MDM activation that may have occurred in previous experiments, and showed similar results to those in figure 5.7. These results, in addition to the results of the Treg-MDM co-culture supernatant experiments in 5.6, suggest that direct contact between MDMs and Treg cells is required in order for the suppressive effect seen on MDM inflammatory responses to *S. pneumoniae* seen in part 4.4.

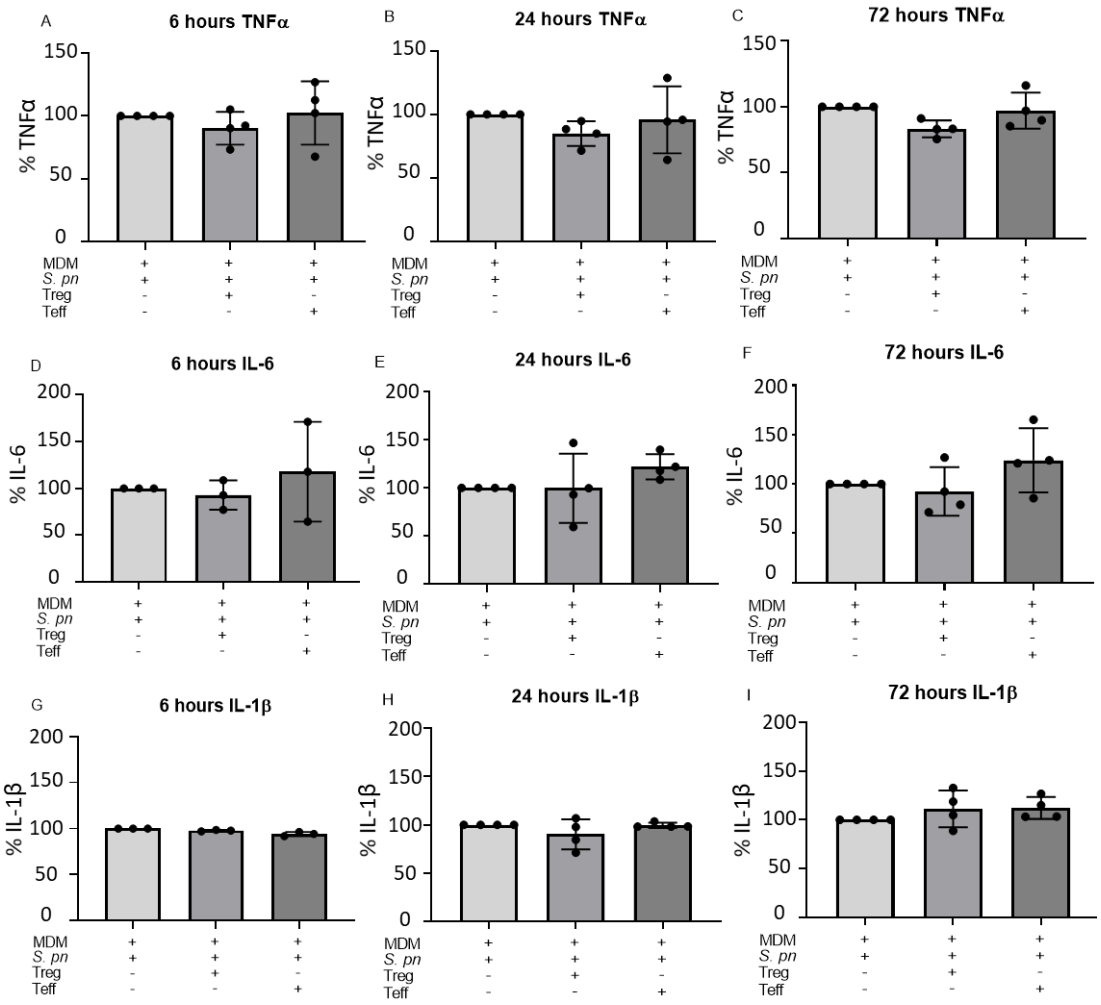

Figure 5.7 TNFα, IL-6 and IL-1β production by *S. pneumoniae*-infected MDMs previously co-cultured in the presence or absence of Treg or Teff cells separated by a transwell insert

MDMs were incubated in the presence or absence of Treg or Teff cells separated from the MDMs by a 3μm-pore transwell insert for 14 hours, before removal of the Treg/Teff cells and infection of the MDMs with MOI 2 TIGR4 *S. pneumoniae* for 6 hours, followed by continued incubation to 72 hours post-infection. Supernatant samples were taken at 6 hours, 24 hours and 72 hours for the detection of TNFα (A, B and C), IL-6 (D, E and F) and IL-1β (G, H and I). Data are converted from the absolute cytokine concentrations measured into a percentage of the concentrations detected in the MDM + *S. pneumoniae* group (without Treg/Teff cells). Graphs show the mean ratio of cytokine measured from 4 biological replicates (cells taken from 4 donors) with error bars showing +/- SD of the mean. Data are paired across conditions for each donor. Statistical analysis was by Friedman test (not statistically significant) with Dunn's multiple comparisons test (not statistically significant).

5.8 Measuring latency associated peptide expression by Treg cells

As direct contact between the Treg cells and the MDMs is necessary for suppression of MDM inflammatory responses to *S. pneumoniae*, the next aim was to determine which mechanism was required for this suppression. Due to evidence implicating TGFβ in Treg cell mediated prevention of susceptibility to invasive pneumococcal pneumonia in mice (Neill *et al*., 2012), that MDMs can induce Treg cells that suppress Th1 cell proliferation through surface TGFβ (Savage *et al*., 2008), and the results in part 5.1 showing exogenous addition of TGFβ to MDMs reduced their inflammatory response to *S. pneumoniae*, the first mechanism explored was the interaction of Treg cell surface-bound TGFβ with MDMs.

The expression of TGFβ on the surface of Treg cells was measured by staining for LAP, part of the TGFβ complex. PBMCs were stained for CD4, CD25 and CD127 as per the protocol for Treg/Teff cell sorting and additionally stained for LAP based on an FMO control (figure 5.8 A). The expression of LAP by $CD25^{+}CD127^{low}$ Treg cells and $CD25^{-}CD127^{high}$ Teff cells were compared. Treg cells had a mean LAP^{+} expression of 8.2%, whereas fewer Teff cells expressed LAP with a mean of 1.7% (figure 5.8 B). Savage *et al*. reported similar percentages of LAP expression of 3-16% by their induced Treg cell line, which were capable of suppressing the proliferation of their Th1 cell line in a surface TGFβ-dependent manner, suggesting that this proportion is capable of suppression (Savage *et al.*, 2008).

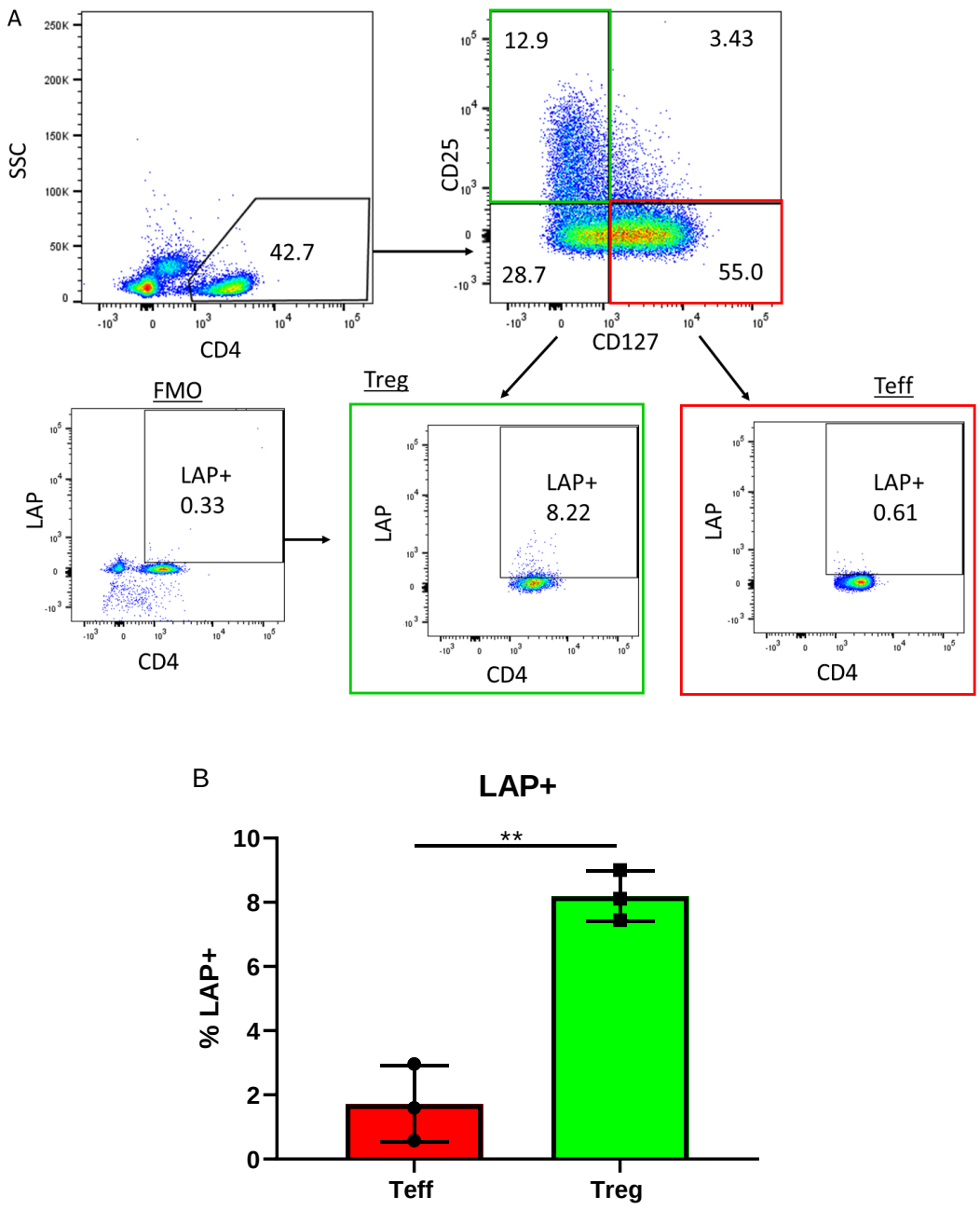

Figure 5.8 LAP expression by CD25^{+}CD127^{low} Treg and CD25^{-}CD127^{+} Teff cells

PBMCs were stained for CD4, CD25, CD127 and LAP (A) and expression of LAP by CD25^{+}CD127^{low} Treg cells and CD25^{-}CD127^{+} Teff cells were compared as determined by the FMO control (B). Graph B shows the mean percentage of LAP^{+} expressing CD4^{+} cells in the Treg and Teff populations from 3 biological replicates (cells taken from 3 donors) with error bars showing +/- SD of the mean. Statistical analysis was by unpaired t test (**P<0.01).

5.9 Effect of neutralising LAP during MDM-Treg/Teff cell co-culture on the inflammatory response of MDMs to *S. pneumoniae*

Neutralisation of LAP by anti-LAP antibody has been found to reduce the suppressive ability of Treg cells and increase Th17 generation in a mouse model of tuberculosis infection (Ye et al., 2011). Therefore, the effect of neutralising LAP during MDM-Treg cell co-culture on MDM inflammatory responses to *S. pneumoniae* was examined, with the hypothesis that the suppressive ability of the Treg cells on the macrophage inflammatory response would be inhibited. MDMs were cultured in the presence or absence of Treg or Teff cells with or without 2μg/ml neutralising anti-LAP antibody, before removal of the Treg/Teff cells and infecting the MDMs in fresh medium. The bacteria were killed after a 6 hours infection by addition of penicillin-streptomycin followed continued incubation of the MDMs for 72 hours. Supernatant samples taken at 6-, 24- and 72 hours post-infection were analysed for concentration of TNFα, IL-6, IL-1β by ELISA. The results were compared against infected MDMs that had been cultured in the absence of Treg or Teff cells or anti-LAP antibody.

Neutralisation of LAP during MDM-Treg cell co-culture did not result in any differences in TNFα production by the MDMs compared to the Treg- co-cultured MDMs without anti-LAP at any of the tested time points (figure 5.9 A, B and C). However, anti-LAP antibody suppressed MDM production of TNFα in the absence of Treg/Teff cell cells, with statistically significant reductions in supernatant TNFα at 6 hours and 24 hours (figure 5.9 A, B and C). The opposite effect was seen in co-culture of MDMs with Teff, with addition of anti-LAP increasing TNFα production by infected MDMs at 6 hours and 24 hours post-infection (figure 5.9 A and B). IL-6 production by MDMs cultured without Treg/Teff cells did not significantly differ statistically in the presence of the anti-LAP antibody compared to absence of anti-LAP, although the trend showed a decrease in the concentration of IL-6 by MDMs in the presence of anti-LAP compared to absence of anti-LAP (figure 5.9 D, E and F). At 24 hours and 72 hours post-infection

there were statistically significant decreases in IL-6 production by infected MDMs co-cultured with Treg cells compared to MDMs cultured in the absence of Treg cells or anti-LAP antibody. These differences were lost when the MDMs were co-cultured with Treg cells in the presence of anti-LAP antibody (figure 5.9 E and F). The largest difference can be seen at 72 hours, where IL-6 production of MDMs co-cultured in the presence of Treg cells was increased in the presence of anti-LAP compared to in the absence of anti-LAP (figure 5.9 F). IL-6 production by MDMs cultured in the presence of Teff cells increased with addition of anti-LAP compared to without the anti-LAP antibody at all time points measured (figure 5.9 D, E and F).

IL-1β production was too low for accurate detection at 6 hours (not shown), and showed no statistically significant differences between conditions at 72 hours (figure 5.9 H). At 24 hours post-infection, there was no statistically significant difference effects of anti-LAP antibody on production of IL-1β by infected MDMs (figure 5.9 G). MDMs that had been cultured in the presence of Treg cells both in the absence and in the presence of anti-LAP antibody, showed a statistically significant decrease in IL-1β production compared with MDMs that had been cultured alone in the absence of anti-LAP antibody. However, this was less statistically significant when the MDMs were cultured with anti-LAP (figure 5.9 G). There was no statistically significant difference in IL-1β production by MDMs cultured in the presence of Teff cells in the absence of anti-LAP antibody compared with those cultured in the presence of anti-LAP antibody at P=0.1807 (figure 5.9 G), although a trend towards increased IL-1β production in the presence of anti-LAP could be seen which is similar to the results for TNFα and IL-6.

To summarise these results, TNFα production by MDMs seemed to be affected by the anti-LAP antibody regardless of the presence of Treg or Teff cells, and anti-LAP did not affect suppression of TNFα production by Treg cell co-culture. Anti-LAP did however cause an increase in the TNFα production by MDMs that had been co-

cultured with Teff cells. The results for IL-6 suggested that the presence of anti-LAP antibody prevented Treg cell suppression of IL-6 production by MDM at 72 hours post-infection, and also increased IL-6 production by MDMs co-cultured with Teff cells at all time points. Lack of Treg ability to suppress IL-6 with anti-LAP treatment may be a factor influencing increased Th17 as shown by Ye and colleagues (Ye et al, 2011). The presence of anti-LAP antibody had no statistically significant effect on IL-1β production by MDMs cultured alone, MDMs co-cultured with Treg cells, or MDMs co-cultured with Teff cells. This experiment was only performed on cells from one donor with 5 technical replicates; therefore, to confirm these findings the experiment should be repeated with cells from further donors.

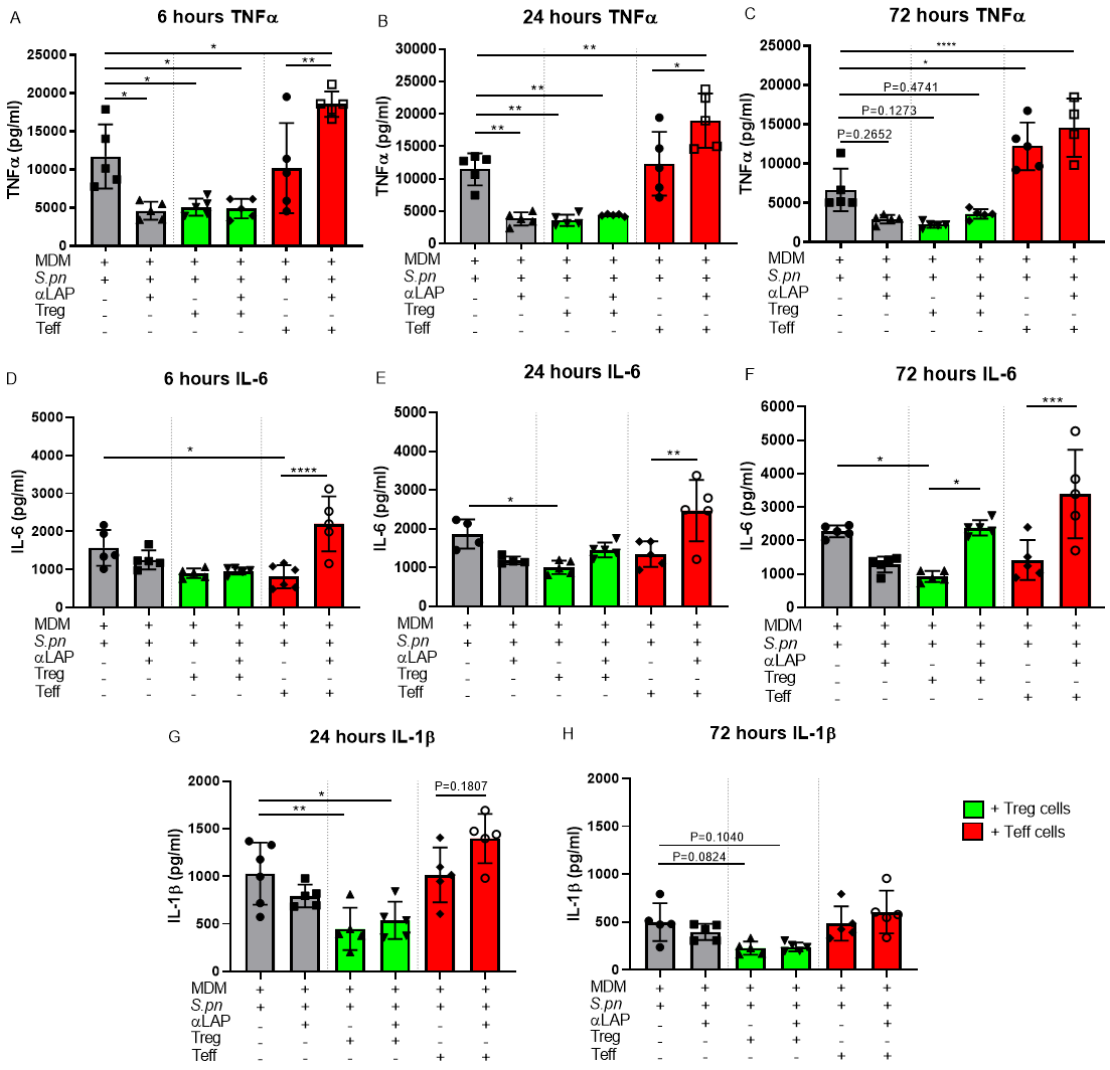

Figure 5.9 TNFα, IL-6 and IL-1β production by *S. pneumoniae*-infected MDMs cultured in the presence or absence of Treg or Teff cells with or without an anti-LAP neutralising antibody

MDMs were cultured in the presence or absence of Treg or Teff cells with or without an anti-LAP neutralising antibody, before removal of the Treg/Teff cells and infection of the MDMs with MOI 2 TIGR4 *S. pneumoniae* in fresh medium without anti-LAP. Supernatant samples were taken at 6 hours (A and D), 24 hours (B, E and G) and 72 hours (C, F, and H) post-infection for detection of TNFα (A, B and C), IL-6 (D, E and F) and IL-1β by ELISA (G and H). 6 hours IL-1β data were omitted due to undetectable IL-1β levels. Graphs show the mean concentration of cytokine detected from 5 technical replicates from 1 donor (a sample each from 5 wells per condition containing cells from 1 donor) with error bars showing +/- SD of the mean. Statistical analysis was by one-way ANOVA (p<0.01 H; p<0.001 E; p<0.0001 A, B, C, D, F, G) with Tukey's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.10 Effect of blocking MDM TGFβ receptors during MDM-Treg/Teff cell co-culture on TNFα, IL-6 and IL-1β production by *S. pneumoniae*-infected MDMs

Activin receptor-like kinase 5 (ALK5) is a TGFβ-1 receptor present on the surface of activated macrophages (Chen *et al*., 2008). To test the hypothesis that Treg cells are suppressing MDM inflammatory responses to *S. pneumoniae* through Treg surface TGFβ, MDM-Treg/Teff co-culture experiments were repeated whilst blocking ALK5 to assess whether the suppressive effect seen with Treg cell co-culture is lost. MDMs were cultured in the presence or absence of Treg or Teff cells with or without the addition of an ALK5 inhibiting antibody (ALK5i) for 14 hours, before removal of the Treg/Teff cells and infection of the MDMs with *S. pneumoniae* in fresh medium. The bacteria were killed after 6 hours by addition of penicillin-streptomycin and the MDMs allowed to incubate until 72 hours post-infection. Supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for the detection of TNFα, IL-6 and IL-1β by ELISA.

Overall, the results showed that addition of ALK5i had little effect on MDM inflammatory responses to *S. pneumoniae*. The presence of Treg cells during culture resulted in statistically significant reductions in TNFα, IL-6 and IL-1β production by the MDMs at 24 hours (figure 5.10 B, E and G) and 72 hours (figure 5.10 C, F and H) post-infection, however this suppression was not lost when the MDM-Treg cell co-culture had been additionally incubated with ALK5i, suggesting that blocking the MDM ALK5 receptor does not result in loss of Treg-mediated suppression. Contrary to the trend seen in part 5.9 where TNFα, IL-6 and IL-1β production by MDMs cultured without Treg/Teff cells was generally decreased by the neutralising anti-LAP antibody (figure 5.9), production of these cytokines was statistically not significantly increased by ALK5 inhibition of MDMs cultured in the absence of Treg/Teff cells. ALK5 inhibition also had little to no effect on the production of TNFα, IL-6 or IL-1β by MDMs co-

cultured with Teff (figure 5.10), whereas anti-LAP increased these responses for MDMs co-cultured with Teff (figure 5.9).

The experiment was repeated once with similar results; however further repeats should be attempted before confirming that blocking the ALK5 receptor has no effect on Treg cell suppression of the MDMs.

