Dendritic cells and the regulation of lung type-2 immune responses

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Contents

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List of abbreviations

A.f.	*Aspergillus fumigatus*
AAI	Allergic airway inflammation
AB-PAS	Alcian blue periodic acid schiff
Ag	Antigen
AHR	Aryl hydrocarbon receptor
ALDH	Aldehyde dehydrogenase
AREG	Amphiregulin
BAC	Bacterial artificial chromosome
BAL	Bronchoalveolar lavage
BMDC	Bone marrow derived dendritic cell
BTLA	B and T lymphocyte attenuator
CCA	Circulating cathodic Ag
CD	Cluster of differentiation
cDC	classical or conventional dendritic cell
CDP	Common dendritic cell precursor
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTV	Cell trace violet
DAMP	Damage associated molecular pattern
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cell
dLN	draining lymph node
DNA	Deoxyribonucleic acid
DTr	Diphtheria toxin receptor
DTx	Diphtheria toxin
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESAM	Endothelial cell-selective adhesion molecule
FACS	Fluorescence assisted cell sorting
FCS	Fetal calf serum
FEV	Forced expiratory volume
FSC	Forward scatter
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GSK	GlaxoSmithKline
GWAS	Genome wide association studies
H&E	Haematoxylin and Eosin
HDM	House dust mite
HLA-DR	Human leukocyte Ag - DR isotype
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
HSD	honestly significant difference
HVEM	Herpesvirus entry mediator
ICOS	Inducible T cell costimulator
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin

i.v.	Intravenous
i.t.	Intratracheal
i.n.	Intranasal
IL-1RA	IL-1 receptor antagonist
IP-10	Interferon induced protein 10
KLRG1	Killer cell lectin-like receptor subfamily G member 1
L	Ligand
LAG3	Lymphocyte-activation gene 3
LAP	Latency associated peptide
LN	Lymph node
LPS	Lipopolysaccharide
LSHTM	London school of hygiene and tropical medicine
LUMC	Leiden university medical centre
MCP-1	Macrophage chemoattractant protein 1
MHC	Multiple histocompatibility complex
MIP	Macrophage inflammatory protein
MPP	Multipotent progenitor
MRC	Medical research council
NET	Neutrophil extracellular trap
NK	Natural killer
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PDGF-BB	Platelet-derived growth factor BB
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
R	receptor
RALDH2	Retinaldehyde dehydrogenase 2
RA	Retinoic acid
RAG	Recombination-activating genes
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RBC	Red blood cell
RNA	Ribonucleic acid
RT	Room temperature
S.c.	Subcutaneous
S. mansoni	*Schistosoma mansoni*
SEA	Schistosome egg Ag
ssRNAseq	Single cell RNA sequencing
SSC	Side scatter
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TLR	Toll like receptor
TNF	Tumour necrosis factor
TSLP	Thymic stromal lymphopoietin
UVRI	Uganda virus research institute
YFP	Yellow fluorescent protein

Abstract

Allergic diseases and parasitic infections, such as asthma and schistosomiasis, are characterised by type-2 inflammation. It is understood that dendritic cells (DCs) are critical to drive type-2 inflammatory responses in allergic and helminthic diseases, via induction of T helper 2 (Th2) cells. In addition to inflammatory responses, regulatory responses arise during allergic airway inflammation (AAI), with dampening of inflammation by T regulatory cells (Tregs) critical. DCs have been shown to promote Treg expansion. However, the interaction between DCs and Tregs during ongoing AAI is not well understood, particularly with respect to AAI driven by fungal allergens. In this thesis we begin by elucidating Treg responses during fungal, *Aspergillus fumigatus* (*A.f.*) driven AAI, revealing an expansion of pulmonary Tregs alongside the development of the inflammatory response. We go on to show that Tregs specifically expand at the site of AAI – lung tissues and the airways – whilst increasing their expression of the suppressive cytokine IL-10. Previous reports have shown Tregs to be unstable during inflammation, gaining an inflammatory phenotype or losing expression of the characteristic transcription factor Foxp3. During *A.f.* driven AAI we found Tregs to be stable, however a small proportion of these cells began to express the Th2 associated cytokine IL-4. By sorting and performing *in vitro* suppression assays on Tregs from control or *A.f.* driven AAI we found that IL-4 expressing Tregs had reduced suppressive capacity. We next moved on to ask how Treg expansion was controlled during *A.f.* driven AAI, focusing on the role of DCs due to their published importance in this role. By systematically depleting DC subsets we reveal that type-2 conventional DCs (cDC2s) that express the lectin MGL2 are required for expansion of Tregs during *A.f.* driven AAI. In addition, we reveal DC expression of the integrin $\alpha v\beta 8$, critical for activating the suppressive cytokine TGFβ, is also required for lung Treg expansion. Moving on from AAI, we go on to investigate the regulation of pulmonary type-2 immune responses in parasite infection, focusing on the trematode *Schistosoma mansoni* (*S. mansoni*). We provide novel insights into lung immunological responses to *S. mansoni* at both lung migratory and adult, egg producing (patent) stages of its life cycle in both a mouse model and during human infection. During murine pulmonary schistosomiasis we reveal type-2 dominated inflammatory responses at both lung migratory and patent stages of infection, in addition to an expansion of cDC2s. Utilising sputum as a proxy for lung responses in human *S. mansoni* infection we observe trends for an increase in inflammatory cytokines during lung migratory infection, which are not observed in endemic patent *S. mansoni* infection. With the exception of an increase in Th cells during endemic patent *S. mansoni* infection, there were no significant changes in sputum granulocytes or lymphocytes measured. In line with our murine studies, we reveal consistent increases in cDCs, and in particular cDC2s during human lung migratory and endemic patent infection. Going back to the murine model, we reveal cDC2s to contribute to driving type-2 inflammatory responses to *S. mansoni* infection. Taken together this work provides novel insights into the regulation of pulmonary type-2 inflammation, centring on the role of cDC2s in promoting both Th2 and Treg responses.