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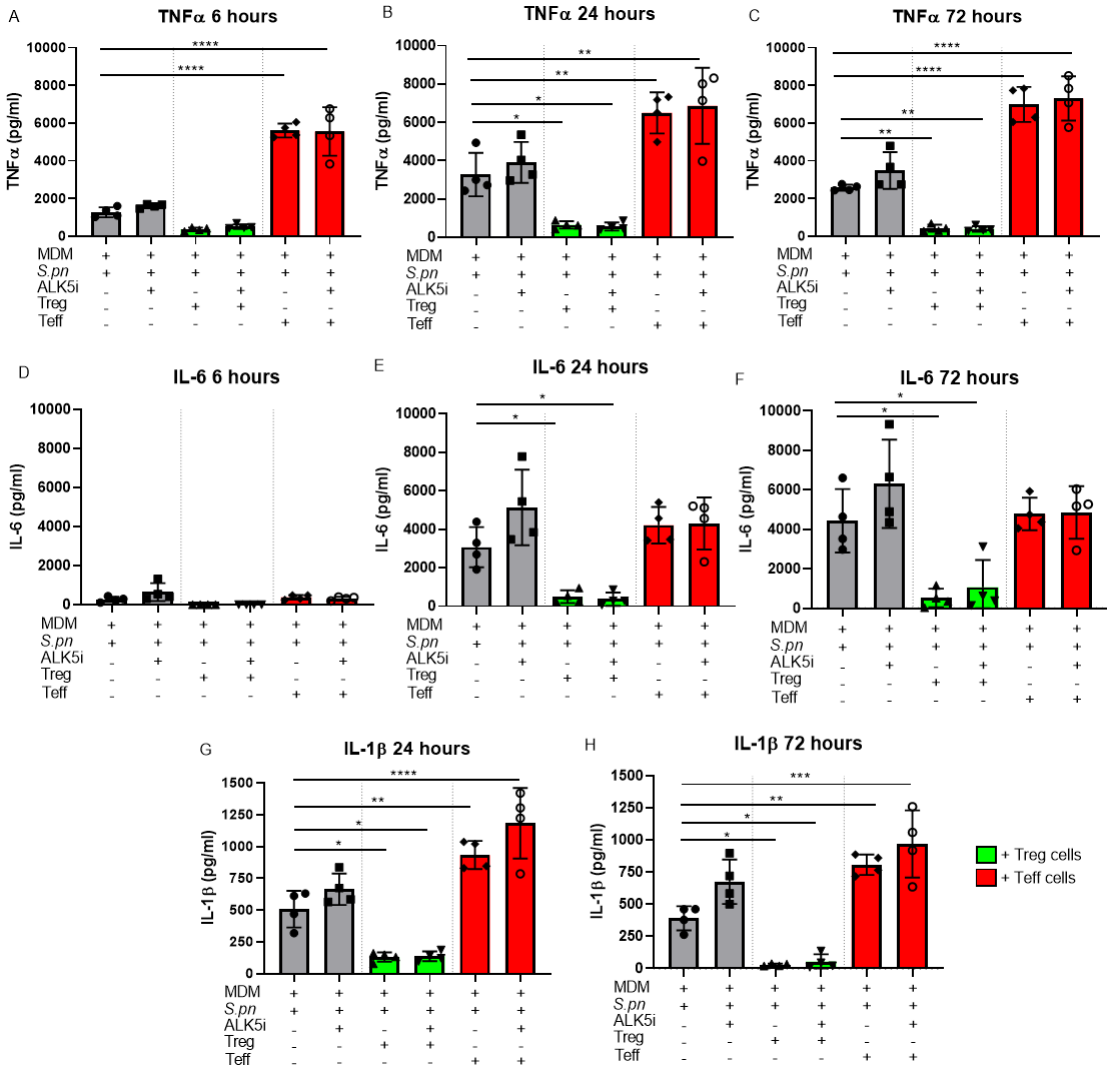

Figure 5.10 TNFα, IL-6 and IL-1β production by *S. pneumoniae*-infected MDMs cultured in the presence or absence of Treg or Teff cells with or without an ALK5 neutralising antibody

MDMs were cultured in the presence or absence of Treg or Teff cells with or without 1µg/ml ALK5 neutralising antibody (ALK5i), before removal of the Treg/Teff cells and infection of the MDMs with MOI 2 TIGR4 *S. pneumoniae* in fresh medium. Supernatant samples were taken at 6 hours, 24 hours and 72 hours post-infection for detection of TNFα (A-C), IL-6 (D-F) and IL-1β (G, H) by ELISA. 6 hours IL-1β data were omitted due to undetectable IL-1β levels. Graphs show the mean concentration of cytokine detected from 4 technical replicates from 1 donor (a sample each from 4 wells per condition containing cells from 1 donor), with error bars showing +/- SD of the mean. Statistical analysis was by one-way ANOVA ($P<0.0001$ A-C and E-G) with Tukey's multiple comparisons test (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$), excluding D which due to non-normality of the data was analysed by Kruskal-Wallis ($P<0.01$) with Dunn's multiple comparisons test (not statistically significant).

5.11 Cytokine production and CD25 and Foxp3 expression by $CD4^+$ cells incubated in the presence of MDMs

The experiments so far have used $CD4^+CD25^+CD127^{low}$ Treg cells sorted directly from the blood of volunteers. Observations so far have shown that, although these Treg cells suppress MDM inflammatory responses in a cell-contact dependent manner and do not make IL-10, MDM-Teff cell co-culture results in IL-10 production, a cytokine which is capable of reducing MDM pro-inflammatory cytokine production upon *S. pneumoniae* infection. Therefore, the effects of the MDM co-culture system on the cytokine production of $CD4^+$ T cells and their expression of CD25 and Foxp3 was examined.

MACS-purified $CD4^+$ cells were either stained immediately or were incubated with MDMs for 48 hours followed by a 6 hour incubation with Golgistop. Cells were stained for IL-2, IL-10, IL-17A, IFNγ and TNFα and analysed by flow cytometry. All cytokines showed increased expression by $CD4^+$ cells after incubation with MDMs, with a mean of 4% of $CD4^+$ cells expressing IL-10, 0.6% IL-17A, 9% TNFα, 3% IL-2, and 6% IFNγ at 48 hours (figure 5.11.1 A). Of note, after 48 hours $CD4^+$ T cell expression of most cytokines was highly variable between donor cells, for example TNFα expression varied between 0.4% and 20% between donors. Although trends towards increased cytokine production from 0 hours to 48 hours with MDM co-culture, there were no statistically significant increases in any of the cytokines tested (figure 5.11.1 B).

Collectively, these results show that both IL-10 and a range of pro-inflammatory cytokines are produced by $CD4^+$ T cells in co-culture with MDMs.

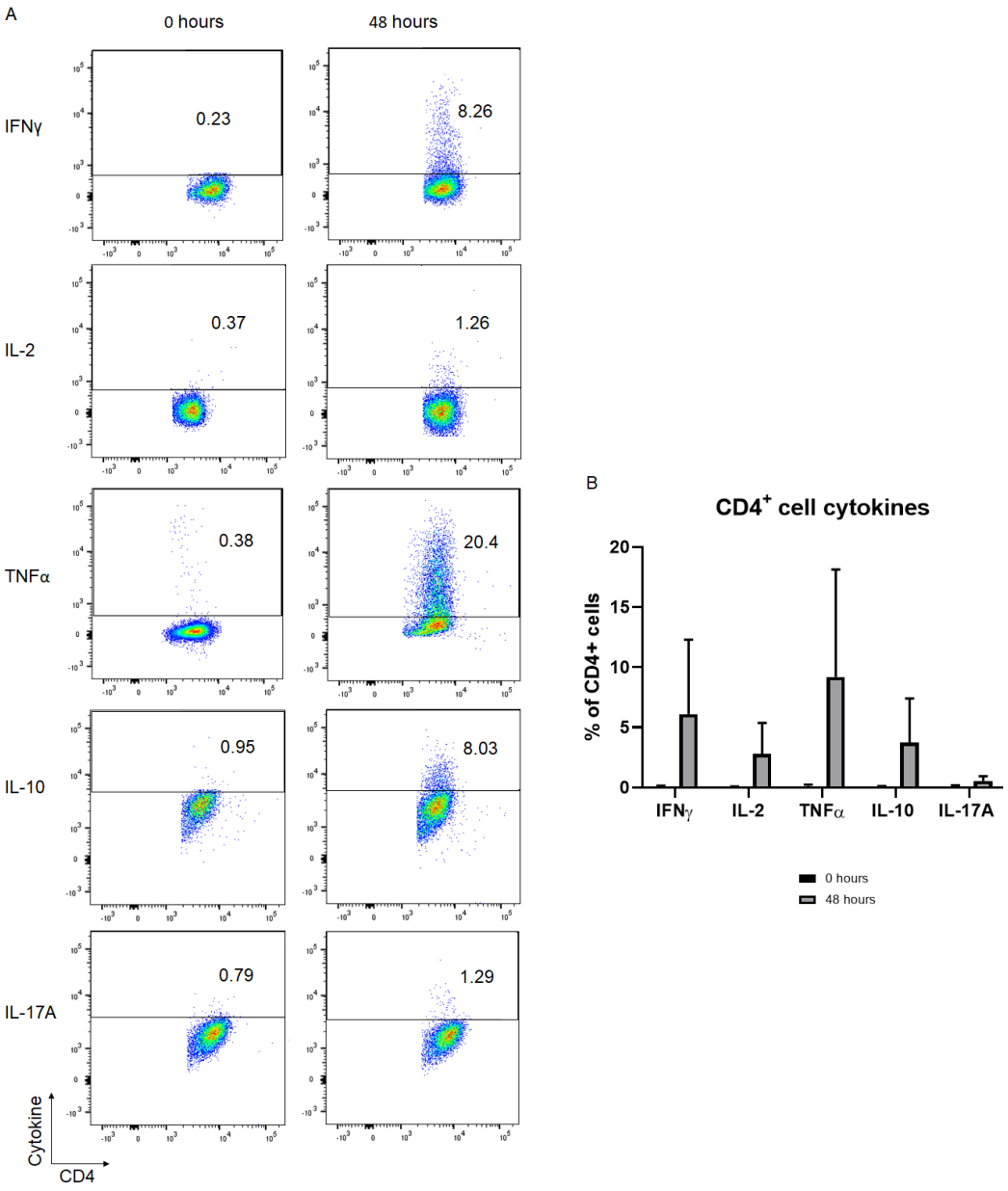

Figure 5.11.1 Cytokine expression by CD4⁺ cells incubated with MDMs

Expression of IFNγ, TNFα, IL-2, IL-10 and IL-17A by CD4 cells with or without 48 hour MDM co-culture (A) and the mean percentage of CD4⁺ cells expressing each cytokine from 4 biological replicates (cells taken from 4 donors) +/- SD of the mean (B). Cytokine-positive populations were gated based on visibly higher expression of fluorochrome than the majority population with the same gate used for the infected and uninfected group per cytokine. Statistical analysis was by two-way ANOVA (interaction factor not significant, row factor (cytokine) not significant, row factor (time) $p<0.001$) with Sidak's multiple comparisons test (not significant between any condition).

The supernatants taken from the 48 hour MDM-$CD4^+$ T cell co-culture were analysed for concentrations of the cytokines TNFα, IL-6, IL-10, TGFβ and IL-17A by ELISA. Undetectable or very low levels of all tested cytokines except TGFβ were present in the supernatants of MDMs cultured without $CD4^+$ T cells (figure 5.11.2). By contrast, TNFα, IL-6, IL-10, TGFβ and IL-17A were all detected in the supernatants of MDM-$CD4^+$ T cell co-culture (figure 5.11.2). TNFα and IL-10 were increased in the supernatants of MDM-$CD4^+$ T cell co-culture compared to MDMs cultured without $CD4^+$ T cells. TGFβ concentration was similar in both culture conditions and had the highest concentrations of all the cytokines tested.

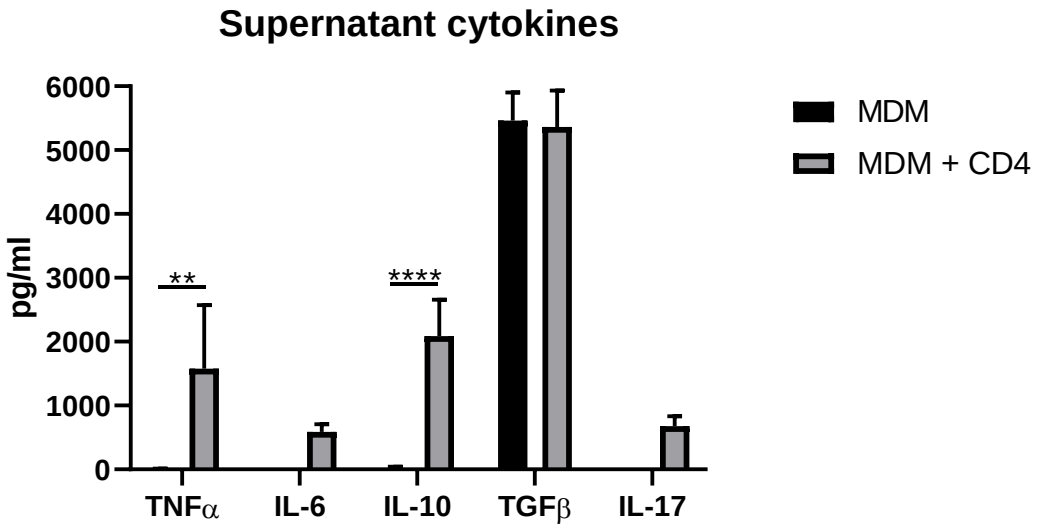

Figure 5.11.2 Cytokine concentrations in the supernatant of MDMs cultured with or without CD4$^+$ T cells

MDMs were cultured for 48 hours in the presence or absence of CD4$^+$ T cells. The supernatants were analysed for the concentration of TNFα, IL-6, IL-10, TGFβ and IL-17 by ELISA. Graph shows mean concentrations from 3 biological replicates (cells taken from 3 donors) with error bars showing +/- SD of the mean. Statistical analysis was by two-way ANOVA (interaction factor $p<0.01$) with Sidak's multiple comparisons test (**$p<0.01$; ****$p<0.0001$).

The expression of CD25 and Foxp3 by $CD4^+$ T cells cultured with MDMs for 48 hours was investigated. $CD4^+$ T cells incubated without MDMs showed little difference in CD25 and CD127 expression, whereas $CD4^+$ cells cultured in the presence of MDMs showed an increase in expression of CD25 and reduction in expression of CD127, with approximately 55% of $CD4^+$ cells falling into the $CD25^+CD127^{low}$ gate (figure 5.11.3 A). A distinct population of $CD25^+Foxp3^+$ cells was identified upon MDM-$CD4^+$ T cell co-culture, constituting approximately 15% of all $CD4^+$ T cells (figure 5.11.3 B).

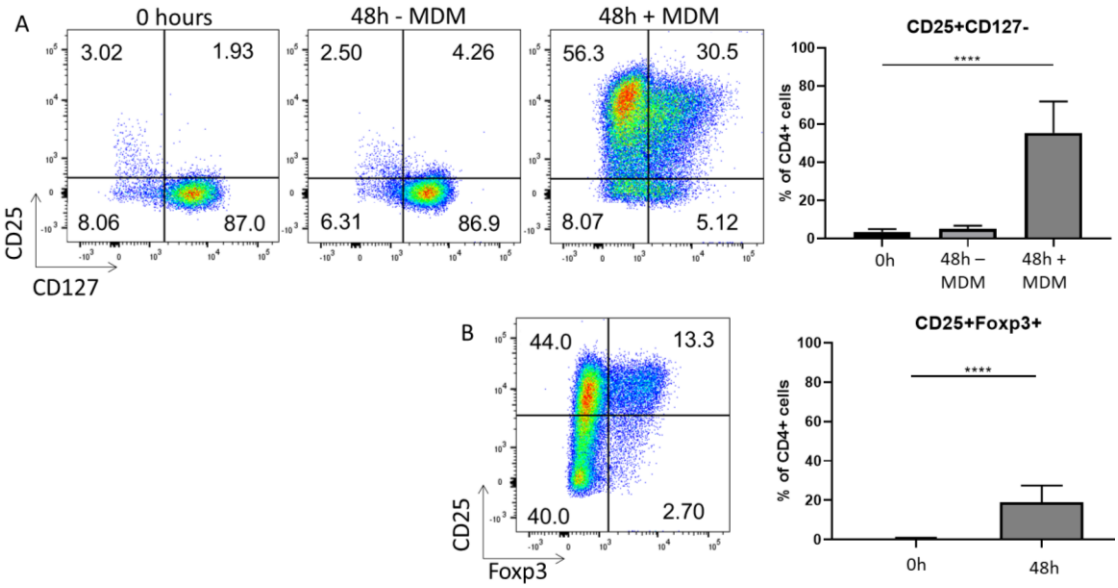

Figure 5.11.3 CD25, CD127 and Foxp3 expression by CD4$^+$ T cells after incubation with MDMs

MACS purified CD4$^+$ T cells were incubated in the presence or absence of MDMs for 48 hours then stained for CD25, CD127 and Foxp3 expression. The percentage of CD25$^+$CD127^{low} cells after 48 hours incubation in the presence or absence of MDMs was compared (A), and a population expressing high levels of Foxp3 and CD25 after 48 hours incubation with MDMs was compared with that at 0 hours was identified (B). Gating for the CD25$^+$CD127^{low} and CD25$^+$Foxp3$^+$ populations was determined visually and applied uniformly across all donors. Graphs show the mean percentage from 7 biological replicates (cells taken from 7 donors), with error bars showing +/- SD of the mean. Statistical analysis in A was by one-way ANOVA (p<0.0001) with Dunnett's multiple comparisons test (***p<0.001) and in B was by unpaired t test (****p<0.0001).

5.12 $CD4^+$ T cell differentiation in MDM co-culture in the presence or absence of *S. pneumoniae* infection

To assess whether the differentiation of $CD4^+$ T cells after T cell culture with the addition of *S. pneumoniae* resulted in a similar distinct $CD25^+Foxp3^+$ population as seen in figure 5.11.3, these experiments were repeated with the addition of a 6 hour *S. pneumoniae* infection during the 48 hour MDM-$CD4^+$ T cell co-culture. The $CD4^+$ T cells were then collected and stained for CD4, CD25 and Foxp3 expression. A lower percentage of $CD4^+$ T cells (approximately 5%) expressed both CD25 and Foxp3 when infected with *S. pneumoniae* compared to uninfected cells (figure 5.12).

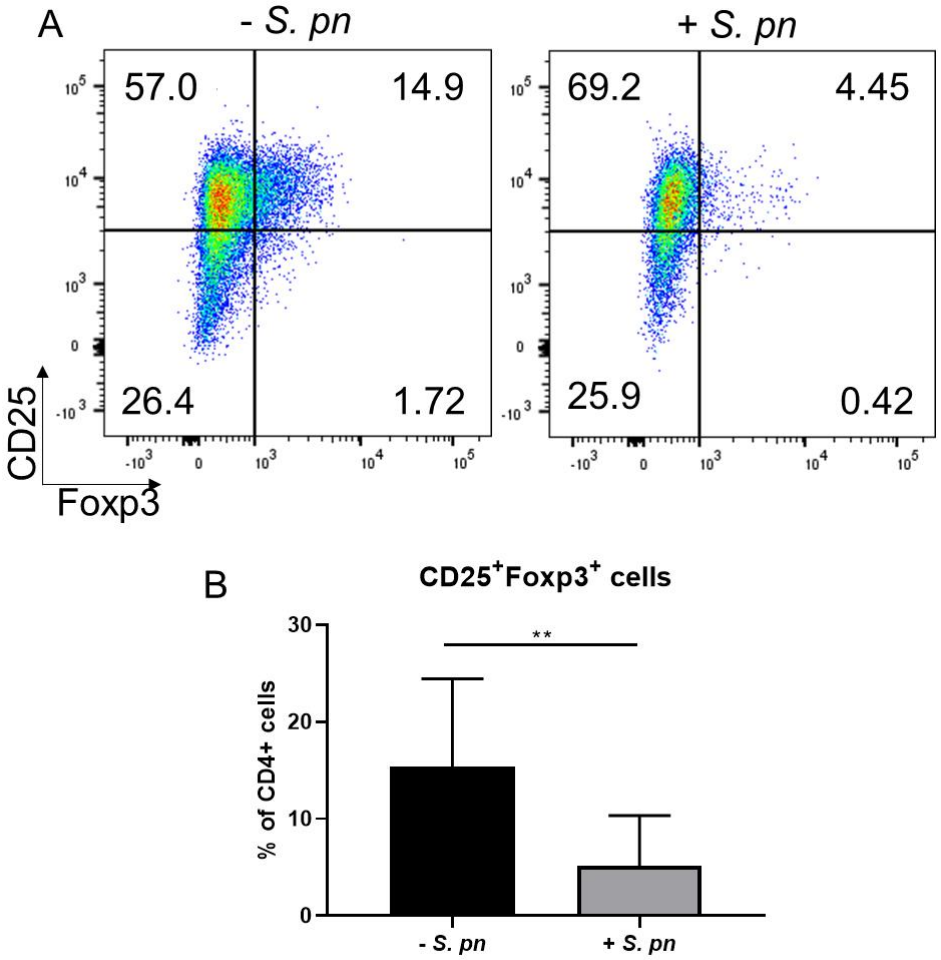

Figure 5.12 CD25 and Foxp3 expression by CD4⁺ T cells after MDM co-culture with or without *S. pneumoniae* infection

CD4⁺ T cells were cultured in the presence of MDMs for 48 hours with or without MOI 2 TIGR4 *S. pneumoniae* infection. The CD4⁺ T cells were collected and stained for CD4, CD25 and Foxp3 and analysed by flow cytometry for 1 representative donor (A) and for 10 donors combined (B). Gating for the CD25⁺Foxp3⁺ populations was determined visually and applied uniformly across all donors. Graph B shows the mean of 10 biological replicates (cells taken from 10 donors) +/- SD of the mean and was analysed by unpaired t test (**p<0.01).

5.13 Cytokine production by $CD4^+$ T cells co-cultured with MDMs with or without *S. pneumoniae* infection

IL-17A, IL-10, IFNγ, TNFα and IL-2 expression by $CD4^+$ T cells co-cultured with MDMs with or without *S. pneumoniae* infection was examined by flow cytometry. MACS-sorted $CD4^+$ T cells were incubated on MDMs for 48 hours with or without a 6 hours infection with *S. pneumoniae*. In the infected groups, *S. pneumoniae* were added to the MDM+$CD4^+$ T cell co-culture for 6 hours, followed by addition of penicillin-streptomycin, followed by continued co-culture for 42 hours. IFNγ, IL-2, TNFα, IL-10 and IL-17A positive $CD4^+$ T cells were detected in both infected and non-infected groups (figure 5.13.1 A). No statistically significant differences were seen in expression of any cytokines when comparing $CD4^+$ T cells incubated in the presence or in the absence of *S. pneumoniae* infection (figure 5.13.2 B).

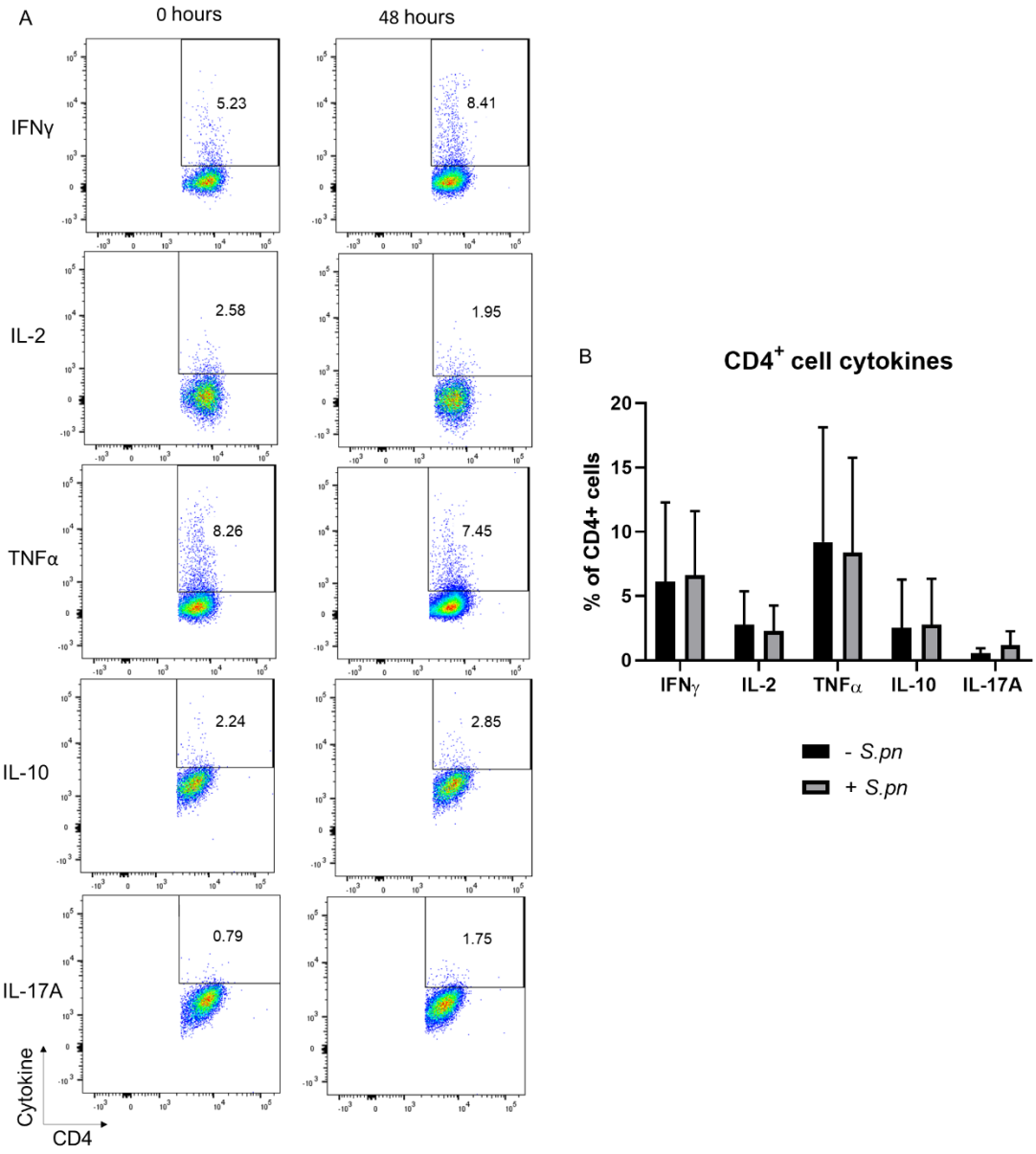

Figure 5.13.1 Cytokine expression by CD4⁺ T cells co-cultured with MDMs with or without *S. pneumoniae* infection

Expression of IL-17A, IL-10, IFNγ, TNFα and IL-2 by CD4⁺ T cells with or without MOI 2 TIGR4 *S. pneumoniae* infection were analysed by flow cytometry. Cytokine-positive populations were gated based on visibly higher expression of fluorochrome than the majority population with the same gate used for the infected and uninfected group per cytokine (A). Graph B shows the mean expression of cytokine from 4 biological replicates (cells taken from 4 donors) with bars showing +/- SD of the mean. Statistical analysis was by two-way ANOVA (interaction factor, column factor and row factor not significant) with Tukey's multiple comparisons test (not significant between uninfected and infected groups for any cytokine).

The expression of IL-17A, IL-10, IFNγ, and IL-2 by the $CD25^{+}Foxp3^{-}$ and $CD25^{+}Foxp3^{+}$ fractions of $CD4^{+}$ T cells with or without *S. pneumoniae* infection were compared. The aim was to see if the $CD25^{+}Foxp3^{+}$ fraction produced less pro-inflammatory cytokine than $CD25^{+}Foxp3^{-}$, as an indication as to whether the $CD25^{+}Foxp3^{+}$ fraction could be a regulatory subset. In addition, IL-10 is reportedly produced by both $Foxp3^{-}$ and $Foxp3^{+}$ Treg cells, therefore the production of IL-10 by these fractions was also examined. By contrast, IL-2 is not produced by Treg cells, and its inclusion in the panel aimed to provide further confirmation for a Treg subset. The effect of infection with *S. pneumoniae* on the expression of these cytokines by each fraction was also investigated.

Contrary to the hypothesis, it was found that the $CD25^{+}Foxp3^{+}$ fractions of $CD4^{+}$ T cells generally expressed the highest levels of pro-inflammatory cytokines, although these differences were not significant for any cytokine (figure 5.13.2). In addition, *S. pneumoniae* infection did not result in significant changes in expression of any cytokine compared to the uninfected groups (figure 5.13.2). IFNγ showed a trend towards increase in the $CD25^{+}Foxp3^{+}$ fraction compared to the $CD25^{-}Foxp3^{-}$ fraction in the uninfected group, and a trend towards increase upon *S. pneumoniae* infection compared to the uninfected group, although these differences were not statistically significant (figure 5.13.2 A). IL-2 expression had a slight trend in increase from the $CD25^{-}Foxp3^{-}$ fraction to the $CD25^{+}Foxp3^{+}$ fraction, although this was not statistically significant, and there were no also no significant differences in IL-2 expression upon *S. pneumoniae* infection compared to the uninfected group (figure 5.13.2 B). There were no statistically significant differences in IL-10 expression between the $CD25^{+}Foxp3^{-}$ and $CD25^{+}Foxp3^{+}$ fractions or between the infected or uninfected groups (figure 5.13.2 C). IL-17A showed a trend towards increased expression in the $CD25^{+}Foxp3^{+}$ fraction compared to the $CD25^{+}Foxp3^{-}$ fraction, and between the

infected and uninfected groups in the $CD25^{+}Foxp3^{+}$ fraction, but these differences were not statistically significant (figure 5.13.2 D).

IL-2 was expressed by approximately 2-5% of the $CD4^{+}$ T cells in MDM co-culture. This is considerably lower than in other studies, for example IL-2 production has been reportedly detected in over 50% of T cells stimulated with autologous APCs and antigenic challenge after 40 hours in culture (Sojka et al., 2004). IL-2 is produced at low levels in resting/naïve $CD4^{+}$ T cells, and produced in large amounts by activated $CD4^{+}$ T cells (Liao et al., 2013). The lack of IL-2 production and low IFNγ production may indicate that the $CD4^{+}$ T cells became anergic T cells. Anergy is a hyporesponsive state that occurs when T cells are activated through the TCR without co-stimulatory signals. T cell anergy is a mechanism of peripheral tolerance when full immune activation is not desirable, such as in response to self-antigens. The anergic state can occur without strong CD28 stimulation and in the presence of anti-CD3 antibody (Harding et al., 1992; Schwartz, 2003; Zheng et al., 2008). Anergic T cells are still capable of producing cytokines, which could explain the expression of other cytokines seen in these results (Zheng et al., 2008). Prolonged culture and more stimulation may promote increased IL-2 production in these CD4+ MDM co-culture experiments.

In summary, the $CD25^{+}Foxp3^{+}$ population expressed IFNγ, IL-2 and IL-17A at generally higher percentages than the $CD25^{+}Foxp3^{-}$ population, indicating that overall this may not be a regulatory group. It is possible that these increased percentages are expressed by a small portion of the $CD25^{+}Foxp3^{+}$ population and are activated conventional T cells, while a proportion of the $CD25^{+}Foxp3^{+}$ population are genuine regulatory cells. *S. pneumoniae* infection resulted in a trend towards increase in these cytokines, but was not statistically significant. The low IL-2 expression by all cells could indicate a state of T cell anergy. This may have been due to weak CD28 signalling, as no anti-CD28 antibody was added to the co-culture (Powell et al., 1998).

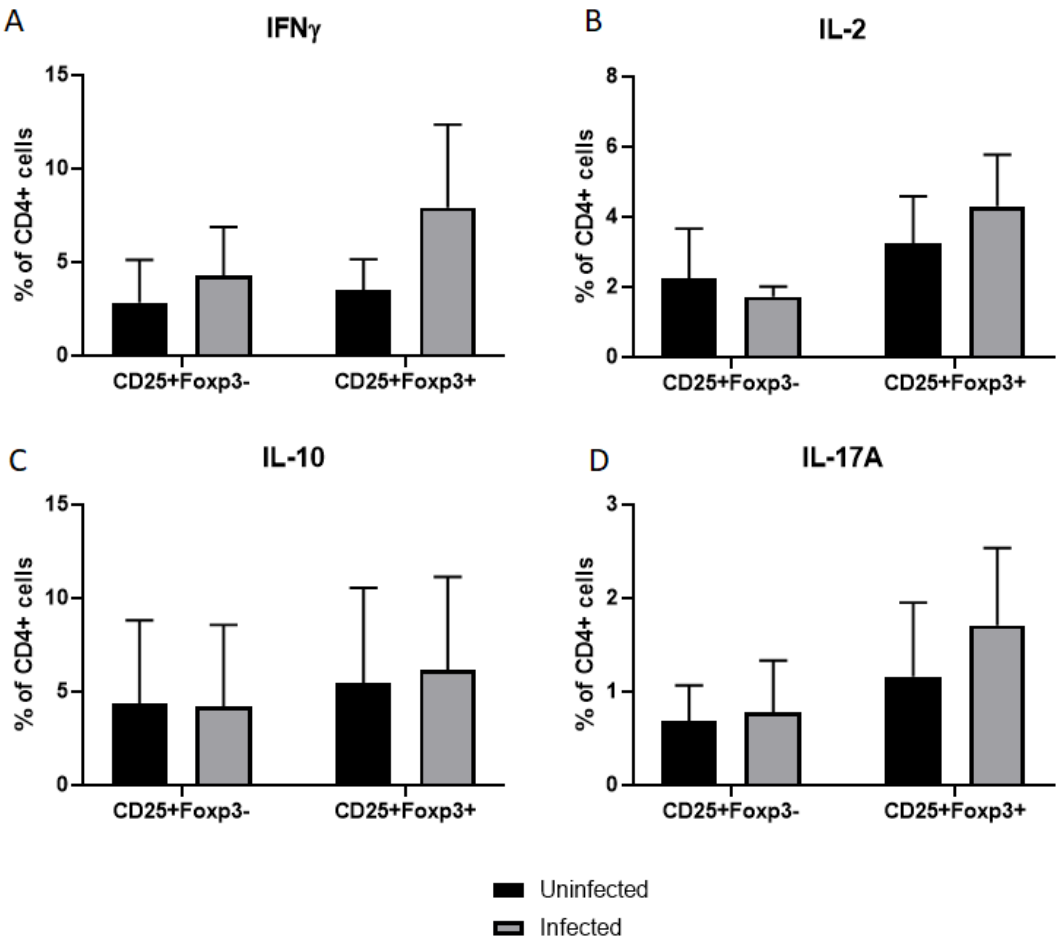

Figure 5.13.2 Cytokine production by CD25/Foxp3$^{-/+}$ fractions of CD4^{+} T cells after MDM co-culture with or without *S. pneumoniae* infection

CD4^{+} T cells were cultured in the presence of MDMs for 48 hours, with or without MOI 2 TIGR4 *S. pneumoniae* infection, followed by a 6 hour incubation with Golgistop and stained for intracellular cytokines. Production of IFNγ (A), IL-2 (B), IL-10 (C), and IL-17A (D) by CD25/Foxp3 fractions were analysed by flow cytometry. Cytokine-positive populations were gated based on visibly higher expression of fluorochrome than the majority population with the same gate used for the infected and uninfected group per cytokine (shown in figure 5.13.1 A). Graphs show the mean percentage of CD4^{+} T cells expressing each cytokine from 4 biological replicates (cells taken from 4 donors), with bars showing +/- SD of the mean. Statistical analysis was by two-way ANOVA (interaction factor not statistically significant in A-D) with Tukey's multiple comparisons test (not significant between any groups).

5.14 Chapter 5 Summary

Exogenous IL-10 and TGFβ suppressed MDM inflammatory responses to *S. pneumoniae*. However, no IL-10 was detected during MDM-Treg cell co-culture, whereas IL-10 was detected during MDM-Teff co-culture. Teff cells expressed IL-10, IL-2, IFNγ and IL-17A when stimulated with PMA, ionomycin and Golgistop, whereas Treg cells did not express statistically significant levels of these cytokines. Repeating the MDM-Treg/Teff co-culture experiments using Treg/Teff supernatant did not result in suppression of MDM inflammatory responses upon subsequent *S. pneumoniae* infection. Similarly, repeating the MDM-Treg/Teff co-culture experiments separating the Treg/Teff cells from the MDMs by a transwell insert did not result in suppression of MDM inflammatory responses upon subsequent *S. pneumoniae* infection. These results suggest that Treg cell suppression of MDM inflammatory responses to *S. pneumoniae* was cell-contact dependent, and independent of cytokines produced as a result of MDM-Treg cell co-culture.

Preliminary experiments investigated if Treg cell surface TGFβ was involved in the suppressive effects seen on MDMs. A small but statistically significant percentage of Treg cells expressed surface TGFβ compared to Teff cells as measured by LAP expression. LAP is increased upon Treg activation, so it is likely that LAP levels were higher after co-culture with MDMs in the presence of Treg cells, but this was not measured in these experiments. Blocking ALK5 receptors or neutralising LAP had little effect on Treg suppressive capability with the exception of a potential effect of blocking LAP on Treg cells suppression of MDM IL-6 production. Addition of the LAP-neutralising antibody increased TNFα and IL-6 production by MDMs co-cultured with Teff and decreased TNFα and IL-6 production by MDMs infected with *S. pneumoniae* in the absence of Treg/Teff cells. This effect on MDMs alone may confound the effect seen with Treg/Teff cells. It is likely that this antibody resulted in more active TGFβ in the supernantant, as there is a high concentration of LAP-associated TGFβ present

in the serum supplementing the culture medium, and removal of LAP activates TGFβ. This could have a suppressive effect on the MDMs, and may explain why MDMs infected with *S. pneumoniae* in the absence of T cells but in the presence of anti-LAP had decreased production of inflammatory cytokine. Whether total $CD4^+$ T cells upregulated CD25 and Foxp3 and the resulting cytokine profiles after MDM co-culture was investigated. After 48 hours in MDM-co-culture, $CD4^+$ T cells expressed detectable IL-2, IFNγ, TNFα, IL-10 and IL-17A. The majority of $CD4^+$ T cells expressed CD25 after MDM co-culture, and approximately 15% expressed both CD25 and Foxp3.

The CD25 and Foxp3 expression and cytokine profiles of $CD4^+$ T cells after MDM co-culture with or without *S. pneumoniae* infection was investigated. A smaller population of $CD25^+Foxp3^+$ cells resulted in the presence of *S. pneumoniae* compared to the uninfected group. IL-17A, IL-10, IFNγ, TNFα and IL-2 expression did not differ statistically between uninfected and infected $CD4^+$ T cells in MDM co-culture, although a trend towards increased IL-17A in the infected group could be seen.

The cytokine expression within the $CD25^+Foxp3^-$ and $CD25^+Foxp3^+$ fractions of uninfected and infected MDM-$CD4^+$T cell co-culture was examined. There were no statistically significant differences in cytokine expression between uninfected and infected cell cultures, although a trend towards higher expression in infected groups was observed in IFNγ, IL-2 and IL-17A expression. Similarly, there were no statistically significant differences in cytokine expression between the $CD25^+Foxp3^-$ and $CD25^+Foxp3^+$ fractions, although a trend towards higher expression in $CD25^+Foxp3^+$ fractions was observed in IFNγ, IL-2 and IL-17A expression. IL-10 expression was variable between donors, and did not differ between fractions or upon *S. pneumoniae* infection. IL-2 expression was low, which may indicate that the cells were anergic.

To summarise:

- IL-10 and TGFβ suppressed MDM inflammatory responses to *S. pneumoniae*
- IL-10 was made by FACS-sorted Teff cells but not by FACS-sorted Treg cells
- Contact between FACS-sorted Treg cells and MDMs was required for suppression of MDM inflammatory responses to *S. pneumoniae*
- Neutralising LAP may affect Treg suppression of MDM IL-6 production upon *S. pneumoniae* infection, but more data are required
- Inhibiting ALK5 receptors had little effect on the ability of Treg cells to suppress MDM inflammatory responses to *S. pneumoniae*
- $CD4^+$ T cells increased expression of CD25 and Foxp3 in MDM co-culture but the $CD25^+Foxp3^+$ population produced both IL-10 and pro-inflammatory cytokines
- Fewer $CD4^+$ T cells were present in the $CD25^+Foxp3^+$ fraction after MDM co-culture with *S. pneumoniae* infection but increases in cytokine expression were not statistically significant compared to uninfected cells
- Low IL-2 detection suggested that these $CD4^+$ T cells after MDM co-culture may be anergic T cells

Chapter 6: Detection of T cell subsets in a human model of *S. pneumoniae* exposure and in lung tissue

6.1 Chapter 6 Introduction

This chapter investigates whether Treg cells can be identified in human samples after exposure to *S. pneumoniae* or within normal lung tissues. The recruitment time of Treg cells to UV-killed *S. pneumoniae* injected into the skin of healthy volunteers was examined. Due to the low number of cells collected these experiments were limited to measurement of surface markers on the recruited cells, using $CD4^+CD25^+CD127^{low}$ cells to define the potential Treg cell population. In addition to the skin challenge experiments, immunofluorescence staining for CD4 and Foxp3 was used to detect the presence of potential Treg cells in human normal lung sections. As it was not possible to assess the suppressive capabilities of the cells isolated from the site of UV-killed *S. pneumoniae* injection or the cells stained in the human lung, the $CD4^+CD25^+CD127^{low}$ and $CD4^+Foxp3^+$ cells are described as potential Treg cells.

Aims:

- Quantify the blood flow to the site of intradermal injection of UV-killed *S. pneumoniae* into the forearm of healthy volunteers over 48 hours
- Determine the proportions and cell numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD4^+CD25^+CD127^{low}$ cells recruited to the site of intradermal injection of UV-killed *S. pneumoniae* in healthy volunteers
- Examine CD45RA and CCR6 expression by the recruited $CD4^+$ cells
- Quantify the concentrations of cytokines present at the site of injection
- Identify $CD4^+Foxp3^+$ cells in human normal lung sections

6.2 Inflammatory response to intradermal injection of UV-killed *S. pneumoniae* into the skin of healthy volunteers

Healthy volunteers were given intradermal injections of UV-killed *S. pneumoniae* suspended in 100µl of saline into the forearm and the inflammatory response was followed over 48 hours by Laser Doppler scans of the injection site (figure 6.2 A). The Doppler scan works by detecting laser scatter caused by the concentration and velocity of red blood cells at the scanned area and recording the frequency of reflected light as a colour-coded image. A set level of background pixels below a fixed threshold are removed from each image which allows comparison of only the inflamed areas. The blood flow is quantified by multiplying the number of pixels by the mean blood flow signal and is referred to as arbitrary "perfusion units".

The perfusion units calculated prior to injection and at 4 hours, 24 hours and 48 hours after injection were compared. Blood flow at 4 hours and 24 hours post-injection was increased compared to the baseline, with blood flow at 24 hours having the highest increase (figure 6.2 B). At the 48 hour time point, blood flow was no longer increased compared to the baseline and the inflammation had visibly lessened as seen on the Doppler images (figure 6.2 A and B). The ethics approval in place restricted volunteers to two visits to the laboratory, so despite the peak in blood flow at 24 hours, it was decided that further experiments involving the recruitment of $CD4^{+}CD25^{+}CD127^{low}$ cells to the site of injection would be carried out at 4 hours and 48 hours post-injection to give a larger span of the time points and to include the possibility that Treg cells would be more numerous during resolution of inflammation. This is the reason for the discrepancy in the number of volunteers for the 24 hour time point seen in figure 6.2 B.

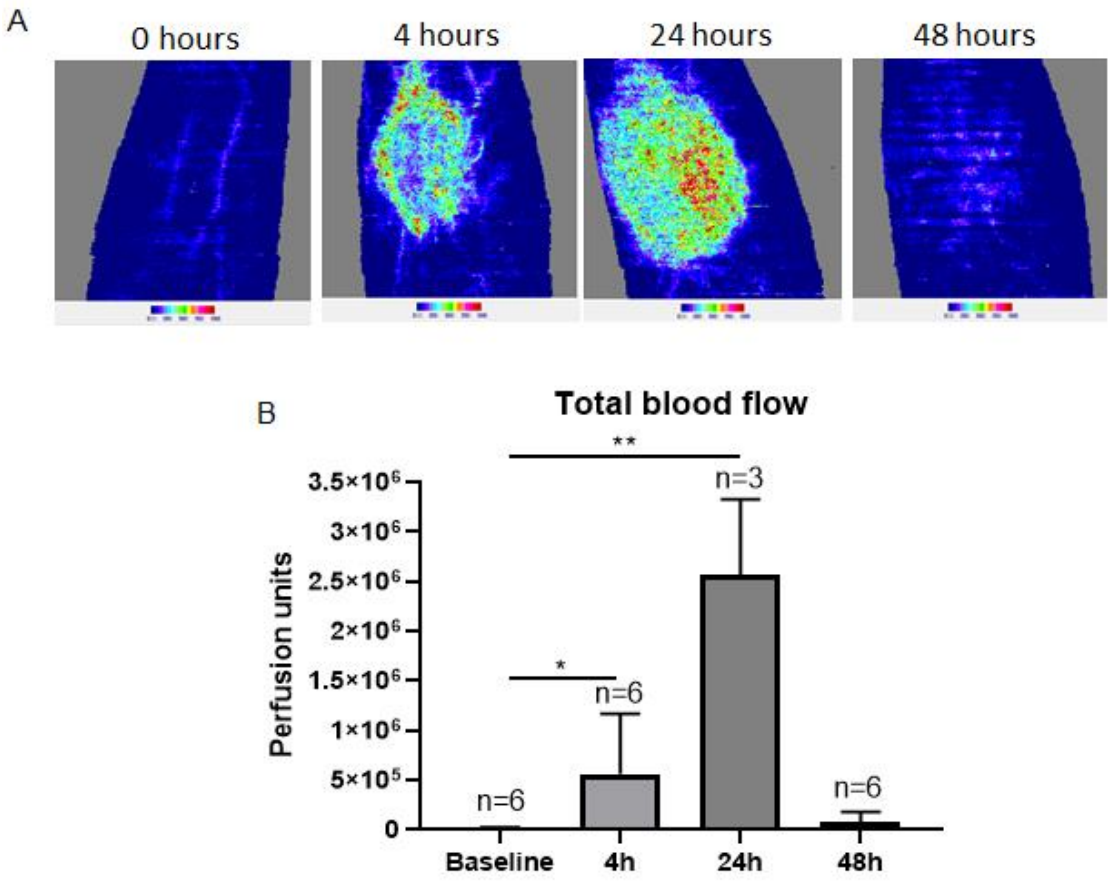

Figure 6.2 Blood flow to the site of intradermal injection of UV-killed *S. pneumoniae* in the forearms of healthy volunteers

The inflammatory response to intradermal injections of $7.5x10^5$ UV-killed TIGR4 *S. pneumoniae* into the forearm of healthy volunteers was followed over 48 hours by Laser Doppler scans of the injection site (A) and blood flow to the site of injection was compared (B). Blood flow measured in arbitrary perfusion units was calculated by multiplying the number of valid pixels (above 300 perfusion units) by the mean blood flow signal from data acquired by the Laser Doppler moorLDI software (version 5). Figure A shows representative images of Doppler scans at each time point from one volunteer. Graph B shows the mean blood flow from 6 volunteers for the baseline, 4 hour and 48 hour time points and 3 volunteers for the 24 hour time points, with error bars showing +/- SD of the mean. Statistical analysis in B was by Kruskal-Wallis test (p<0.01) with Dunn's multiple comparisons test (*p<0.05, **p<0.01).

6.3 Recruitment of $CD3^+$, $CD4^+$, $CD8^+$ and $CD4^+CD25^+CD127^{low}$ cells to the presence of UV-killed *S. pneumoniae* injected into the skin of healthy volunteers

Healthy volunteers were given intradermal injections of UV-killed *S. pneumoniae* suspended in 100µl of saline into each forearm. At 4 hours and 48 hours post-injection, a blister was raised at the site of injection by applying a suction chamber connected to a negative pressure instrument, and the blister fluid aspirated for flow cytometry analysis of the T cell subsets using surface markers to detect $CD3^+$, $CD4^+$, $CD8^+$ and $CD4^+CD25^+CD127^{low}$ cells. Cell gating was established on cells from whole blood taken prior to injection (figure 6.3.1 A). Dead cells and doublets were also excluded from analysis. The percentage of $CD3^+$ cells was increased in the 48 hour blister compared to the 4 hour blister and compared to the 0 hour blood sample (figure 6.3.1 B). The proportion of $CD3^+$ cells expressing CD4 and CD8 in the 0 hour blood were a mean of 63% CD4 and 29% CD8 (figure 6.3.1 C). The 4 hour blister fluid cells also had a higher proportion of $CD4^+$ cells to $CD8^+$ cells, although this difference was statistically less significant than that seen in the blood, with a mean proportion of 55% $CD4^+$ and 36% $CD8^+$ (figure 6.3.1 C). However, in the 48 hours blister no difference could be seen in the percentage of $CD4^+$ compared to CD8+ cells, with a mean proportion of 42% $CD4^+$ and 41% $CD8^+$ (figure 6.3.1 C). Of note, the $CD4^+/CD8^+$ proportion in the 48 hour blister fluid cells was variable between donors, with some donors having higher a higher proportion of $CD4^+$ cells, some having a higher proportion of $CD8^+$ cells, and some showing little difference between the $CD4^+$ and $CD8^+$.

The $CD4^+$ cells were examined for expression of $CD25^+$ and $CD127^{low}$ to identify the potential Treg cell population. The $CD4^+CD25^+CD127^{low}$ cell gate was established on the 0 hour blood sample and applied to the 4 hour and 48 hour blister samples. The percentage of $CD4^+$ cells in the $CD4^+CD25^+CD127^{low}$ cell gate statistically was

significantly higher in the 48 hour blister sample than that in the 0 hour blood sample or the 4 hour blister sample, with mean percentages of $CD4^+$ cells having $CD4^+CD25^+CD127^{low}$ being 6.4% in the blood, 15% in the 4 hour blisters and 40% in the 48 hour blisters (figure 6.3 D). Ideally, the $CD4^+CD25^+CD127^{low}$ cells would have been additionally stained for $Foxp3^+$ expression as the most reliable marker of Treg cells; however the low cell counts made the extra step of intracellular staining likely to result in too few cells for analysis.

The percentages of $CD3^+$, $CD4^+$, $CD8^+$, and $CD4^+CD25^+CD127^{low}$ cells established by flow cytometry were applied to the numbers of cells counted by haemocytometer within each blister sample to determine the number of cells per ml of each cell type present at 4 hours and 48 hours post-injection. The number of $CD3^+$ (figure 6.3.2 A) and $CD8^+$ (figure 6.3.2 B) statistically were significantly higher in the 48 hour blister compared to the 4 hour blister. $CD4^+$ cells were close to statistically significant increase at 48 hours compared to 4 hours (p=0.0625, figure 6.3.2 C). The number of $CD4^+CD25^+CD127^{low}$ cells was significantly higher in the 48 hour blister compared to the 4 hour blister (figure 6.3.2 D). An overall comparison of the numbers of each cell types can be seen in figure 6.3.2 E.

To summarise, the percentage and number of $CD3^+$ cells statistically were significantly increased in the 48 hour blister compared to the 4 hour blister. $CD4^+$ cells were the predominant fraction of the $CD3^+$ cells at 4 hours, whereas a clear CD4 or CD8 majority was not apparent at 48 hours. The percentage and number of $CD4^+CD25^+CD127^{low}$ cells was increased in the 48 hours blisters compared to the 4 hours blisters and compared to 0 hours blood.

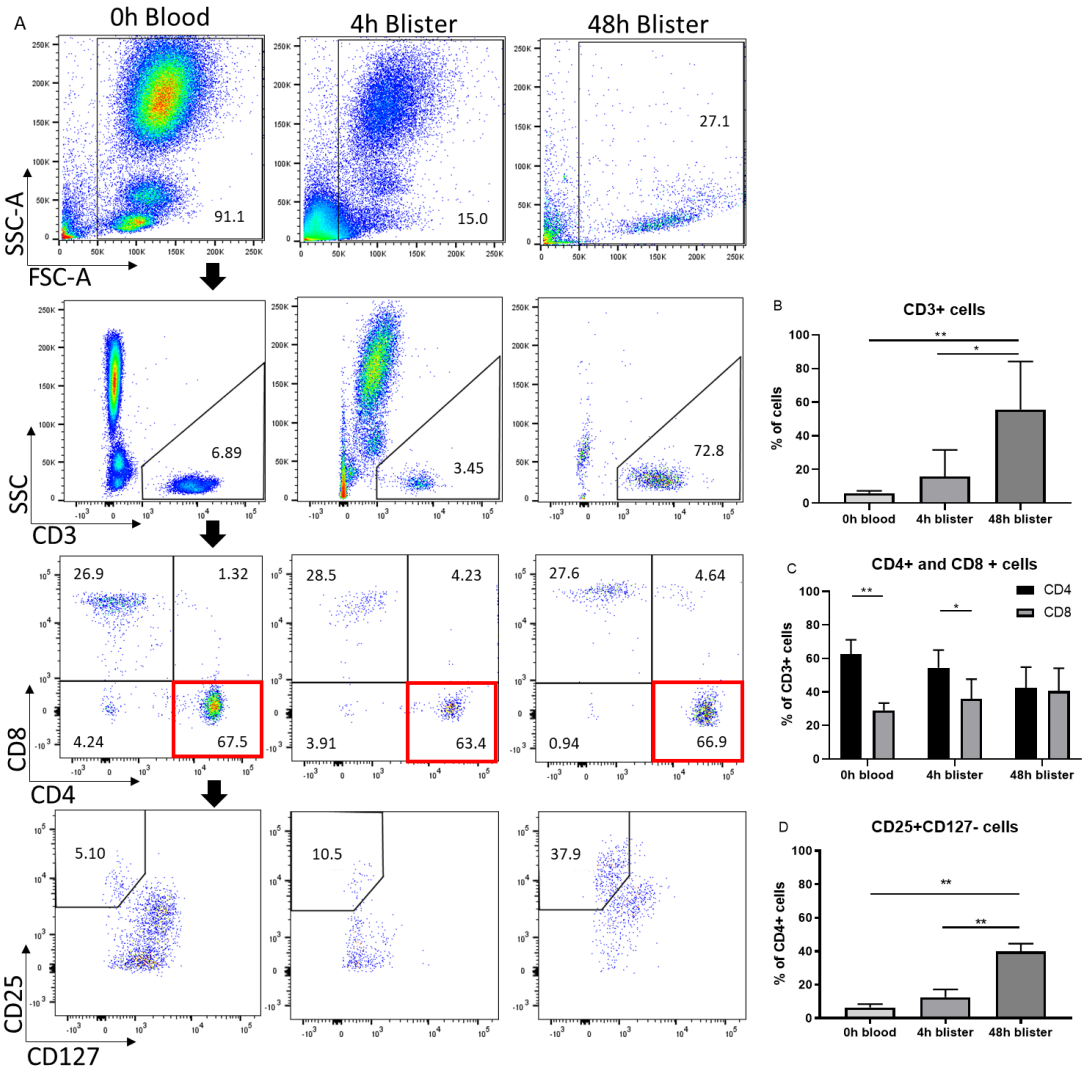

Figure 6.3.1 Gating strategy and percentages of CD3$^+$, CD4$^+$, CD8$^+$ and CD25$^+$CD127^{low} cells in blister fluid at the site of intradermal *S. pneumoniae* injection and in whole blood

Cells were isolated from blister fluid at the site of intradermal injection of 7.5x10^5 UV-killed TIGR4 *S. pneumoniae* at 4 hours and 48 hours post-injection or cells were isolated from whole blood prior to injection. Cell gates for CD3$^+$, CD4$^+$, CD8$^+$, and CD4$^+$CD25$^+$CD127^{low} cells (A) were based on the blood sample. Expression of CD3$^+$ (B), CD4$^+$ and CD8$^+$ (C), and CD25$^+$CD127^{low} (D) cells as a percentage of their parent population was compared in the blood and blister fluid cells. Graphs show the mean percentages from 6 biological replicates (6 volunteers). Statistical analysis in B and D was by one-way ANOVA (p<0.01 B and D) with Tukey's multiple comparisons test (*p<0.05, **p<0.01). Statistical analysis in C was by two-way ANOVA (interaction factor p<0.05) with Tukey's multiple comparisons test (*p<0.05, **p<0.01).

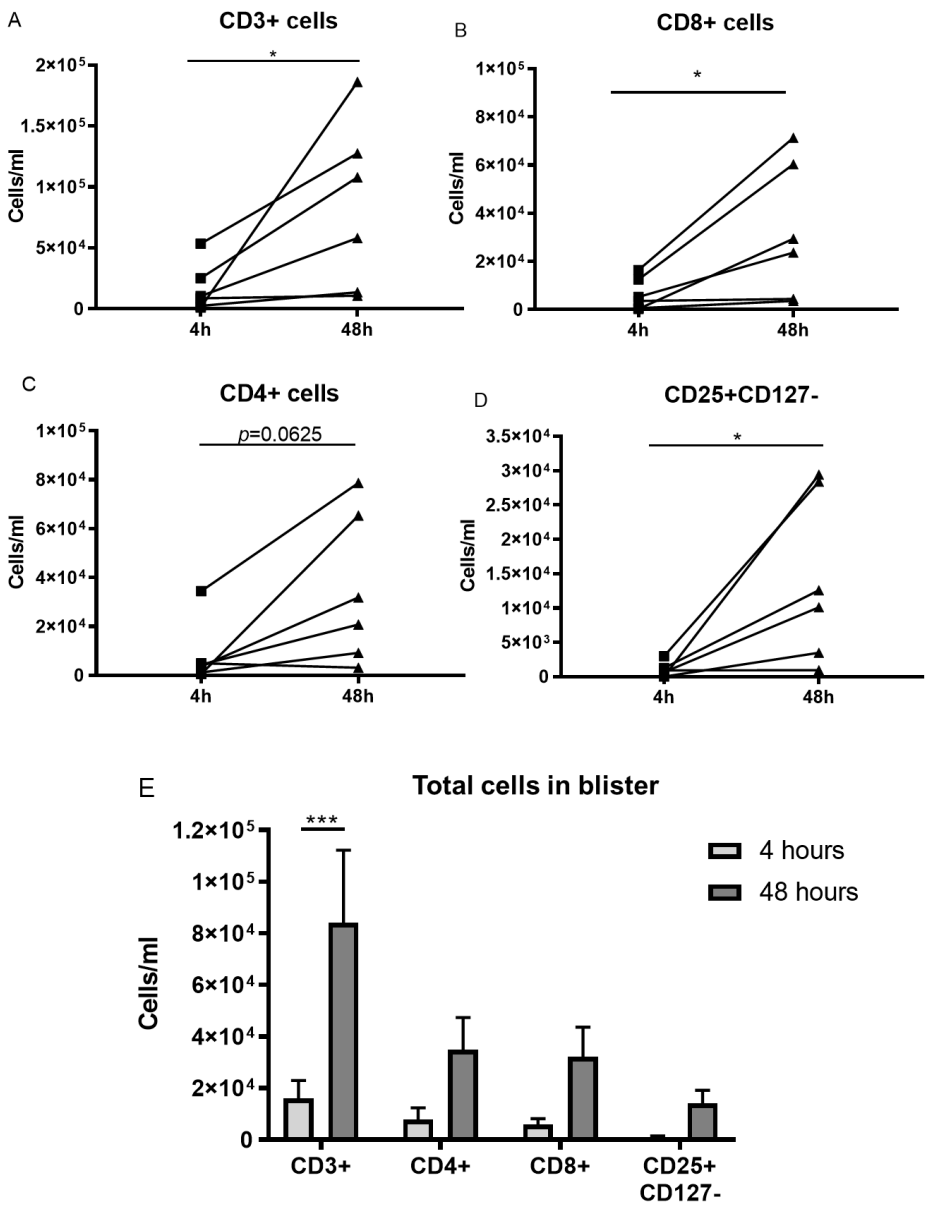

Figure 6.3.2 CD3$^+$, CD4$^+$, CD8$^+$ and CD25$^+$CD127^{low} cell counts in blister fluid at the site of *S. pneumoniae* intradermal injection

The number of cells per ml were calculated using cells counted by haemocytometer in the 4 hour and 48 hour blister fluid and the percent expression of CD3 (A), CD8 (B), CD4 (C) and CD4$^+$CD25$^+$CD127^{low} cells (D) based on the flow cytometry analysis in figure 6.3.1. An overall comparison of the cell numbers is shown in E. Graphs A-D show the absolute cell numbers from 6 donors, with each data point representing 1 donor. Graph E shows the mean cell numbers from 6 biological replicates (6 volunteers) +/- SD of the mean. Statistical analysis in A-D was by Wilcoxon matched-pairs signed rank test (*p<0.05). Statistical analysis in E was by two-way ANOVA (interaction factor not statistically significant, row factor "time" p<0.01; column factor "cell type" p<0.001) with Sidak's multiple comparisons test (***p<0.001).

6.4 Separating the $CD4^+$ T cells present in the blister fluid into resting T cell, activated T cell and Treg populations

$CD4^+$ T cells in the 0 hour blood samples, 4 hour blister samples and 48 hour blister samples were separated into $CD25^{low}CD127^+$ resting T cells, $CD25^+CD127^+$ activated T cells and $CD25^{++}CD127^{low}$ potential Treg cells (figure 6.4 A). The majority of the $CD4^+$ T cells in the 0 hour blood samples fell into the resting T cell gate with a mean of 57% of $CD4^+$ cells being $CD25^{low}CD127^+$, indicating a predominate resting T cell type in the blood (figure 6.4 B). The percentages of resting T cells in the 4 hour and 48 hour blister samples were statistically significantly lower than those seen in the 0 hour blood samples, with a mean of 28% in the 4 hour blister samples and a mean of 20% in the 48 hour blister samples (figure 6.4 B). A trend towards increased percentages of activated T cells could be seen from the 0 hour blood samples to the 4h blister samples, with means of 31% in the blood and 42% in the 4 hour blister, however these differences wrere not statistically significant (figure 6.4 B). Conversely, a trend towards decreased proportions of activated T cells could be seen in the 48 hour blister compared to the 0 hour blood and 4 hour blister samples, with a mean of 21% activated T cells at 48 hours, which was close to being statistically significantly lower than the 4 hour blister sample at p=0.06 (figure 6.4 B). As also shown in part 6.3, the increase in the percentage of $CD25^{++}CD127^{low}$ potential Treg cells constituting the $CD4^+$ T cell population was statistically significant between the 0 hour blood samples and the 48 hour blister samples (figure 6.4 B). The numbers of cells per ml of resting T cells, activated T cells and potential Treg cells showed a trend towards increase for all three populations between the 4 hours and 48 hours blister samples, but these increases were not statistically significant (figure 6.4 C). Within the 4 hour blister and within the 48 hour blister samples, the number of cells per ml of resting T cells, activated T cells and potential Treg cells did not statistically significantly differ between these three T cell population types (figure 6.4 C).

The reliability of the data particularly for the 4 hour blister is questionable due to the small number of cells isolated from the blister at this time point allowing small differences in the number of events in each gate to cause large differences in the overall percentages representing each population and is reflected in the large variation in data between donors (figure 6.4 A and B).

Overall these data indicate that while the resting T cell is the predominant $CD4^+$ T cell type in the 0 hour blood, activated T cells predominate in the 4 hour blister samples, and the potential Treg type are the majority cell type for most donors in the 48 hour blister, which indicates a progression from an initial pro-inflammatory response at 4 hours towards resolution at 48 hours.

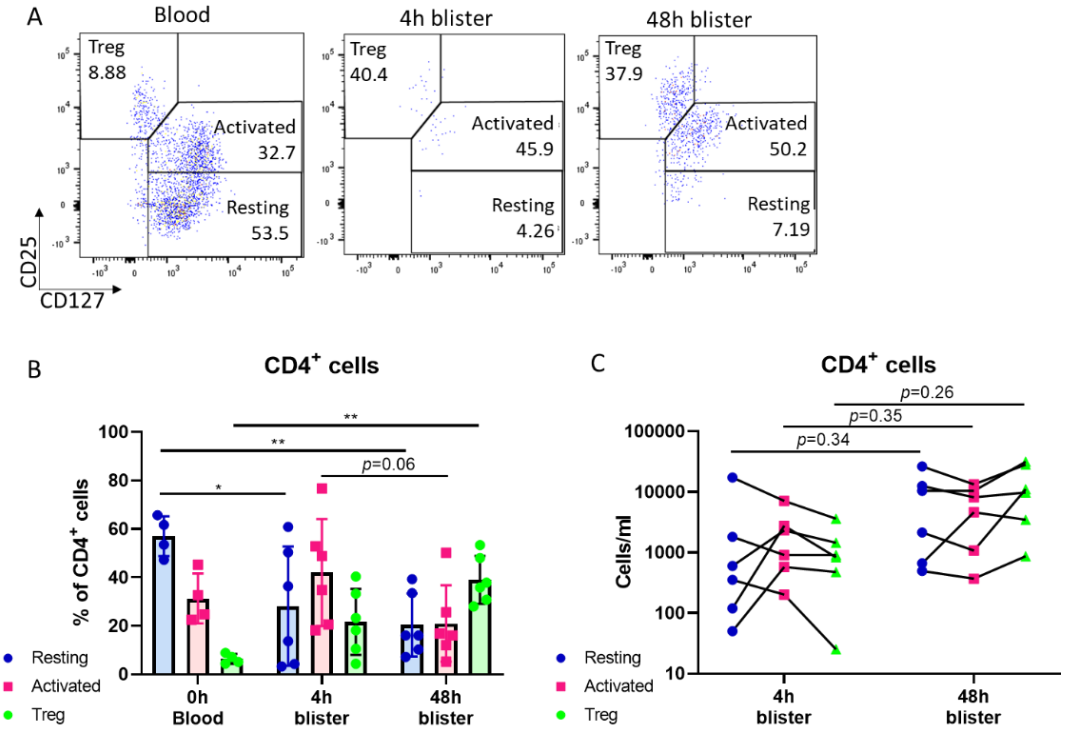

Figure 6.4 Separating the CD4⁺ T cells into resting T cell, activated T cell and Treg populations based on CD25 and CD127 expression

CD4⁺ T cells in the 0 hour blood, 4 hour blister and 48 hour blister samples were separated into $CD25^{low}CD127^{+}$ resting T cells ("resting"), $CD25^{+}CD127^{+}$ activated T cells ("activated), and $CD25^{++}CD127^{low}$ Treg cells ("Treg") after exclusion of cell debris, dead cells and doublets and selection of $CD3^{+}CD4^{+}$ cells (A). The percentages of resting T cells, activated T cells and Treg cells constituting the CD4⁺ T cell populations in the 0 hour blood, 4 hour blister and 48 hour blister samples were compared (B). The numbers of resting T cells, activated T cells and Treg cells in the 0 hour blood, 4 hour blister and 48 hour blister samples were compared (C). Figure A shows representative flow cytometry plots from 1 donor. Figure B shows data from 4 donors for the blood samples and 6 donors for the 4 hour and 48 hour blister samples, with each point representing the value for each donor, bars showing the mean of all donors, and error bars showing SD of the mean. Figure C shows data from 6 donors. Statistical analysis in B was by two-way ANOVA (interaction factor $p<0.001$; row factor "0h blood vs 4h blister vs 48h blister" not significant; column factor "resting vs activated vs Treg" not significant) with Tukey's multiple comparisons test (*$p<0.05$; **$p<0.01$). Statistical analysis in C was by repeated measures two-way ANOVA (interaction factor $p<0.05$; row factor "4h blister vs 48h blister" not significant; column factor "resting vs activated vs Treg" not significant) with Tukey's multiple comparisons test (not significant between any groups).

6.5 CD45RA expression by $CD4^+$ T cell subsets recruited to the site of intradermal injection of UV-killed *S. pneumoniae*

Naïve T cells express CD45RA which is lost upon antigen experience, resulting in effector and memory T cells with CD45RO expression (Akbar et. al., 1988). Around 90-95% of circulating $Foxp3^+$ cells are $CD45RO^+$ (Taams *et. al*., 2001). The CD45RA expression of $CD4^+$ and $CD25^+CD127^{low}$ cells in the blister fluid was examined and compared to cells taken from whole blood samples prior to *S. pneumoniae* intradermal injection. A mean of 42% of $CD4^+$ cells taken from the blood were $CD45RA^+$, whereas $CD45RA^+$ cells only constituted 4% and 8% of $CD4^+$ cells in the blister fluid at 4 hours and 48 hours respectively (figure 6.5.1 B). In the whole blood samples, a mean of 10% of $CD4^+CD25^+CD127^{low}$ cells expressed CD45RA, whereas 0.84% and 0.06% in the 4 hour and 48 hour blister fluid were $CD45RA^+$, respectively (figure 6.5.1 B). These results suggest that the $CD4^+$ cells and $CD4^+CD25^+CD127^{low}$ recruiting to the injection site at both 4 hours and 48 hours post-injection are predominantly antigen-experienced effector/memory cells. The low CD45RA expression also indicates that the $CD4^+$ cells isolated from the blister are a different population to peripheral blood cells, which are >40% $CD45RA^+$ (figure 6.5.1 A).

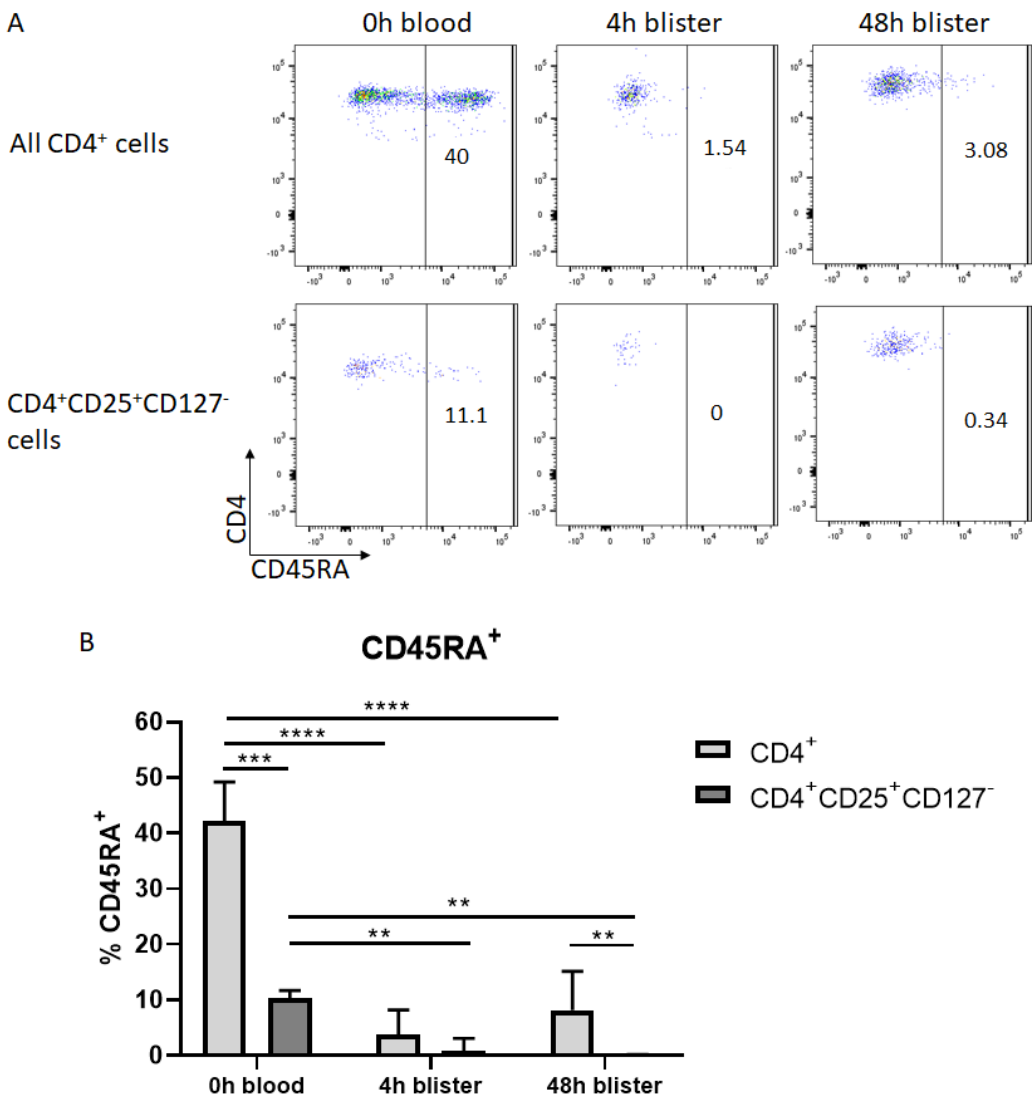

Figure 6.5.1 CD45RA expression by CD4$^+$ and CD4$^+$CD25$^+$CD127^{low} cells in the blister fluid at the site of *S. pneumoniae* intradermal injection and in whole blood prior to injection

Cells isolated from blisters raised at the site of intradermal injection of 7.5x10^5 UV-killed TIGR4 *S. pneumoniae,* or cells taken from whole blood prior to injection were stained for CD3, CD4, CD25, CD127 and CD45RA for analysis by flow cytometry. Gating for CD45RA$^+$ cells was based on the CD4$^+$CD45RA$^+$ population in whole blood (A). The percentage of CD4$^+$ and CD25$^+$CD127^{low} cells that were CD45RA$^+$ detected at 0 hours in the blood and at 4 hours and 48 hours in the blister fluid were compared (B). Graph B shows the mean percentage of CD45RA$^+$ cells detected from 6 donors, with error bars showing +/- SD of the mean. Statistical analysis in B was by two-way ANOVA (p<0.0001 interaction factor; p<0.0001 row factor "time"; p<0.0001 column factor "% of cells") with Tukey's multiple comparisons test (**p<0.01, ***p<0.001, ****p<0.0001).

The level of CD45RA expression by $CD4^+$ T cell subsets was compared between the 0 hour blood sample and the 4 hour and 48 hours blister samples. $CD4^+$ T cells in the 0 hour blood samples, 4 hour blister samples and 48 hour blister samples were separated into $CD25^-CD127^+$ resting T cells, $CD25^+CD127^+$ activated T cells and $CD25^{++}CD127^{low}$ potential Treg cells, and the CD45RA expression of these T cell subsets was classified as high expression ("$CD45RA^{++}$") moderate expression ("$CD45RA^+$") or CD45RA negative (figure 6.5.2 A).

In the 0 hour blood samples, a mean of 42% of all $CD4^+$ T cells were $CD45RA^{++}$, a mean of 43% had intermediate CD45RA expression, and a mean of 15% were $CD45RA^-$ (figure 6.5.2 B). When separating the $CD4^+$ T cells in the 0 hour blood into resting, activated and potential Treg populations, a mean of 63% of resting T cells were $CD45RA^{++}$, a mean of 25% of resting T cells were $CD45RA^+$, and a mean of 12% of resting T cells were $CD45RA^-$ (figure 6.5.2 B). Of the activated T cells in the 0 hour blood, a mean of 11% of activated T cells were $CD45RA^{++}$, a mean of 72% of activated T cells were $CD45RA^+$, and a mean of 19% of activated T cells were $CD45RA^-$ (figure 6.5.2 B). Of the potential Treg cells in the 0 hour blood, a mean of 17% of potential Treg cells were $CD45RA^{++}$, a mean of 69% of potential Treg cells were $CD45RA^+$, and a mean of 15% of potential Treg cells were $CD45RA^-$ (figure 6.5.2 B). In the 4 hour blister samples, a mean of 13% of all $CD4^+$ T cells were $CD45RA^{++}$, a mean of 52% had intermediate CD45RA expression, and a mean of 36% were $CD45RA^-$ (figure 6.5.2 B). Of the 4 hour blister sample resting T cells, a mean of 22% of resting T cells were $CD45RA^{++}$, a mean of 42% of resting T cells were $CD45RA^+$, and a mean of 37% of resting T cells were $CD45RA^-$ (figure 6.5.2 B). Of the activated T cells in the 4 hour blister sample, a mean of 10% of activated T cells were $CD45RA^{++}$, a mean of 54% of activated T cells were $CD45RA^+$, and a mean of 36% of activated T cells were $CD45RA^-$ (figure 6.5.2 B). Of the potential Treg cells in the 4 hour blister sample, a mean of 1% of potential Treg cells were $CD45RA^{++}$, a

mean of 79% of potential Treg cells were $CD45RA^{+}$, and a mean of 20% of potential Treg cells were $CD45RA^{-}$ (figure 6.5.2 B). In the 48 hour blister samples, a mean of 8% of all $CD4^{+}$ T cells were $CD45RA^{++}$, a mean of 69% had intermediate CD45RA expression, and a mean of 24% were $CD45RA^{-}$ (figure 6.5.2 B). Of the 48 hour blister sample resting T cells, a mean of 27% of resting T cells were $CD45RA^{++}$, a mean of 46% of resting T cells were $CD45RA^{+}$, and a mean of 28% of resting T cells were $CD45RA^{-}$ (figure 6.5.2 B). Of the activated T cells in the 48 hour blister sample, a mean of 13% of activated T cells were $CD45RA^{++}$, a mean of 59% of activated T cells were $CD45RA^{+}$, and a mean of 29% of activated T cells were $CD45RA^{-}$ (figure 6.5.2 B). Of the potential Treg cells in the 48 hour blister sample, a mean of 0.1% of potential Treg cells were $CD45RA^{+}$, a mean of 79% of potential Treg cells were $CD45RA^{+}$, and a mean of 21% of potential Treg cells were $CD45RA^{-}$ (figure 6.5.2 B).

Combining these data with the data in part 6.4 indicate that a large proportion (approximately 42%) of $CD4^{+}$ T cells in the 0 hour blood sample highly express CD45RA, especially those in the resting T cell population, which constitute ~57% of the $CD4^{+}$ T cell subsets, of which ~63% are $CD45RA^{++}$. The remaining resting T cells were represented by moderate CD45RA expression (25% of resting T cells) or $CD45RA^{-}$ (~12% of resting T cells). Activated T cells with moderate CD25 expression represent ~31% of the 0 hour blood samples, however a smaller percent (~11%) of these cells highly express CD45RA, with the majority expressing moderate levels of CD45RA (~72%) followed by $CD45RA^{-}$ (19%). Finally, ~6.4% of blood $CD4^{+}$ T cells were in the Treg gate, and the majority of these expressed moderate levels of CD45RA (~69%), followed by those highly expressing CD45RA (~17%), with $CD45RA^{-}$ representing the smallest percentage (~15%).

The 4 hour blisters contained a lower proportion of resting T cells and a higher proportion of activated T cells compared to the 0 hour blood samples, with the 4 hour blisters containing 28% resting T cells and 42% activated T cells compared to 57%

resting T cells and 31% activated T cells in the blood. Unlike the blood samples, the majority (~42%) of the resting T cells in the 4 hour blister sample expressed moderate levels of CD45RA, followed by $CD45RA^{-}$ (37%), and the smallest proportion were $CD45RA^{++}$ (22%). The majority (~54%) of the 4 hour blister activated T cells expressed moderated levels of CD45RA, followed by $CD45RA^{-}$ cells (~36%) and ~10% highly expressed CD45RA. A larger proportion of the $CD4^{+}$ T cells in the blister samples fell into the Treg gate (~22%) than in the blood sample (~6.4%). The majority of the 4 hour blister potential Treg cells expressed moderate levels of CD45RA (~79%), followed by $CD45RA^{-}$ cells (~20%) and only 1% expressed high levels of CD45RA. A higher proportion (~22%) of resting T cells express high levels of CD45RA than activated T cells (~10%).

For most donors, the majority (~39%) of $CD4^{+}$ T cells in the 48 hour blister fluid fell into the Treg gate, The majority (~79%) of these potential Treg cells in the 48 hour blisters expressed moderate CD45RA levels, followed by $CD45RA^{-}$ cells (~21%), and only 0.1% highly expressed CD45RA. These data are similar to the 4 hour blister data, but differ from the 0 hour blood samples in that a substantial proportion (~17%) of the blood Treg cells expressed high levels of CD45RA. The resting T cells and activated T cells in the 48 hour blister samples constituted ~21% and 20% of the total $CD4^{+}$ T cells, respectively. Of the resting T cells, most (~46%) expressed moderate levels of CD45RA, ~29% were $CD45RA^{-}$ and ~27% highly expressed CD45RA. Of the activated T cells, most (~59%) expressed moderate levels of CD45RA, ~29% were $CD45RA^{-}$ and ~13% highly expressed CD45RA. A higher proportion (~27%) of resting T cells express high levels of CD45RA than activated T cells (~13%).

To summarise, over half of the blood $CD4^{+}$ T cells are resting T cells containing a large fraction of $CD45RA^{++}$ cells, 30% of the $CD4^{+}$ T cells are activated T cells with a majority expressing moderate CD45RA levels, and around 6% of blood $CD4^{+}$ T cells are Treg cells, which predominantly express moderate levels of $CD45RA^{+}$, and with

approximately equal proportions around (15-17%) either highly expressing CD45RA or are $CD45RA^-$. A smaller proportion of the 4 hour blister $CD4^+$ T cells are resting T cells, and there are increased proportions of activated T cells and potential Treg cells, with all 3 subtypes predominantly expressing moderate levels of CD45RA, an increase in $CD45RA^-$ cells and a decrease in $CD45RA^{++}$ cells. In the 48 hour blister, the majority of $CD4^+$ T cells fall into the Treg gate, and roughly equal percentages constitute the resting T cell and activated T cell groups. The majority of cells in all groups express moderate levels of CD45RA, and hardly any in the Treg group (~0.1%) express high levels of CD45RA. These data are summarised in table 6.5.

	0h blood	**4h blister**	**48h blister**
All $CD4^+$ T cells			
$CD45RA^{++}$	42.2	12.9	8.2
$CD45RA^+$	43.1	52.0	68.5
$CD45RA^-$	15.0	35.8	24.4
Activated	**31.3**	**42.0**	**21.0**
$CD45RA^{++}$	10.5	10.4	12.6
$CD45RA^+$	71.5	54.4	58.6
$CD45RA^-$	18.6	36.1	29.0
Resting	**57.1**	**28.2**	**20.4**
$CD45RA^{++}$	63.3	21.9	26.9
$CD45RA^+$	25.0	41.7	45.8
$CD45RA^-$	11.9	36.7	27.8
Treg	**6.4**	**21.7**	**39.0**
$CD45RA^{++}$	17.1	1.0	0.1
$CD45RA^+$	69.3	79.3	79.4
$CD45RA^-$	15.1	19.7	20.9

Table 6.5 CD45RA expression of $CD4^+$ T cell subsets in the blood and blister samples

Percentages of $CD45RA^{++}$, $CD45RA^-$ and CD45RA intermediate ("$CD45RA^+$") $CD4^+$ T cells within the whole $CD4^+$ T cell population ("All $CD4^+$ T cells") resting T cell ("resting"), activated T cell ("activated") and potential Treg ("Treg") populations of the 0 hour blood samples, 4 hour blister samples and 48 hour blister samples.

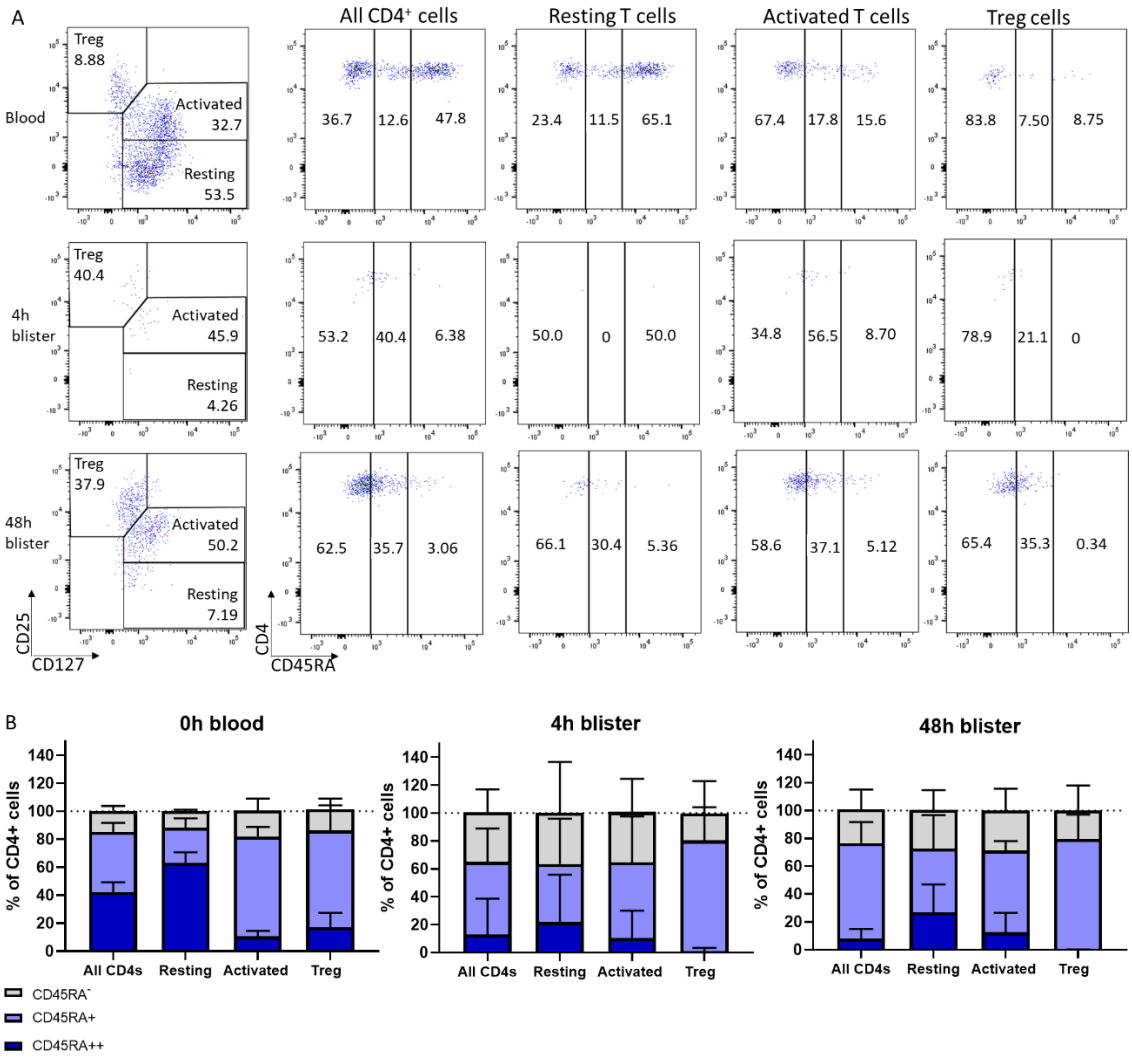

Figure 6.5.2 CD45RA expression by CD4⁺ subsets in the blood sample and blister fluid

CD4⁺ T cells in the 0 hour blood samples, 4 hour blister samples and 48 hour blister samples were separated into $CD25^{-}CD127^{+}$ resting T cells ("resting"), $CD25^{+}CD127^{+}$ activated T cells ("activated") with moderate CD25 expression, and $CD25^{++}CD127^{low}$ potential Treg cells ("Treg") with high CD25 expression, and the CD45RA expression of these T cell subsets was classified as CD45RA high expression ("$CD45RA^{++}$"), CD45RA moderate expression ("$CD45RA^{+}$") or $CD45RA^{-}$ (A).The percentages of CD4+ T cells and the resting T cell, activated T cell and Treg cell subtypes expressing high levels of CD45RA, moderate levels of CD45RA or are CD45RA- in the 0 hour blood samples, 4 hour blister samples, and 48 hour blister samples are shown (B). Data in A are representative flow cytometry plots from 1 donor. Data in B are from 6 donors combined, with error bars showing SD of the mean.

6.6 CCR6 expression of cells recruited to the *S. pneumoniae* injection site

CCR6 is reported to be expressed on Treg, Th17, Th22, and Th17-derived Th1 cells (Kleineweitfeld et. al. 2005; Yamazaki et. al., 2008; Trifari et. al., 2009; Annunziato et. al., 2007), though not on classical Th1 or Th2 cells (reviewed by Eyerich and Zielinski, 2014). CCR6 is essential for the recruitment of Treg and Th17 cells to sites of inflammation (Liao et al., 1999; Yamazaki et al., 2008).

The expression of CCR6 on the $CD4^+$ cells isolated from the blister fluid was compared to $CD4^+$ cells in the blood. Live, single cells were selected for $CD3^+$, $CD4^+$ and $CCR6^+$ expression with gating based on the 0 hour blood sample (figure 6.6.1 A). In both the 4 hour and 48 hour blister, a large proportion of $CD4^+$ cells were $CCR6^+$, with mean percentages of 80% and 86% respectively, whereas a mean of 13% of 0 hours blood $CD4^+$ cells were $CCR6^+$ (figure 6.6.1 B). The number of $CD4^+CCR6^+$ cells per ml was also increased from 4 hours to 48 hours in the blister fluid (figure 6.6.1 C).

The same $CCR6^+$ gate was applied to the $CD4^+CD25^+CD127^{low}$ cell population detected in the blood and the 4 hour and 48 hour blister fluid. A mean of 2% of $CD4^+CD25^+CD127^{low}$ cells in 0 hour blood were $CCR6^+$, whereas 89% and 91% of $CD4^+CD25^+CD127^{low}$ cells were $CCR6^+$ in the blisters at 4 hours and 48 hours, respectively (figure 6.6.2 A). The number of $CCR6^+$ $CD4^+CD25^+CD127^{low}$ cells per ml was also increased from 4 hours to 48 hours in the blister fluid (figure 6.6.2 B).

To summarise, the majority of $CD4^+$ cells and $CD4^+CD25^+CD127^{low}$ cells recruiting to the injection site were $CCR6^+$ at both 4 hours and 48 hours post-injection.

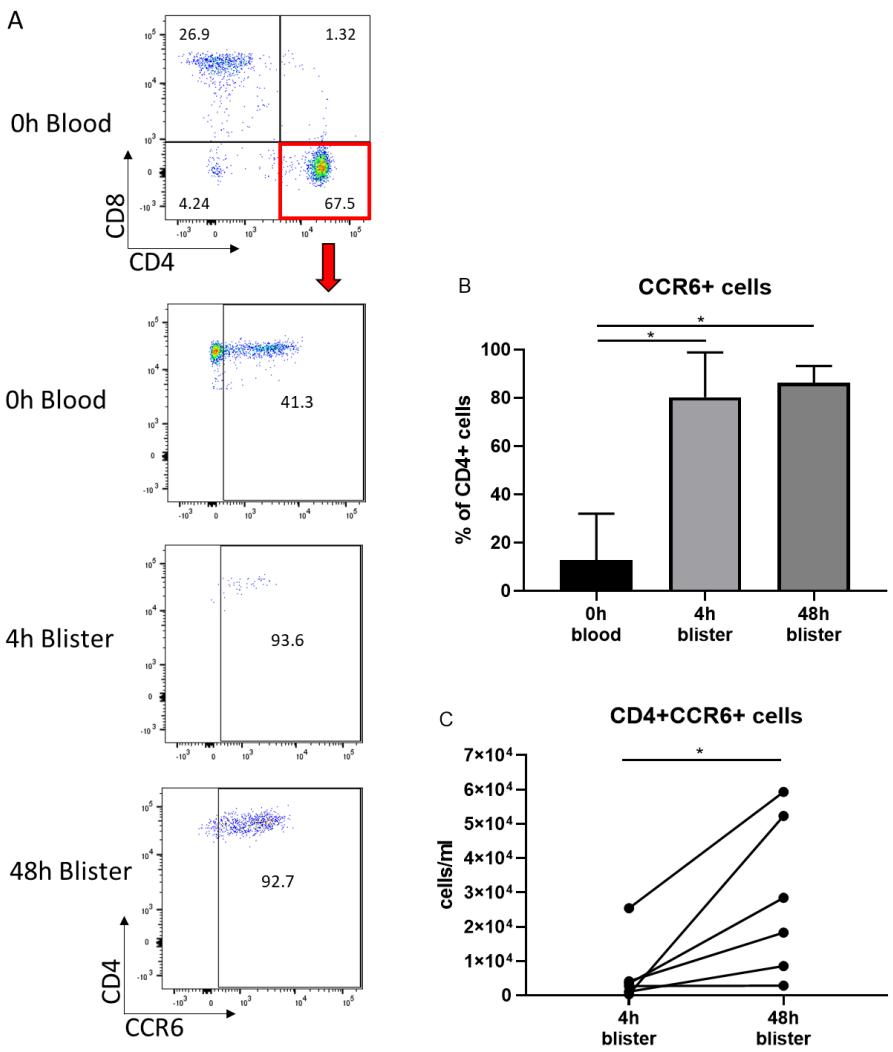

Figure 6.6.1 CCR6 expression by CD4⁺ cells in blister fluid at the site of UV-killed *S. pneumoniae* intradermal injection and in peripheral blood prior to injection

Peripheral blood cells from volunteers prior to intradermal injection of $7.5x10^5$ UV-killed TIGR4 *S. pneumoniae* into the forearm and cells taken from the fluid from blisters raised at the site of injection after 4 hours and 48 hours were analysed for CCR6 expression by $CD4^+$ cells. $CD4^+CCR6^+$ cells were gated based on visible positive populations from the blood (A), the percentages of $CD4^+$ cells expressing CCR6 were compared (B) and the number of $CD4^+CCR6^+$ cells per ml in the blister fluid compared per donor (C). Graph B shows the mean percentage from 6 donors, with error bars showing +/- SD of the mean. Graph C shows the absolute cell counts from 6 individual donors, with each data point representing 1 donor. Statistical analysis in B was by Kruskal-Wallis test ($p<0.01$) with Tukey's multiple comparisons test (*$p<0.05$). Statistical analysis in C was by Wilcoxon test (*$p<0.05$) with data paired across time points for each donor.

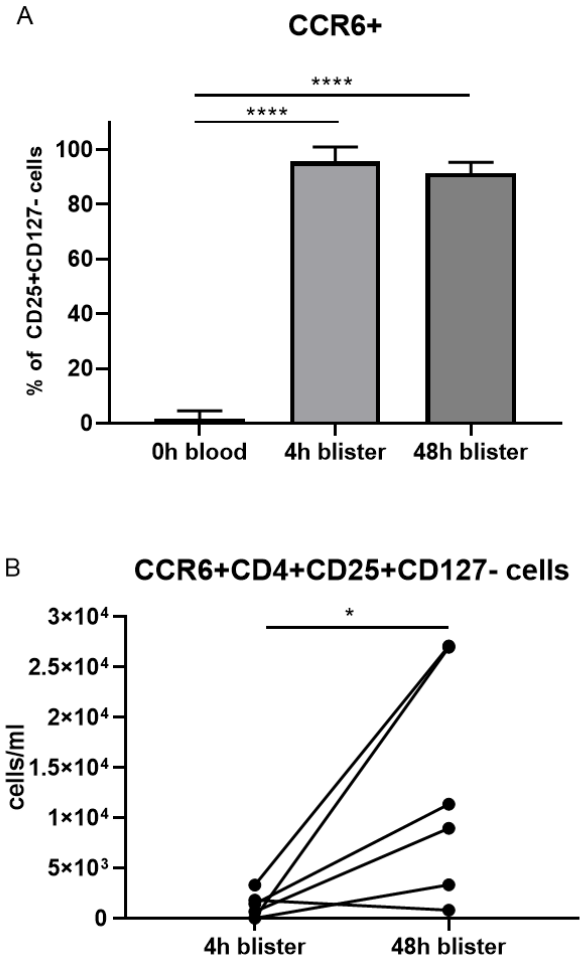

Figure 6.6.2 CCR6 expression by $CD4^+CD25^+CD127^{low}$ cells from blister fluid at the site of intradermal injection of UV-killed *S. pneumoniae* and from peripheral blood prior to injection

Cells taken from peripheral blood from volunteers prior to intradermal injection of $7.5x10^5$ killed TIGR4 *S. pneumoniae* into the forearm and cells taken from the fluid from blisters raised at the site of injection after 4 hours and 48 hours were analysed for CCR6 expression by $CD4^+CD25^+CD127^{low}$ cells. $CCR6^+$ cells were gated based on visible positive populations from the blood as shown in figure 6.5.1. The percentages of $CD4^+CD25^+CD127^{low}$ cells expressing CCR6 (A) and the number of $CCR6^+CD4^+CD25^+CD127^{low}$ cells per ml in the blister fluid were compared (B). Graph A shows the mean percentage from 6 biological replicates (6 donors), with error bars showing +/- SD of the mean. Graph B shows the absolute cell counts from 6 individual donors, with each data point representing 1 donor. Statistical analysis in A was by one-way ANOVA (*p<0.0001) with Tukey's multiple comparisons test (****p<0.0001). Statistical analysis in B was by paired t test (*p<0.05) with data paired across time points for each donor.

6.7 Enumerating granulocytes and agranulocytes in the blister fluid

Visible clusters of granulocytes and agranulocytes could be seen in the flow cytometry plots of cells taken from the blister fluid based on side-scatter and forward-scatter. The majority of granulocyte populations in blood are neutrophils and the predominant $CD3^-$ agranulocytes are monocytes. Therefore, the proportions and cell numbers of these populations were analysed as an indication of the presence of monocytes and neutrophils in the blisters. Using a whole blood sample to determine gates, live cells were separated into granulocytes and agranulocytes based on side-scatter (area) and forward-scatter (area), and the agranulocytes were separated into $CD3^-$ and $CD3^+$ populations (figure 6.7 A). Dead cells and doublets were excluded (not shown). The percentages of granulocytes and agranulocytes were similar between the blood samples and the 4 hour blister fluid cells, with granulocytes as the predominant cell type and with no statistically significant differences in the proportions of these cell types between the blood and 4 hour blister (figure 6.7 B). However, the 48 hour blister fluid contained higher percentages of agranulocytes than granulocytes and these proportions were statistically significantly different from both the blood samples and the 4 hour blister samples (figure 6.7 B). These results suggest that granulocytes (likely neutrophils) are the predominant cell type in the early response to the intradermal *S. pneumoniae* challenge, and by 48 hours post-injection the neutrophil response has declined and agranulocytes (likely monocytes and lymphocytes) predominate. To separate the lymphocytes from the rest of the agranulocyes, the CD3 expression in this population was analysed. There were no statistically significant differences in $CD3^-$ compared to $CD3^+$ populations in either the 4 hour blister fluid samples or the 48 hour blister fluid samples, although a trend towards increase in both $CD3^-$ and $CD3^+$ cells was observed from 4 hours to 48 hours (figure 6.7 C).

Numbers of granulocytes, agranulocytes and $CD3^-$ agranulocytes in the 4 hours and 48 hours blister fluid were compared. Granulocyte numbers showed a trend towards decrease in numbers from 4 hours to 48 hours with the difference close to statistical significance at p=0.063(figure 6.7 D), which correlates with the decrease in their percentage of the total cell population (figure 6.7 B).The numbers of agranulocytes (figure 6.7 E) and $CD3^-$ agranulocytes (figure 6.7 F) generally showed a trend towards increase from 4 hours to 48 hours, although this was not statistically significant and was not the case for all donors. Of note, although red blood cells were eliminated as much as possible during preparation of the cells for flow cytometry, it is likely that the $CD3^-$ agranulocyte populations contained red blood cells that survived the lysis step. This would give an overestimate of the $CD3^-$ agranulocyte populations and was likely to have been the case with the donor that had very high cell counts at 4 hours as can be seen in figure 6.6 D-F. Therefore, the value of using scatter profiles to analyse cell populations is limited and is used here only as an indication of the potential cell populations that could be examined in more detail in future work by addition of further markers.

In summary, these preliminary data indicate that neutrophils likely predominated in the early response to the intradermal *S. pneumoniae* injections at 4 hours post-injection, whereas at 48 hours post-injection neutrophil numbers declined and agranulocytes numbers increased. The agranulocyte populations contained both $CD3^+$ lymphocytes and $CD3^-$ cells which were likely monocytes.

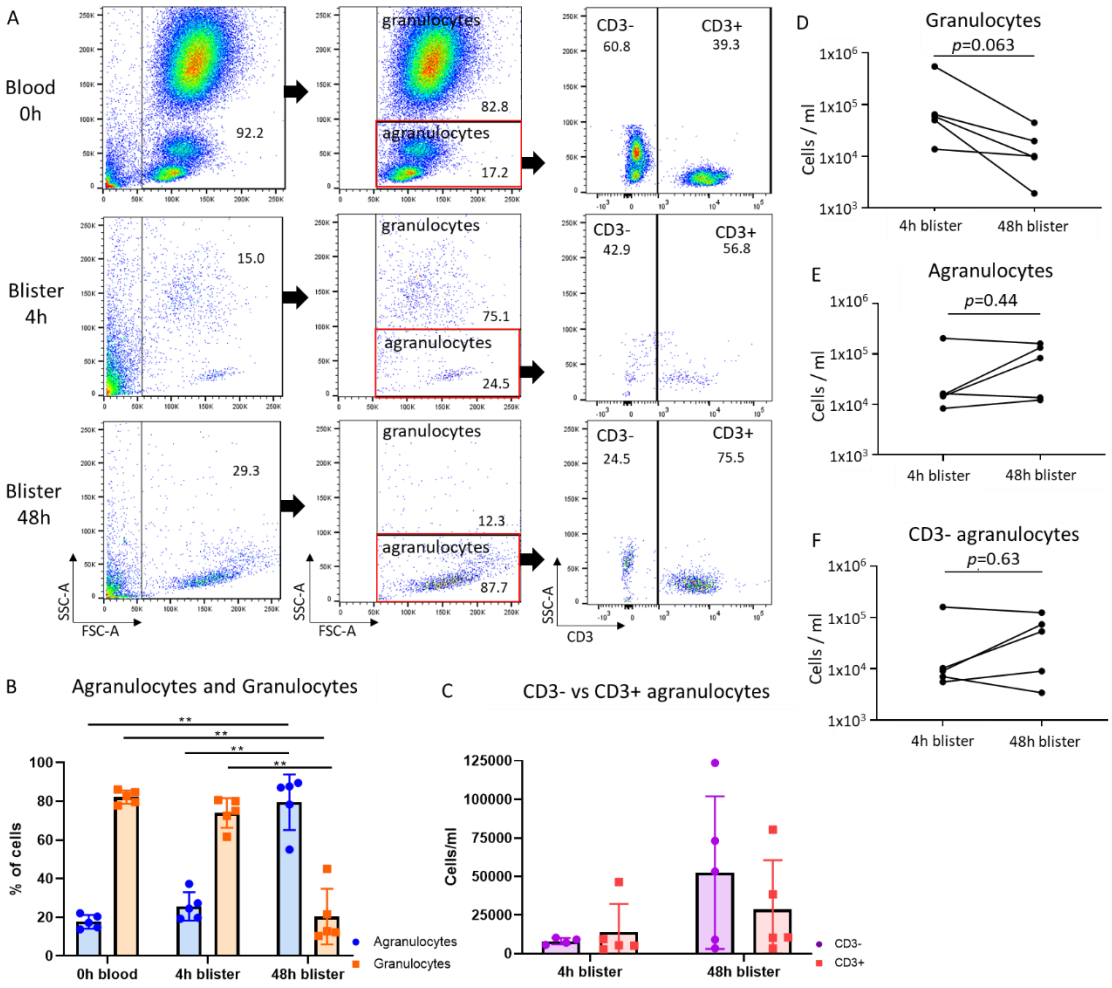

Figure 6.7 Enumerating granulocytes and agranulocytes in the blister fluid

Granulocytes, agranulocytes and $CD3^-$ agranulocytes in whole blood samples prior to *S. pneumoniae* intradermal injection and in the 4 hour and 48 hour post-injection blister fluid were analysed by flow cytometry based on their side-scatter and forward-scatter profiles (A). The percentages of granulocytes and agranulocytes in blood and in the blister fluid (B), numbers of $CD3^-$ and $CD3^+$ agranulocytes in blister fluid (C), and the numbers of granulocytes (D), agranulocytes (E) and $CD3^-$ agranulocytes (F) in the blister fluid were compared. Figure A shows the gating strategy on 1 representative donor used for analysis. Figures B-F show data from 5 donors, with each point representing data from one donor. Statistical analysis was by two-way repeated measures ANOVA in B (row factor "time" not significant, column factor "cell type" $p<0.001$, interaction factor $p<0.0001$), by mixed-effects model ANOVA in C (row factor "time", column factor "cell type" and interaction factor not significant), and by Wilcoxon matched-pairs signed rank test in D-F). Post-test in B and C was Tukey's multiple comparisons test (**$p<0.01$). Error bars in B and C show SD of the mean.

6.8 Concentration of cytokines detected in the fluid of blisters raised at the site of UV-killed *S. pneumoniae* intradermal injection

The concentration of TNFα, IL-6, IL-1β, CXCL8, IL-17, IL-22 and IL-10 in the blister fluid was detected by ELISA. There was a statistically significant decrease in the concentrations of TNFα and CXCL8 in the blister fluid from 4 hours to 48 hours (figure 6.8 A and B). IL-6 concentration was approximately 100-fold higher than that of TNFα and did not show statistically significant differences between 4 hours and 48 hours post-injection at p=0.1053, although a trend towards decreasing concentration was seen from 4 hours to 48 hours (figure 6.8 C). The concentrations IL-1β was close to statistically significant decrease at 48 hours compared to 4 hours at p=0.0669 (figure 6.8 D). Concentrations of IL-17 (E) and IL-22 (F) were significantly increased from 4 hours to 48 hours. Detection of IL-10 was variable across donors, and the concentration did not differ between 4 hours and 48 hours (figure 6.8 G). These data suggest an overall trend towards decreasing inflammation from 4 hours to 48 hours, compatible with the changes seen in blood flow between these time points (figure 6.2) and with decreased granulocyte infiltration and increased T cell recruitment.

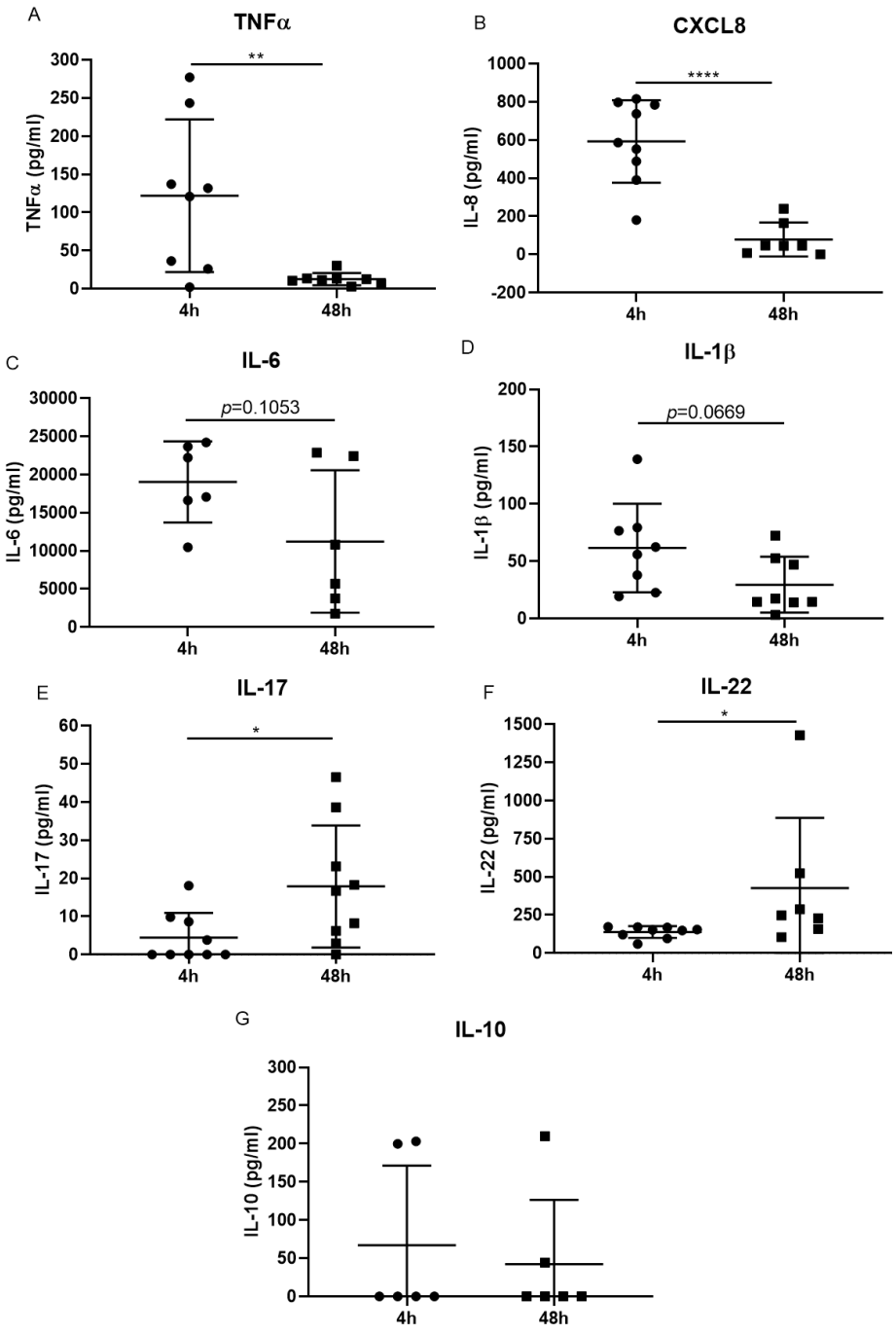

Figure 6.8 Cytokine concentrations in the fluid of blisters raised at the site of *S. pneumoniae* intradermal injection

The concentration of TNFα (A), CXCL-8 (B), IL-6 (C), IL-1β (D), IL-17 (E) IL-22 (F) and IL-10 (G) in the blister fluid at the site of intradermal injection of $7.5x10^5$ UV-killed TIGR4 *S. pneumoniae* was detected by ELISA. Graphs show the mean concentration of cytokines detected from 8 donors with each data point representing 1 donor, and error bars showing +/- SD of the mean. Statistical analysis was by unpaired t test in A-D, and by Mann-Whitney test in E-G due to non-normality of the data (*p<0.05, **p<0.01***p<0.001, ****p<0.0001).

6.9 Immunofluorescence staining of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells in human normal lung

The intradermal *S. pneumoniae* experiments suggested that $CD4^+CD25^+CD127^{low}$ cells are increased at the site of *S. pneumoniae* challenge in the skin at 48 hours. However, the co-culture experiments in part 4.4 suggested that Treg cells interacting with MDMs prior to infection results in a statistically significant reduction in the inflammatory response of those MDMs upon subsequent infection with *S. pneumoniae*. The presence of Treg cells already present in normal lung was investigated by immunofluorescence (IF) staining for CD4 and Foxp3.

The CD4 and Foxp3 antibodies and their secondary antibodies were first tested on cytospins of PBMCs to ensure positive staining of both $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells (figure 6.9 A, B and C). The presence of Treg cells in the lungs was then investigated by immunofluorescence staining of normal human lung for CD4 and Foxp3 expression. Both $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells were found in lung sections taken from 3 donors (figure 6.9 D, E and F). Overall by visual observation the majority of these cells were $CD4^+Foxp3^-$ (figure 6.9 G).

Although $CD4^+Foxp3^+$ cells were located in the lung, further analysis to prove their suppressive ability should be conducted before describing them as Treg cells. Additional work to quantify the ratio of $CD4^+Foxp3^-$ to $CD4^+Foxp3^+$ cells would be useful as this was not included in the current experimental design.

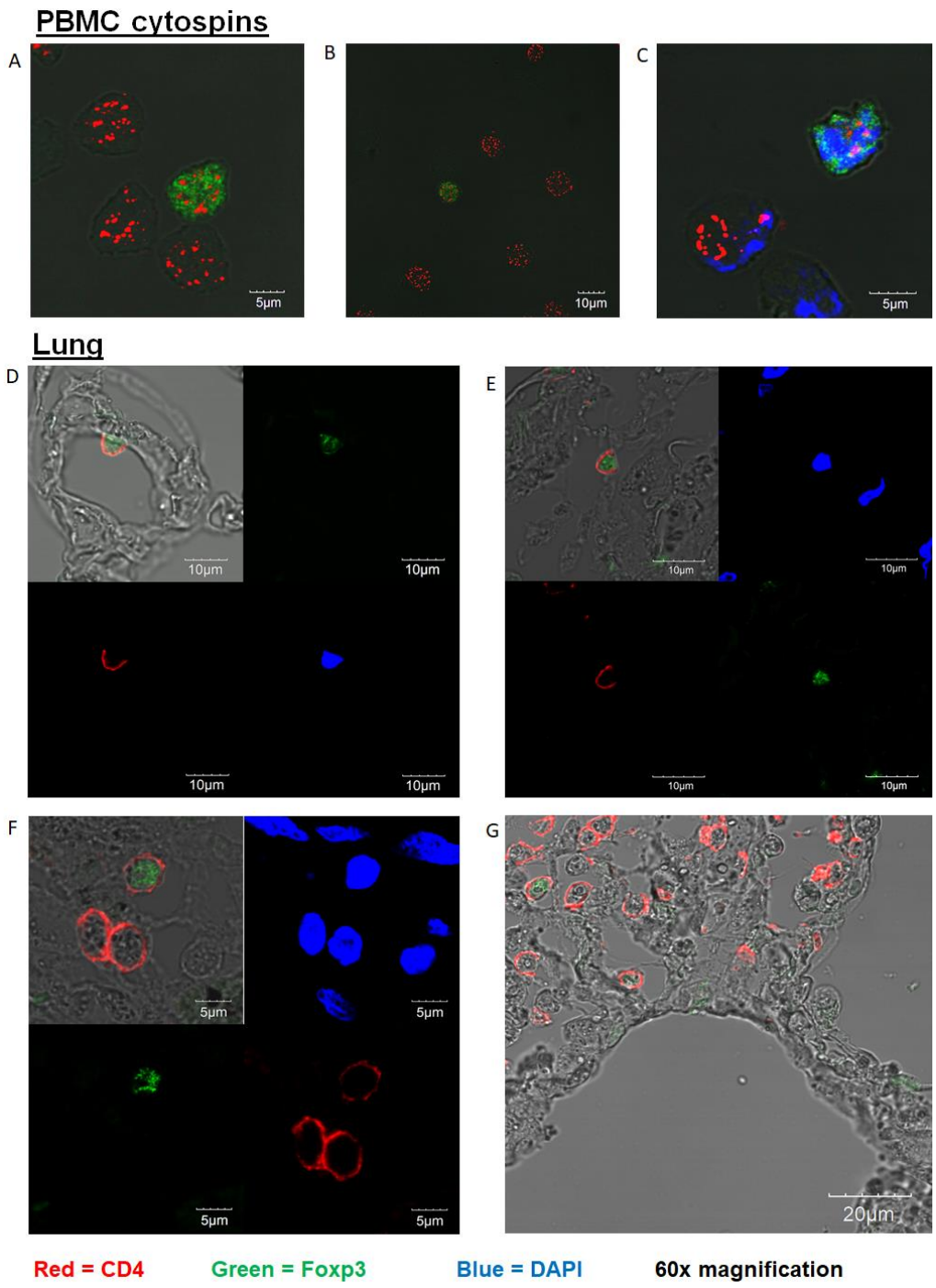

Figure 6.9 IF staining of PBMC and human lung for CD4 and Foxp3

PBMC cytospins (A-C) and normal human lung (D-G) were stained for CD4, Foxp3 and DAPI and viewed by confocal microscopy at 60x magnification. CD4 is stained red, Foxp3 is stained green and DAPI is blue. Figures A, B and G, and the top left panels in D-F show images without blue DAPI in order to clearly visualise the green Foxp3. A and B show a representative image each from 2 donors. D-F show a representative image each from 3 separate donors. Figure G is an example of the distribution of $Foxp3^-$ compared to $Foxp3^+$ cells from one donor.

6.10 Chapter 6 summary

Blood flow to the site of intradermal injection of UV-killed *S. pneumoniae* into the forearms of healthy volunteers was increased at 4 hours and 24 hours post-injection compared to the baseline and was decreased by 48 hours. The numbers of CD3, CD8 and $CD4^{+}CD25^{+}CD127^{low}$ cells detected in the blister fluid was increased at 48 hours compared to 4 hours (CD4 was close to statistical significance at p=0.0625). Approximately 15% of CD4 cells were $CD25^{+}CD127^{low}$ in the 4 hour blister and 40% in the 48 hour blister – which are large percentages compared to the mean 6.4% found in the peripheral blood prior to injection. The vast majority of both $CD4^{+}$ and $CD4^{+}CD25^{+}CD127^{low}$ cells either expressed CD45RA at moderate levels or were $CD45RA^{-}$, suggesting an antigen-experienced effector/memory phenotype. The predominant $CD4^{+}$ T cell subtype in the 0 hour blood samples were resting T cells containing a large fraction of $CD45RA^{++}$ cells, whereas the 4 hour blisters contained larger proportions of activated T cells with moderate CD45RA expression, and the largest subtype for most donors in the 48 hour blister samples were the potential Treg cells, indicating progression from the early highly inflammatory response at 4 hours towards resolution at 48 hours. The moderate CD45RA expression may indicate increased proliferation and increased cytokine production of these T cells compared to the $CD45RA^{-}$ memory cells and the $CD45RA^{++}$ naïve/resting cells. As could be expected, the post-injection blister fluid contained a lower proportion of resting T cells than in peripheral blood prior to *S. pneumoniae* injection.

Means of >80% of the $CD4^{+}$ cells and >89% of $CD4^{+}CD25^{+}CD127^{low}$ cells in the blister fluid were $CCR6^{+}$, suggesting tissue-homing/resident Treg, Th17 or Th22 phenotypes were dominant. Analysis of granulocytes and agranulocytes based on scatter profiles indicated that neutrophils likely predominated in the 4 hour blisters, and their numbers declined at 48 hours, at which time agranulocytes (likely monocytes and lymphocytes) predominated. The reduced presence of granulocytes correlated with the reduced

CXCL8 concentration in the 48 hour blisters. Further analysis of the agranulocyte markers would be beneficial, in particular surface markers indicating highly pro-inflammatory and low/anti-inflammatory macrophage phenotypes along with better Treg characterisation would add to the data in this thesis regarding Treg-macrophage interaction.

Although examination of cytokine profiles by individual cell types by intracellular staining was not possible, the cytokines present within the blister fluid were examined. TNFα and IL-8 showed statistically significant decrease in the 48 hour blister compared to the 4 hour blister. Trends suggested lowered IL-6 and IL-1β from 4 hours to 48 hours although this was not statistically significant. Conversely, IL-17 and IL-22 increased from 4 hours to 48 hours. There was no difference in IL-10 concentration between 4 hours and 48 hours, and not all donors had detectable IL-10 in the blister fluid. Overall, the generally lowered pro-inflammatory cytokine concentrations along with the lowered blood flow detected with the Doppler scan images suggest that the inflammatory response is lowered at 48 hours compared to a peak at 24 hours, and these decreases coincided with large increases in antigen-experienced effector/memory phenotype $CD4^{+}CD25^{+}CD127^{low}$ cells.

Experimental design did not allow for intracellular staining for transcription factors or cytokine profiles of the cells recruited to the intradermal injection of *S. pneumoniae* and additionally cells could not be FACS sorted to perform a suppression assay to determine whether the $CD25^{+}CD127^{low}$ fraction were definite Treg cells. This was due to the low numbers of cells collected from the blister fluid. However, these experiments have helped develop a useful model of the human inflammatory response to *S. pneumoniae* which could be used in the future to determine Treg cell responses if a more specific surface marker profile for Treg cells becomes available.

Immunofluorescence staining for CD4 and Foxp3 found both $CD4^{+}Foxp3^{-}$ and $CD4^{+}Foxp3^{+}$ cells in human normal lung sections. This could indicate the presence of

lung resident Treg cells that are capable of rapidly modulating responses to *S. pneumoniae* upon infection, or influencing macrophage inflammatory potential prior to infection. Further work to assess the suppressive ability of these cells in precision lung slice models could help determine whether they are Treg cells. In addition, the current experimental design did not include quantification of the proportions of $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells, and future work should also investigate whether there are differences in these CD4/Treg populations in the lungs from subjects with lung diseases that predispose to pneumonia (e. g. COPD).

In conclusion:

- Blood flow to the site of intradermal injection of UV-killed *S. pneumoniae* peaks at 24 hours and is resolving at 48 hours
- $CD3^+$, $CD4^+$, $CD8^+$ and $CD4^+CD25^+CD127^{low}$ cells are increased at 48 hours compared to 4 hours
- The proportions of agranluocytes increases and the proportion of granulocytes decreases from the 0 hour blood samples to the 48 hour blister samples
- The majority of $CD4^+$ and $CD4^+CD25^+CD127^{low}$ cells are antigen-experienced cells expressing moderate/low CD45RA and express $CCR6^+$, suggesting a tissue-homing/resident Treg or Th17 or Th22 phenotype
- TNFα and IL-8 concentration in the blisters decreased from 4 hours to 48 hours, whereas IL-17 and IL-22 were increased
- $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ were both detected in human normal lung

Chapter 7: Discussion

The experiments in this thesis have investigated the effects of Treg cells on macrophage inflammatory responses to *S. pneumoniae* and begun to identify whether Treg cells are likely to be present at the site of *S. pneumoniae* infections. The major findings were:

1. Anti-inflammatory effects of human Treg cells on the inflammatory responses of macrophages to *S. pneumoniae,* but not to the Gram negative antimicrobial resistant pathogen *A. baumannii*
2. Treg cells reduced the inflammatory response of macrophages to *S. pneumoniae* when cultured prior to infection, and to a lesser extent when added simultaneously with infection
3. Teff cells reduced macrophage pro-inflammatory responses when added during *S. pneumoniae* infection, but the anti-inflammatory effect took longer than that seen with Treg cells
4. Suppression of pro-inflammatory cytokine production requires a mechanism involving cell contact between Treg cells and macrophages
5. Immunofluorescent staining for $CD4^+$ and $Foxp3^+$ T cells in human lung sections identified that Treg cells are present in normal human lung
6. A human infection model involving intradermal injection of killed *S. pneumoniae* in healthy volunteers detected recruitment of T cell subsets to the site of pneumococcal challenge

7.1 Effects of Treg or Teff cells on MDM inflammatory responses to *S. pneumoniae*

7.1.1 Data obtained using monocyte-derived macrophages

All *in vitro* macrophage experiments used MDMs differentiated from monocytes in the presence of M-CSF. Supernatant TNFα, IL-6 and IL-1β as three of the main cytokines upregulated by *S. pneumoniae*-infected MDMs were used as the readout of MDM pro-inflammatory responses (Tomlinson et al., 2014). Macrophage development *in vivo* is thought to be driven principally by M-CSF, which is why this method for MDM culture was used (Hume, 2006). However, the main contributor to the inflammatory response during initial *S. pneumoniae* infections are tissue resident macrophages such as alveolar macrophages. Alveolar macrophages may respond differently than MDMs to *S. pneumoniae* and Treg/Teff co-culture *in vitro*, and it would be informative to investigate whether similar results obtained with MDMs are replicated using alveolar macrophages. Several differences and similarities between alveolar macrophages and MDMs have been reported. Alveolar macrophages are commonly thought to have predominant anti-inflammatory properties due to prostaglandin E2 (PGE2), TGFβ and IL-10 production, however freshly isolated alveolar macrophages have been found to have greatly increased expression of genes associated with pro-inflammatory responses, which were higher than those of MDMs by comparison (Tomlinson et al., 2012). In terms of surface proteins, similar levels of the CD206 mannose receptor and the CD80 co-stimulatory marker on the MDM and alveolar macrophage surface have been reported, whereas CD163 and CD169 and were higher on MDMs (Lescoat et al., 2018). CD163 is considered a marker of macrophages with an anti-inflammatory phenotype, and $CD169^{+}$ macrophages are associated with an immune tolerance phenotype and allow *S. pneumoniae* intracellular replication, suggesting that MDMs could be less efficient in bacterial control than alveolar macrophages (Ercoli et al., 2018). MHC II were particularly

upregulated in alveolar macrophages compared to MDM, suggesting increased antigen presentation capacity of alveolar macrophages (Tomlinson et al., 2012). However, isolation and *in vitro* culture of AMs would require obtaining fresh human bronchoalveolar lavage fluid, and the conditions required for co-culture with T cells have not been established in our laboratory making these experiments technically challenging.

It would also would have been interesting to see if MDMs differentiated with GM-CSF as opposed to M-CSF are affected differently by Treg/Teff co-culture and *S. pneumoniae* infection. MDMs differentiated in the presence of M-CSF have been shown to have a more anti-inflammatory phenotype, whereas differentiation with GM-CSF promotes a more inflammatory macrophage subtype (Verreck et al., 2004). Despite this, the MDMs differentiated using M-CSF in the experiments in this thesis produced large concentrations of pro-inflammatory cytokines upon *S. pneumoniae* infection, suggesting acquisition of an activated pro-inflammatory macrophage phenotype. MDMs differentiated with GM-CSF are also reported as showing more similarities to alveolar macrophages compared with M-CSF differentiated MDMs (Lescoat et al., 2018), and so repeating these experiments with GM-CSF MDMs would provide interesting additional data on the Treg- co-cultured macrophage response to *S. pneumoniae*.

7.1.2 Optimising MDM-Treg/Teff co-culture conditions

Experiments were carried out to establish the optimal conditions to observe effects of Treg cells on macrophage inflammatory responses to *S. pneumoniae*. Addition of both anti-CD3 and anti-CD28 antibodies in the culture medium resulted in little effect of addition of Tregs on the MDMs inflammatory response. High doses of anti-CD28 antibody may abrogate the suppressive effects of Treg cells (Takahashi et al., 1998), hence anti-CD28 was removed from the experimental method. Subsequently, statistically significant decreases in pro-inflammatory cytokine production was

observed with medium supplemented only with anti-CD3. This is a condition that has been widely used in Treg cultures with APCs (Taams et al., 2005; Abraham et al., 2008; Shevach, 2018). Whether Treg cells could suppress macrophage responses in the absence of anti-CD3 antibodies was not investigated in the current experiments. A previous study found that anti-CD3 antibody during Treg/Teff-monocyte co-culture was required for Treg effects on monocyte activation (Taams et al., 2005). Additionally, culturing monocytes in the presence of anti-CD3 antibody alone also did not cause monocyte activation (Taams et al., 2005). These suggest that the anti-CD3 antibody is required for Treg effects on macrophage / monocytes but its presence alone does not affect the monocytes.

Evidence suggests that anti-CD3 antibodies can encourage preferential Treg activity/expansion (Belghith et al., 2003; Valle et al., 2015). In this thesis, Teff cells were sometimes suppressive towards MDM pro-inflammatory cytokine production, and experiments examining $CD4^+$ T cell differentiation also included anti-CD3 antibody in the medium and in which high levels of Foxp3 were observed. However, it has been reported that anti-CD3 therapy does not promote the conversion of conventional T cells into regulatory cells *in vivo*, and instead promotes proliferative expansion of existing Treg populations, suggesting that Teff cells were not being converted to Treg cells by anti-CD3 stimulation (Nishio et al., 2010; Penaranda et al., 2011). In the experiments investigating $CD4^+$ T cell differentiation, Treg cells would have been present which may have been preferentially expanded to account for the increased $Foxp3^+$ population.

The suppressive ability of Treg cells isolated by the sorting protocol used in this thesis could be better determined by using an improved suppression assay or by more detailed phenotyping of the Treg. A more appropriate protocol for determining the ability of the Treg cells to suppress Teff proliferation may include labelling of the Teff cells with cell trace violet to determine a diluting effect of the tracer through rounds of

proliferation, and by use of APCs to better stimulate Teff proliferation. All cells should be stained with Foxp3 in this protocol in order to separate Treg from Teff cells during flow cytometry analysis. Assessment of the methylation of the TSDRs would also be useful in confirming Treg identity. It would also be beneficial to examine the expression of markers highly expressed by Treg cells such as CTLA4, TNFR2, CD73, CD39 and GARP to help confirm Treg identity and also to help determine which surface mechanisms may be utilised by the Treg to suppress macrophage responses.

The ratio of Treg/Teff cells to MDMs used in these experiments was 1 Treg/Teff per 3 MDMs. Using fewer Treg cells in culture with MDMs resulted in loss of statistically significant suppression of pro-inflammatory cytokine production. In the peripheral blood of healthy volunteers, monocytes and Treg cells constitute approximately 5% and 0.01% of leukocytes, respectively. Therefore, the ratio used in the *in vitro* experiments are unlikely to be realistic *in vivo*. However, it is difficult to predict the increases in these cell numbers upon infection, particularly when considering the contribution of peripheral Treg cells differentiating from naïve $CD4^+$ T cells upon antigen exposure, the proportion of monocytes differentiating into macrophages, and sparse data on the presence of tissue-resident Treg cells. In addition, the $CD25^+CD127^{low}$ population of cells recovered from the *S. pneumoniae* skin blister constituted approximately 40% of $CD4^+$ T cells, indicating that there may be increased proportions of Treg cells present at infection sites. However, the purpose of the *in vitro* work was to assess whether Treg cells could have an anti-inflammatory effect on macrophages, hence the experiments used a relatively high Treg to macrophage ratio. The magnitude of the decrease in pro-inflammatory cytokine production seen *in vitro* was large, and may be too great an effect to be beneficial *in vivo* whereas less marked modulation of inflammatory responses by a lower Treg to macrophage ratio could be beneficial to the host.

7.1.3 IL-10 and TGFβ reduce MDM inflammatory responses to *S. pneumoniae*

Exogenous addition of IL-10 or TGFβ to MDMs prior to infection with *S. pneumoniae* reduced supernatant TNFα, IL-6 and IL-1β levels. The IL-10 dosage was determined based on reports that 100ng/ml of exogenous IL-10 caused maximal suppression of pro-inflammatory cytokine production in the monocytic cell line THP-1 (Murthy et al., 2000). In the same study by Murthy et al., 1ng/ml IL-10 was also sufficient to show statistically significant reduction in pro-inflammatory cytokine production. 1ng/ml is also similar to the concentration of IL-10 produced by Teff-MDM co-culture in the *in vitro* experiments in this thesis and to the IL-10 concentrations reportedly produced by APC-Treg cell culture by others (Kryczek et al., 2006). The dosage of TGFβ was more difficult to determine. TGFβ is reported to have both pro- and anti-inflammatory effects (Vaday et al., 2001; Wahl et al., 1987; Wiseman et al., 1988; McCartney-Francis et al., 1990; Celada et al., 1992). Initially, experiments used the same 1ng/ml and 100ng/ml dosages as those used in IL-10 experiments. However, at 100ng/ml, very variable results were obtained, with occasional suppression and occasional enhancement of the macrophage pro-inflammatory cytokine production. Based on findings of statistically significant anti-inflammatory effects on monocytes/macrophages at 1-50ng/ml, the upper dosage was decreased to 50ng/ml (Bogdan et al., 1992; Hamon et al., 1993; Imai et al., 2000). These doses lead to more consistent effects on MDM TNFα, IL-6 and IL-1β responses, with statistically significant reduction in TNFα and IL-6 and a trend towards reduced IL-1β reduction with 50ng/ml TGFβ.

7.1.4 Treg cells reduced MDM inflammatory responses to *S. pneumoniae*

When MDMs were cultured with Treg cells prior to infection, there was statistically significant reduction in TNFα, IL-6 and IL-1β from 6 hours post-infection. This suppression required contact between the MDMs and Treg cells, as incubation of the

MDMs in Treg conditioned media or incubating the MDMs and Treg cells separated by transwell inserts were not able to cause suppression of MDM inflammatory responses. Notably, Treg cells did not make the anti-inflammatory cytokine IL-10. However, Treg cells did not have to be present during the MDM infection in order to reduce the inflammatory response of the MDMs, as removal of the Treg cells from the culture prior to infection still resulted in a reduced inflammatory response by the MDMs upon infection. In fact, delaying the infection by 24 hours after removing the Treg cells retained the suppressed inflammatory response of the MDMs. Treg cells also decreased the inflammatory response of MDMs when added simultaneously to infection, however this decrease was not as statistically significant as when Treg cells had been incubated with MDMs prior to infection. These data reflect similar findings from Taams and colleagues, who reported reduced pro-inflammatory cytokine production from monocytes cultured with Treg cells and stimulated with LPS, and from Roquilly and colleagues who found that Treg cells reduced APC responses in *E. coli* pneumonia (Taams et al., 2005; Roquilly et al., 2017). These data suggest that co-incubation with Tregs shifts MDMs towards a less inflammatory phenotype, at least in response to *S. pneumoniae*. This effect could have important implications for host immunity, tilting macrophage responses to causing a less harmful inflammatory response but at the potential cost of poorer control of the invading *S. pneumoniae*.

7.1.5 Investigating the Treg cell-contact-dependent mechanism of suppression

A number of Treg cell surface components were considered for the cell-contact mechanism by which Treg cells suppressed MDM inflammatory responses to *S. pneumoniae*. Membrane-bound TGFβ was the first considered as exogenous soluble TGFβ had anti-inflammatory effects on MDMs responses to *S. pneumoniae* and because TGFβ is implicated in protective Treg responses in mouse IPD models (Neill et al., 2012). Furthermore, *S. pneumoniae* neuraminidases can activate latent TGFβ

by removing sialic acids from LAP (Gratz et al., 2017), which could aid in Treg-promoted immune evasion in carriage or control of inflammation. In the current experiments, Treg cells were found to have approximately 8% expression of LAP, compared to around 2% for Teff cells. These data are similar to another report which also found LAP^+ Treg cells to be suppressive (Savage *et al.*, 2008). LAP expression is increased on activated Treg cells, with 64% LAP^+ cells after 24 hours (Nakamura et al., 2001), although whether LAP expression was increased on Tregs after incubation with macrophages was not confirmed in this thesis. However, a neutralising antibody for LAP did not reverse the suppressive ability of Treg cells on MDM TNFα or IL-1β production, although a loss of suppression of IL-6 production was seen at 72 hours post-infection. The antibody appeared to generally lower the response of the infected control MDMs, which could have masked any relative increase in cytokine production by anti-LAP treated Treg cells. This result was however only taken from the cells from one donor, and further repeat experiments are required in order to confirm if this is a consistent result. Similarly, blocking TGFβ signalling through inhibition of the ALK5 receptor did not prevent Treg suppression of MDM pro-inflammatory cytokine production. MDMs without Treg/Teff co-culture showed trends towards increased cytokine production with ALK5 inhibition, suggesting that the lack of reversal of Treg suppression was not masked by general effect of the antibody on the MDMs as could have been the case with LAP neutralisation. Both neutralisation of LAP and inhibition of ALK5 were likely to have had effects on the MDMs through preventing the effects of soluble TGFβ present in the medium. These experiments could be repeated in serum-free medium or by including a pre-incubation step with a neutralising antibody or the TGFβ-inhibiting peptide P17 on Treg cells alone.

Other candidates for blocking Treg surface TGFβ-mediated suppression are αVβ8 integrins and GARP. αVβ8 integrins are present on the surface of Treg cells but not

on other T cells, and are required for TGFβ1 activation. A blocking antibody against β8 immunosuppression by human Treg cells in a model of graft-vs-host disease (Stockis et al., 2017), and αvβ8-mediated TGF-β activation by Treg cells has been shown to be essential for suppression of T-cell-mediated inflammation (Worthington et al., 2015). GARP is a transmembrane protein forming part of the Treg surface TGFβ complex. Forced expression of GARP in non-Treg cells allows presentation of latent TGF-β1 on the surface but does not activate it (Stockis, Colau et al., 2009). Although not sufficient for TGFβ activation, GARP is required, and GARP-blocking antibodies have been generated that inhibit Treg suppression of Teff proliferation (Cuende et al., 2015). Therefore, blocking αVβ8 or GARP may be another approach to examine the effect of Treg surface TGFβ as a suppressive mechanism to suppress MDM inflammatory responses.

Besides TGFβ, other molecules implicated in Treg contact-dependent mechanisms of suppression include CTLA4 and TNFR2. CTLA4 is commonly investigated as a contact-dependent mechanism of suppression used by Treg cells, but no cell signalling pathway triggered through CTLA4 has yet been discovered. CTLA4 is believed to downregulate CD80 and CD86 ligands on APCs, leading to reduced CD28 co-stimulation of Teff cells (Qureshi et al., 2011). Whether CTLA4 has a direct inhibitory effect on macrophages that downregulates the inflammatory response to *S. pneumoniae* would be important to investigate.

TNFR2 is expressed on Treg cells and is another contact-dependent method of suppression. TNFR2 can only be fully initiated by membrane-bound TNFα (Grell et al., 1995). TNFR2 knockout exacerbates collagen-induced arthritis in mouse models, whereas TNFR1 knockout ameliorates disease (Kontoyiannis et al., 1999; Piguet et al., 1992). Polymorphisms in the TNFR2 gene correlate with autoimmunity, for example in rheumatoid arthritis, Crohn's disease and systemic lupus erythematosus (Barton et al., 2001; Sashio et al., 2002; Komata et al., 1999). The MDM-Treg/Teff

co-culture experiments could be repeated with TNFα inhibitors, of which a range are in clinical use and with differential effects on TNFRs. Infliximab has been shown to inhibit soluble TNFα and membrane-bound TNFα, whereas etanercept has less stable interaction with membrane-bound TNFα (Kirchner et al., 2001). Adalimumab, but not etanercept, has been shown to bind to membrane TNFα on monocytes in rheumatoid arthritis patients and enhanced binding to TNFR2 on Treg cells, resulting in Treg expansion (Nguyen and Ehrenstein, 2016).

7.1.6 Effect of Treg cells on MDM phagocytosis of *S. pneumoniae*

No statistically significant difference was seen in the intracellular bacterial numbers in macrophages incubated with Treg cells, with Teff cells or incubated alone, although a trend towards decreased internal bacteria was seen with Treg or Teff cell co-culture compared to macrophages cultured alone. The protocol used to measure internalised bacteria was the antibody protection method of killing of extracellular bacteria with gentamicin followed by lysing the macrophages and counting the resulting CFUs. Very low numbers of bacteria were released from the MDMs, making the counting of CFUs prone to error and smaller differences between MDM populations could have been missed. In addition, whether the rate of bacterial killing once internalised was increased/decreased by Treg/Teff co-culture was not determined. Some donors had increased CFU in the supernatant with increasing number of Treg cells in the co-culture, which could correspond with decreased phagocytosis or bacterial killing. Teff cell number had little effect on bacterial CFU. However, overall the data on *S. pneumoniae* CFU during T cell / MDM co-culture experiments were inconsistent across donors and the overall results were statistically not significant. These experiments could be repeated using fluorescently labelled *S. pneumoniae* to visualise the number of internalised bacteria by microscopy as a perhaps more sensitive measure of phagocytosis. There is evidence that Treg cells can increase the phagocytic capacity of macrophages (Tiemessen et al., 2007). The study by

Tiemessen and colleagues found this increase in phagocytosis to be partly IL-10-dependent and their Treg-macrophage co-culture duration was 40 hours, compared to 14 hours for the experiments in this thesis with no IL-10 produced in this time. Therefore, it is possible that longer co-culture allowing time for IL-10 production may result in a statistically significant effect on phagocytosis of *S. pneumoniae*.

7.1.7 Treg cells did not reduce MDM pro-inflammatory cytokine production in response to *A. baumannii*

Experiments co-culturing MDMs with Treg/Teff cells prior to infection were repeated using the Gram-negative pneumonia pathogen *A. baumannii*. Although Treg- co-cultured MDMs had a lowered inflammatory response when infected with *S. pneumoniae*, this was not the case when Treg- co-cultured MDMs were instead infected with *A. baumannii*. *A. baumannii* provoked a much larger production of inflammatory cytokines at the same MOI as *S. pneumoniae*, but reducing the MOI and consequently decreasing pro-inflammatory cytokine concentration still did not allow Treg- co-culture to suppress cytokine production compared to infected MDMs without co-culture. These results suggest that Treg cells cannot modulate the macrophage inflammatory response to all pathogens. Interestingly, similar experiments using Treg-monocyte co-culture found that these monocytes had reduced pro-inflammatory cytokine production upon exposure to LPS (Taams et al., 2005). LPS is one of the major virulence factors of *A. baumannii* stimulating macrophage inflammatory responses when recognised by TLR4 and CD14. However, whether the dose of soluble LPS used in the study by Taams and colleagues (50ng/ml) was comparable to the effective LPS dose when whole bacteria were incubated MDMs in the co-culture experiments in this thesis is unknown and very hard to characterise; presentation of LPS on a whole bacterium could have a more potent pro-inflammatory effect compared to an equivalent quantity of soluble LPS. Furthermore, the exact chemical composition of LPS influences pro-

inflammatory potency, and there may be differences in LPS structure between the strain of *A. baumannii* used for the experiments in this thesis and that used by Taams et al. The data in this thesis helps support the importance of using a more physiologically relevant form of a pro-inflammatory stimulus compared to purified chemical products.

7.1.8 $CD4^+$ T cell differentiation and cytokine production in MDM co-culture

Teff cells in co-culture with MDMs produced IL-10, exogenous IL-10 reduced MDM pro-inflammatory cytokine production upon *S. pneumoniae* infection, and Teff cells added simultaneously with *S. pneumoniae* to MDMs sometimes caused reduced pro-inflammatory responses at later time points compared to MDMs infected alone. From these indications, the possibility that Teff cells could adopt an induced Treg phenotype in the co-culture with MDMs was considered. Therefore, the cytokine profiles and Treg markers of T cells after MDM co-culture were examined. To be more representative of the normal physiological T cell population, total $CD4^+$ T cells were used in these experiments.

A useful control would be to compare these levels to an isotype control to eliminate the possibility of non-specific binding of the antibody. In pro-inflammatory environments, Teff and Treg subsets can produce IL-17 (Beriou et al., 2009). Specifically, IL-6 in combination with TGFβ promotes Th17 differentiation from naïve $CD4^+$ T cells, whereas TGFβ in the absence of IL-6 promotes Treg differentiation (Bettelli et al., 2006). In the co-culture conditions used in this thesis, high concentrations of TGFβ (approximately 5000pg/ml) and low levels of IL-6 (approximately 600pg/ml) were present during culture without infection.

After 48 hours MDM co-culture, most $CD4^+$ T cells expressed CD25, and a distinct population of approximately 15% of $CD4^+$ T cells expressed both CD25 and Foxp3. However, with addition of *S. pneumoniae* infection, $CD25^+Foxp3^+$ cells were

approximately 5%. A trend towards increased IL-17A in the *S. pneumoniae* infected group was seen, however this was not a statistically significant overall. The trend towards increased differentiation of $CD4^+$ T cells into IL-$17A^+$ cells when exposed to *S. pneumoniae* may partly be explained by an increase in the IL-6 concentration in the co-culture upon infection. In addition, Treg cells and to a lesser extent Teff cells express TLRs on their surfaces, particularly TLR2 (Sutmuller et al., 2006; Nyirenda et al., 2011). TLR2 signalling causes loss of suppression and proliferation of Treg cells and drives them to produce adopt a Th17-like phenotype through MyD88, whereas TLR4 and TLR9 are not thought to be involved (Sutmuller et al., 2006; Nyirenda et al., 2011). Triggering of TLR2 signalling on the Treg/Teff surface by *S. pneumoniae* lipoproteins (Tomlinson et al., 2014) may increase IL-17A production and reduce suppressive function of Treg cells thereby partly explaining the reduced levels of Foxp3 expression. It would be beneficial to test this possibility by repeating these experiments with mutant *S. pneumoniae* with deletion of the lipoprotein diacylglyceryl transferase gene (*Δlgt*) which have reduced surface lipoproteins that are required for TLR2 signalling in macrophages (Tomlinson et al., 2014). Conversely, Ply binding to the mannose receptor of human DCs or mouse alveolar macrophages polarises naïve T cells into $Foxp3^+$ cells (Subramanian et al., 2019). Therefore, it would also be beneficial to see if Ply-deficient mutant *S. pneumoniae* decreases Foxp3 expression and cytokine production in the $CD4^+$ T cells differentiated in these MDM co-culture experiments. The co-culture system used in these experiments would be useful for future experiments investigating Treg and Th17 responses during *S. pneumoniae* infection.

The cytokine expression of the $CD25^+Foxp3^-$ and $CD25^+Foxp3^+$ fractions were compared. Generally, the $CD25^+Foxp3^+$ fraction expressed the highest percentages of pro-inflammatory cytokines compared to the $CD25^+Foxp3^-$ fraction, indicating that the $CD25^+Foxp3^+$ cells are probably not a pure regulatory cell population. IL-2 is

produced at low levels in resting/naïve $CD4^+$ T cells, and produced in large amounts by activated $CD4^+$ T cells (Liao et al., 2013). This indicated that the T cells in these experiments may be anergic, which may occur without strong CD28 stimulation and in the presence of anti-CD3 antibody (Harding et al., 1992; Schwartz, 2003; Zheng et al., 2008). It would be interesting to see if addition of anti-CD28 antibody affects the production of cytokines by the T cells in this co-culture.

An important addition to this model would be to assess the suppressive ability of fractions of the differentiated $CD4^+$ T cells. The levels of $Foxp3^+$ expression did not correlate with the CD25 expression, with a distinct subpopulation expressing Foxp3 whereas the majority of cells expressed CD25. Further analysis of Treg-associated surface markers expressed by these cells may allow a FACS sorting strategy and investigation of the suppressive ability of each sub-population. In particular, if a specific mechanism of cell contact-mediated suppression is identified in the experiments comparing undifferentiated peripheral blood FACS-sorted Treg and Teff experiments, it would be interesting to sort differentiated $CD4^+$ T cells based on the surface expression of the identified marker and see if the same mechanism applies to these differentiated $CD4^+$ T cells.

7.2 Examining T cell subsets in normal human lung and in response to intradermal injection of *S. pneumoniae* in healthy volunteers

The MDM co-culture with sorted Treg and Teff populations infected with *S. pneumoniae* indicated that the timing of interaction between these three cell types may influence the magnitude of the inflammatory response by macrophages. Incubating Treg or Teff cells with MDMs prior to addition of *S. pneumoniae* had the largest effect on the inflammatory response of the MDMs to infection, in which Treg cells potently reduced MDM TNFα, IL-6 and IL-1β production, whereas Teff cells generally resulted in similar or increased levels compared to MDMs infected without Treg/Teff co-culture. For this effect *in vivo*, Treg and Teff cells would have to interact with macrophages prior to infection, conditioning their response to subsequent *S. pneumoniae* encounter. Two possible scenarios could lead to this interaction: (a) resident populations of Treg and Teff cells could interact with resident macrophages, attenuating/exacerbating macrophage responses when subsequently exposed to *S. pneumoniae*; or (b) alternatively, Treg cells interacting with macrophages during a predisposing infection such as influenza could attenuate macrophage responses to subsequent *S. pneumoniae* infection. Furthermore, addition of Treg cells to MDMs simultaneously with *S. pneumoniae* infection reduced MDM inflammatory responses (albeit to a lesser extent than pre-incubation) suggesting that Tregs could also alter the inflammatory response to *S. pneumoniae* by recruited cells to the site of infections. In mice models T cells are reported to be recruited to the lungs at the same time as monocyte/macrophages at approximately 48 hours after *S. pneumoniae* infection, suggesting this mechansims could be relevant during the development of pneumonia (Kadioglu et al., 2000; Bergeron et al., 1998).

To assess whether the *in vitro* situations where Tregs suppressed MDM inflammatory responses to *S. pneumoniae* identified by the co-culture experiments could be relevant during human infection, I assessed whether Tregs can be identified in normal human lung tissues and the T cell subsets recruited in response to intradermal injection of *S. pneumoniae* in healthy volunteers.

7.2.1 $CD4^+$ and $Foxp3^+$ cells are present in normal human lung

There is good evidence for the existence of tissue-resident populations of T cells. For example, large numbers of lung-resident T cells reported (Purwar et al., 2010). Amongst these cells isolated by Purwar et al., a population of $CD4^+CD25^+Foxp3^+$ T cells was identified by flow cytometry although the data was not shown in this paper. In this thesis, immunofluorescent staining of a limited number of samples confirmed the presence of both $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells in normal human lung sections. Hence, it is possible that tissue resident Treg cells could help suppress lung macrophage inflammatory responses to *S. pneumoniae* infection. Further work to quantify the suppressive capacity of the $Foxp3^+$ cells would be necessary to confirm these are Treg cells. Ideally, immunofluorescence would identify close interaction of the $Foxp3^+$ cells with lung macrophages, and both cell types could be isolated for *in vitro* study to provide evidence the Treg cells can modulate macrophage inflammatory responses. Future work is required to increase the numbers of samples assessed, identify the relative abundance of Treg to Teff cells in the lung, and their spatial relationship to macrophage populations.

Susceptibility to pneumonia is increased after infection with influenza virus, and prior influenza infection impairs alveolar macrophage responses during *S. pneumoniae* infection leading to a reduced ability to clear bacteria (Loosli, 1979; Sun and Metzer, 2008; Ghoneim et al., 2013). Mouse studies have also found that recovery from a primary pneumonia induced by *Escherichia coli* or influenza A virus resulted in a more severe secondary *E. coli* pneumonia with impaired macrophage and DC antigen

presentation and cytokine production upon secondary infection, associated with increased numbers of peripherally induced Tregs after the initial pneumonia and during the second pneumonia (Roquilly et al., 2017). Depletion of Treg cells during resolution of primary pneumonia did not alter the course of this infection, but restored the effectiveness of bacterial clearance during the second pneumonia. This immune re-programming was local and long lived, with effects lasting at least until 21 days after the primary infection. These findings suggest one mechanism of increased susceptibility to a second pneumonia is increased Treg cell numbers attenuating macrophage and DC responses to infection. The *in vitro* data from experiments in this thesis complement these findings and suggest that Treg cell attenuation of macrophage pro-inflammatory cytokine production could contribute to increased susceptibility to *S. pneumoniae* after viral infection.

7.2.2 Recruitment of T cell subsets in response to intradermal injection of *S. pneumoniae*

For this thesis, a model of intradermal injection of UV-killed *S. pneumoniae* into the skin of healthy volunteers was used to investigate Treg recruitment to the site of *S. pneumoniae* infection. The inflammatory response was measured by Doppler scanning, and blisters were raised at the site of injection to collect recruited cells for flow cytometry analysis and to measure local cytokine concentrations. Using this model statistically significant increases in a $CD4^+CD25^+CD127^{low}$ T cell population post-injection of *S. pneumoniae* were identified, reaching approximately 40% of the $CD4^+$ T cell populations by 48 hours. However, the small number of cells collected prevented intracellular staining and suppression assays from being carried out to establish whether these were true Treg cells. If enough cells could be collected from the blister, intracellular staining for Foxp3 and cytokine production could confirm if the cells have a regulatory phenotype. This could be achieved by increasing the number of bacterial CFU injected into the skin. Although intracellular staining for cytokine

production by the recruited T cells was not possible, the blister supernatants were analysed for cytokine concentrations. Increased concentrations of the Th17 cytokines IL-17 and IL-22 were detected at 48 hours, corresponding with the time-point when T cell proportions were increased, although these cytokines may have been produced by various cell types.

Studies on human responses to infection are sparse and the majority of data is provided from mouse models. The skin is an easily accessible location for conducting experiments examining the human immune response, and the intradermal challenge experiment has the major advantage of allowing the human inflammatory response to *S. pneumoniae* to be assessed at a mucosal site. The model has two main disadvantages. Firstly, for safety reasons it uses killed *S. pneumoniae* rather than live, weakening and shortening the duration of the inflammatory response. Secondly, there maybe differences in immune responses that may occur in the skin compared to the natural site of *S. pneumoniae* infection in the lung/nasopharynx. However, the profiles of the cells collected in the blister fluid in response to *S. pneumoniae* show immune response characteristics that partially overlap between cutaneous and lung/nasal T cell populations. Skin-homing T cells have a $CD45RA^-$ memory phenotype and express cutaneous lymphocyte–associated antigen (CLA), and almost all $CD4^+$ T cells in the blister were $CD45RA^{low}$/$CD45RA^-$ (Picker et al., 1990; Santamaria Babi et al., 1995). The majority (>90%) of lung resident T cells are also $CD45RA^-$, although they do not express CLA (Purwar et al.,2010). It would have been informative to see the proportion of *S. pneumoniae* blister $CD4^+$ cells expressing CLA. The majority of the blister $CD4^+$ T cells were $CCR6^+$, which is expressed by Th17 cells, Th17-derived Th1 cells, Treg cells, and Th22 cells. CCR6 is important for the migration of Th17 and Treg cells to tissue in response to inflammation (Yamazaki et al., 2008; Zhang et al., 2015). Studies show that more than 75% of resident T cells in normal skin express CCR6, whereas approximately 40% of peripheral blood $CD4^+$ T

cells are $CCR6^+$ (Hedrick et al., 2013). Similarly, approximately 70% of nasal $CD4^+$ T cells and 50-60% in the lung express CCR6 (Purwar et al., 2011; Francis et al., 2008), suggesting the proportion of $CCR6^+$ cells in the lung may not be quite as high as those detected in the blister fluid from the skin infection model. Correlating with the predominance of $CCR6^+$ cells, the blister fluid had increased concentrations of IL-17 and IL-22. Skin-homing memory T cells are particularly potent producers of IL-22, and IL-17 production is also typical in response to cutaneous challenges such as bacterial infection and psoriasis (Duhen et al., 2009; Blauvelt and Chiricozzi 2018; Ferran et al., 2013; De Jesús-Gil et al., 2018). Similarly, IL-17 and IL-22 are produced by T cells in response to *S. pneumoniae* and are important in clearance of colonisation and pneumonia (Lundgren et al., 2012; Lu et a., 2008; Engen et al., 2014; Trevejo-Nunez et al., 2016). An interesting overlap between throat and skin immune responses is demonstrated by *Streptococcus pyogenes* throat infection, which is an infectious trigger of psoriasis and is thought to activate tonsillar CLA^+ T cells which migrate to the skin where they encounter antigens that share sequence homology with the streptococcal proteins (Valdimarsson et al., 2009).

The recruitment of macrophages was not examined in the present study. However data on the recruitment of macrophages to *S. pneumoniae* using the same protocol have been collected by another group (Dr. Daniel Marks, UCL) and is being prepared for publication. The baseline number of $CD4^+$ cells in the skin blister without induction of inflammation has been examined in the study by Motwani and colleagues. They found $CD4^+$ cells in the range of $1x10^3$ to $1x10^4$ cells per ml in the baseline skin blister, suggesting the presence of skin resident $CD4^+$ T cells in the blister fluid (Motwani et al., 2016). In their study, $CD4^+$ T cell numbers peaked at 14 hours after intradermal *E. coli* injection at approximately $1x10^4$ to $1x10^5$ cells per ml (Motwani et al., 2016). By comparison, the current experiments found a mean of $3.5x10^4$ $CD4^+$ T cells per ml at 48 hours after *S. pneumoniae* injection, an increase from the mean of

approximately $1x10^4$ cells per ml detected at the 4 hour time-point for the *S. pneumoniae* model and the $1x10^3$ to $1x10^4$ cells per ml pre-infection reported by Motwani and colleagues.

7.3 Additional limitations

The experiments carried out in this thesis were primarily *in vitro* cell culture work on cells isolated from human blood. *In vitro* experiments are useful for showing the results of interactions between specific cells, however physiologically there would be multitudes of different cell types and varying micro-environments regarding the cytokine milieu etc. that would influence how a cell ultimately behaves. In addition to having only select populations of cells interacting *in vitro*, culture conditions will not fully reflect those present *in vivo*; for example, the experiments were performed in tissue culture rather than extracellular fluid, and the medium was supplemented with activating anti-CD3 antibody. Furthermore, most of the experiments were carried out using serotype 4 *S. pneumoniae*, and whether Treg cells suppress MDM responses to other *S. pneumoniae* strains with different capsular serotypes and genetic background requires investigation.

The intradermal *S. pneumoniae* challenge model is useful for studying the human cellular recruitment to the bacteria. Methods in which human responses to infection can be studied are limited, particularly in lung infection where experiments cannot safely and accessibly be carried out. Conducting experiments in the skin allows a practical way in which the human reaction to infection can be investigated. However, care should be taken in extrapolation of the observations obtained in the skin to that which may occur in the lung, as increasing evidence suggests that the immune system has tissue-specific immune responses.

7.4 Summary and future work

Overall the data in this thesis suggest that Treg cells, and to some extent Teff cells, have the potential to reduce macrophage pro-inflammatory responses during *S. pneumoniae* infection. The anti-inflammatory actions of Treg/Teff cells on macrophages are likely to have implications in *S. pneumoniae* colonisation and in pneumonia. Treg-macrophage interaction prior to infection caused the most potent suppression of the macrophage response. These data add to the existing evidence that Treg-macrophage pre-infection interaction causes macrophage unresponsiveness, which *in vivo* occurs upon resolution of a primary pneumonia resulting in increased susceptibility to a *E. coli* second pneumonia (Roquilly et al., 2017). However, the data in this thesis also highlights a difference in the immune response between two pneumonia-causing pathogens – *S. pneumoniae* and *A. baumannii*, the latter of which are resistant to Treg-mediated suppression of macrophage inflammatory responses. Additionally, $CD4^+Foxp3^+$ cells are present in normal human lung along with $CD4^+Foxp3^-$ cells, which may provide a scenario in which Treg cells and macrophages could interact prior to infection. The *in vitro* experiments also indicate that direct contact is required for the suppressed macrophage response by the Treg cells. The work in this thesis has also developed useful *in vitro* co-culture models for studying MDM-Treg/Teff interactions and infection and for examining cellular recruitment to the presence of *S. pneumoniae* in a human intradermal challenge model. The data obtained in this thesis has provided several additional avenues to investigate in the suppression of macrophages by Treg cells in *S. pneumoniae* infection. Furthermore, acquiring more results for some of the presented experiments would strengthen the existing data. Potential future experiments include the following:

1. Identifying the cell contact-dependent mechanisms of suppression used by the Treg cells on the MDMs is important future work for the data obtained in this thesis.

Firstly, more work on confirming the current data on TGFβ as a potential mechanism should be conducted. Subsequently, there are several other Treg surface components that are associated with conferring suppression which can be blocked, such as CTLA4 and TNFR2.

2. It would also be of interest to see whether the Teff mechanism of suppression of MDM responses observed during *S. pneumoniae* infection involved IL-10, which could be deduced by blocking IL-10 and additionally infecting MDMs in the presence of Teff supernatant and culturing Teff cells and MDMs separated by a transwell insert.

3. All *in vitro* experiments in this thesis used MDMs, however it would be very useful to see if the effects of Treg cells can also reduce alveolar macrophage responses to *S. pneumoniae*. These could be obtained from human bronchoalveolar lavage fluid or precision cut lung slices.

4. Very little investigation was carried out on the specific effects of Treg cells on MDM phenotypes, for example levels of expression of mannose and scavenger receptors, CD80/CD86 and TLRs. The protocol used for differentiating MDMs resulted in the cells being irreversibly adhered to the tissue culture plates. Addition of trypsin/EDTA, dispase, ice-cold PBS, or lidocaine treatment could not successfully remove the MDMs. Therefore, analysis by flow cytometry was not possible during the thesis.

5. Similarly, it would be important to know if the Treg cells are affecting MDM gene expression. Removing the Treg cells from the culture 24 hours prior to infection of the MDMs retains the suppressive effect, which indicates that the changes could be post-transcriptional. Addition of actinomycin-D could help confirm whether changes are post-transcriptional. For this experiment, NFκB translocation could be a readout. Transcriptome analysis would provide highly detailed information on how co-culture with Treg cells affects MDMs, and how this interaction may affect transcriptional responses to infection with *S. pneumoniae*.

6. The effect of Treg cells on MDM phagocytosis of *S. pneumoniae* was briefly investigated in this thesis, but the antibody protection protocol used did not allow for an accurate or conclusive readout for the numbers of internalised bacteria. It would be informative to use a more reliable method to investigate whether Treg or Teff cell co-culture affects MDM phagocytosis, for example by flow cytometry (if the MDMs can be lifted off the tissue culture plates) or microscopy using fluorescent *S. pneumoniae*.

7. Currently there are sparse data on the presence and activity of resident lung Treg populations. In a limited number of normal subjects $CD4^+$ and $Foxp3^+$ cells were detected by immunofluorescent staining of normal human lung sections. These investigations can now be expanded to include more subjects as well as to quantify the proportions of these cells in human lungs and whether they are interacting with lung macrophage populations. This may give a physiologically relevant scenario in which Treg cells could affect macrophage inflammatory responses through their interaction prior to infection. It would be interesting to see whether age or lung disease has an effect on the proportions of Treg to Teff cells in the lungs. Ideally, lung Treg and Teff cells should be isolated in order to assess the suppressive ability of the Treg cells. These cells could potentially be isolated from precision cut lung slices.

8. The human intradermal *S. pneumoniae* challenge model has lots of potential for investigating immune responses to the bacteria. The first improvement that may be possible to this model is to increase the dose of *S. pneumoniae* in the intradermal injection, which may allow an increased cellular recruitment into the blister. Several volunteers showing no visible response to the injection in terms of Doppler imaging and cells present in the blister for flow cytometry, and even in responding volunteers, there were few cells present in the blister. This suggests that the dose used at present is at the lower limit of that which reproducibly stimulates an inflammatory response. Increasing the cells collected from the blister could allow intracellular staining and

potentially *in vitro* assays to be carried out on these cells. This model would be useful to investigate differences in proportions of cell populations recruited in those in risk groups for pneumonia, for example the elderly.

9. Whether the Treg interactions with MDMs described in this thesis are important during the development of *S. pneumoniae* infection will require infection models that allow manipulation of Treg cell numbers or activity. The mouse pneumonia model could be used for this, and has previously been used to demonstrate in immune naïve mice an important role for Treg cells in controlling infection (Neill et al., 2012). Another potential model that could be developed and used to investigate the role of Treg cells during *S. pneumoniae* is the precision lung slice model, which has the advantage of using human material (Neuhaus et al., 2017). Furthermore, as all humans have been previously exposed to *S. pneumoniae* on multiple occasions, any resident lung lymphocytes in a precision slice lung model could include T cells that recognise *S. pneumoniae* antigens.

7.5 Conclusion

The host inflammatory response to *S. pneumoniae* is important to clear infection but may also cause detrimental complications if inadequately controlled. The data in this thesis demonstrated that Treg cells reduced macrophage inflammatory responses to *S. pneumoniae* when co-cultured either prior to- or during pneumococcal infection. Direct Treg-macrophage contact was required. The Treg cells do not have to be present during the infection as pre-conditioning the macrophages prior to infection reduced MDM inflammatory responses to *S. pneumoniae*. However, Treg cells can also reduce macrophage inflammatory responses when added to the culture simultaneously with *S. pneumoniae*. Teff cell co-culture resulted in trends towards reduced macrophage inflammatory responses to *S. pneumoniae* in some cases, and increased responses in others. Teff cells can make the anti-inflammatory cytokine IL-10, the exogenous addition of which dampens macrophage inflammatory responses to *S. pneumoniae*. Treg or Teff suppressive effects were not seen with *A. baumannii* infection, indicating an element of pathogen-specificity with this effect. A model of *S. pneumoniae* challenge using intradermal injection of bacteria into the skin of healthy volunteers allowed recruitment of T cell subsets to be observed. T cells were increased at 48 hours post-injection and contained a substantial $CD4^+CD25^+CD127^{low}$ population. IL-17 and IL-22 concentrations were raised at 48 hours. $CD4^+$ and $Foxp3^+$ cells were identified in sections of normal human lung, suggesting resident Treg cells may help control the early response of macrophages to *S. pneumoniae*. Overall, the data indicate that human Treg cells can modulate macrophage responses to *S. pneumoniae in vitro*, which are likely to have important implications in pneumococcal carriage and IPD *in vivo*. Further investigation is required to define the mechanisms involved in Treg cell suppression of MDM inflammatory responses to *S. pneumoniae*, and to demonstrate these interactions can affect the course of disease.

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Appendix – conference abstracts

Oral presentation: "Effects of Regulatory T cells on macrophage inflammatory responses to *Streptococcus pneumoniae.*" Europneumo 2017, Stockholm, Sweden.

Gabriella Szylar, Michael Ehrenstein, Jeremy Brown

Background: The inflammatory immune response to *Streptococcus pneumoniae* can result in serious complications such as ARDS, septic shock, and loss of tissue barrier integrity. Macrophages are an important source of pro-inflammatory cytokines during *S. pneumoniae* infection. Regulatory T cells (Tregs) are a small population of anti-inflammatory cells that have been found to modulate macrophage activity, and to be protective against invasive pneumococcal disease in mice.

Aims: We aim to characterise the effects of Tregs on the macrophage inflammatory response to *S. pneumoniae*, assess the mechanisms of action by which Tregs may modulate the macrophage response, and investigate whether Treg-macrophage interactions may prevent bacterial translocation across endothelial and epithelial tissue barriers.

Results: Co-culture of human monocyte-drived macrophages (MDMs) with Tregs reduced MDM TNFα production upon subsequent infection with *S. pneumoniae* TIGR4 strain . Using a novel human model of *S. pneumoniae* challenge requiring intradermal injection of killed *S. pneumoniae* into the forearm of healthy volunteers, we demonstrated Tregs accumulated at the site of injection within 48 hours. Transwell models of endothelium and epithelium have been established in order to test the effects of *S. pneumoniae*-infected MDM conditioned media with and without Treg co-culture on bacterial translocation across the endothelial monolayer.

Conclusion: Our preliminary data demonstrate Treg accumulation after *S. pneumoniae* challenge in humans and that Tregs have the ability to modulate the MDM inflammatory response to *S. pneumoniae*.

Oral presentation: "Suppression of macrophage inflammatory responses to *Streptococcus pneumoniae.*" British Thoracic Society 2017, London, UK.

Gabriella Szylar, Jeremy Brown

Background: The highly inflammatory immune response to *Streptococcus pneumoniae* infection can result in complications such as sepsis and Acute Respiratory Distress Syndrome. Macrophages are an important source of the inflammatory cytokines that activate epithelial and endothelial cells, resulting in a loss of barrier integrity. Regulatory T cells (Tregs) are a population of anti-inflammatory cells that modulate macrophage activity and are protective against invasive pneumococcal disease in mice.1,2

Aims: To characterise the in vitro effects of Tregs on the macrophage inflammatory response to *S. pneumoniae* and to observe Treg recruitment to the site of intradermal injection of UV-killed S. pneumoniae in a human model.

Results: Preliminary data suggest that co-culture of human monocyte-derived macrophages (MDMs) with CD4+CD25+CD127- Tregs reduced MDM TNFα production by at least 45% (One-way ANOVA $p<0.01$) and IL-6 production by at least 52% (One-way ANOVA $p<0.01$) 72 hours after initial infection with *S. pneumoniae* TIGR4 strain (MOI of 2, ratio of 1 Treg to 3 MDMs). Separation of Tregs from the MDMs during co-culture using transwell inserts prevented the suppressive effects of the Tregs. Using a novel human model of *S. pneumoniae* challenge involving intradermal injection of UV-killed *S. pneumoniae* into the forearm of healthy volunteers, we demonstrated that Tregs accumulated at the site of injection within 48 hours, increasing from undetectable Treg population at 4 hours to constituting approximately 33% of CD4 cells by 48 hours.

Conclusion: Preliminary data suggest that Tregs modulate the MDM inflammatory response to *S. pneumoniae* in a contact-dependent manner, and track to the site of intradermal injection of the UV-killed bacteria in human volunteers.

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Poster presentation: "Regulatory T cells recruit in response to intradermal *Streptococcus pneumoniae* challenge and can modulate macrophage inflammatory responses in vitro." International Symposium on Pneumococci and Pneumococcal Diseases 2018, Melbourne, Australia.

Gabriella Szylar, Ricardo Jose, Jeremy Brown

Macrophages are a main source of pro-inflammatory cytokines in response to *S. pneumoniae*. Conversely, regulatory T cells (Tregs) are a population of anti-inflammatory cells that may provide protection against excessive inflammatory responses. We aim to investigate whether Tregs can recruit to the site of *S. pneumoniae* challenge, and whether Tregs can modulate the macrophage inflammatory response *in vitro*. Using a novel human model of *S. pneumoniae* challenge involving intradermal injection of UV-killed *S. pneumoniae* into the forearm of healthy volunteers, we found an approximate 5-fold increase in the overall number of CD3+ T cells present at the site of injection from 4 to 48 hours, and an approximate 10-fold increase in the number of Tregs, the proportion of which increased from about 13% to 40% of all CD4+ cells. *In vitro*, Tregs isolated from PBMCs were found to reduce the inflammatory response of monocyte-derived macrophages (MDMs) when co-cultured prior to an infection with *S. pneumoniae* compared with MDMs co-cultured with T effectors (Teffs), resulting in the MDMs producing approximately 1.2-fold less TNFα when incubated with Tregs compared to Teffs. This suppressive effect relied on contact between the Treg and the macrophage. In conclusion, an increased population of Tregs is present at the site of intradermal *S. pneumoniae* challenge at 48 hours, and *in vitro* co-culture of Tregs with MDMs reduces the MDM TNFα response upon *S. pneumoniae* infection. These results suggest a potential role for Tregs in limiting inflammatory responses in *S. pneumoniae* infection.

Poster presentation: "Regulatory T cells are present in the lungs and can modulate macrophage inflammatory responses in vitro to *Streptococcus pneumoniae.*" Europneumo 2019, Greifswald, Germany.

Gabriella Szylar, Giuseppe Ercoli, Elisa Ramos-Sevillano, Michael Ehrenstein, Jeremy Brown

Background: Excessive inflammatory responses to *Streptococcus pneumoniae* infection can lead to complications such as sepsis and meningitis, with macrophages being a main producer of inflammatory cytokines such as TNFα and IL-6. Foxp3+ Regulatory T cells (Tregs) are anti-inflammatory cells that protect against excessive inflammation. However, the effects of Tregs on macrophage responses to *S. pneumoniae* are unclear.

Aims: We aim to characterise the effects of Tregs on monocyte-derived macrophage (MDM) inflammatory response to *S. pneumoniae*, assess the mechanisms of action by which Tregs may modulate the MDM response *in vitro*, and determine whether Tregs are present in normal human lung.

Results: Pre-incubating Tregs with MDMs before infecting the MDMs with *S. pneumoniae* reduced MDM production of TNFα by approximately 50% and IL-6 by approximately 65%. Separating the Tregs and MDMs by transwells during pre-incubation abrogated this suppression. Although Latency Associated Peptide (LAP) was detected on the surface of approximately 8% of Tregs, neutralising LAP and blocking the macrophage ALK5 receptor did not prevent suppression. Tregs did not suppress the MDM inflammatory response when these experiments were repeated using the bacterium *Acinetobacter baumannii*. Using Immunofluorescence staining for CD4 and Foxp3, both CD4+Foxp3+ and CD4+Foxp3- cells were found in normal human lung.

Conclusion: *In vitro* Tregs can modulate MDM inflammatory response to *S. pneumoniae* in an MDM-Treg contact-dependent manner, the mechanism of which is yet to be determined. CD4+ cells and Foxp3+ Tregs are present in normal human lung and could be involved in regulating macrophage inflammatory responses during *S. pneumoniae* infection.