Lay abstract

When the immune system responds to allergens or parasitic worms it reacts with a shared response, known as type-2 inflammation. This response is found in the diseases asthma and schistosomiasis, the latter caused by infection with the parasitic worm *Schistosoma mansoni* (*S. mansoni*). Type-2 responses can be characterised by the production of stereotypical immune proteins, called cytokines, such as IL-4, and the expansion of certain types of immune cells such as eosinophils. It is known that dendritic cells (DCs), an immune cell that is able to sense damage and the presence of infectious microbes, can be critical to start off type-2 immune responses. DCs can do this by directing another immune cell, T helper (Th) cells, to change into Th2 cells which go to the tissue and produce cytokines which promote type-2 responses. In addition to promoting inflammation, DCs can cause Th cells to change into T regulatory (Treg) cells, which are able to dampen inflammation by multiple mechanisms, including the production of the regulatory cytokine IL-10. We do not fully understand how DCs are able to cause the expansion of Tregs during type-2 inflammation. We also do not understand if Tregs that arise during type-2 inflammation continue to be suppressive, or if they could change into cells that promote inflammation. In this thesis we first look at how Tregs change during allergic airway inflammation (AAI), showing that they expand in the lungs and express cytokines that are both regulatory (IL-10) and associated with type-2 inflammation (IL-4). Despite their mixed presentation, we find that during AAI Tregs remain able to dampen inflammatory responses, although Tregs that express IL-4 have reduced suppressive ability. We then go on to ask what the role of DCs is in Treg expansion. There are many different types of DCs, called subsets, which have been proposed to have different functions, for instance type-2 conventional DCs (cDC2s) have been shown to be important for promoting type-2 inflammation. Using various methods, we remove each of these subsets one by one, finding that cDC2s, as well as promoting inflammation, are critical for Treg expansion. Moving on from AAI, we go on to investigate the regulation of lung type-2 immune responses in *S. mansoni* infection. Less is known about lung immune response in this disease, so we had to start a bit earlier, utilising a mouse model of the disease to reveal type-2 inflammatory responses in the lung to be characteristic of this infection. We reveal that cDC2s expand during *S. mansoni* infection, and are critical to promote some, though not all, aspects of type-2 inflammation. Next, we ask if similar inflammatory characteristics are seen in human lungs during infection with *S. mansoni.* To sample human lung responses, we take sputum samples from individuals undergoing controlled experimental *S. mansoni* infection, or from individuals naturally infected with *S. mansoni* in Uganda, a country where schistosomiasis is endemic. We find differing inflammatory cell and cytokine responses in the sputum from each study, with one consistent change, namely an increase in sputum DCs, and in particular cDC2s. The increase in sputum cDC2s during human infection is in line with our mouse studies and suggests cDC2s play a critical role in dictating lung immune responses in schistosomiasis. Taken together this work provides novel insights into the regulation of lung type-2 inflammation, centring on the role of cDC2s in promoting both pro and anti-inflammatory responses.

Declaration

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Contributions

Dr Somaia Alsafri and Harry Isitt performed and/or analysed some of the histology presented in chapter 3.

Alice Costain performed a number of schistosome infections in chapter 5.

Sheila Brown and Dr Peter Cook performed *Ccr2*$^{-/-}$ and *Batf3*$^{-/-}$ experiments in chapter 4.

Dr Kevin Couper provided VertX IL-10 reporter mice for experiments in chapter 3.

Prof Mark Travis provided DEREG mice for Treg depletion experiments in chapter 3, and CD11c$\Delta\alpha$vβ8 mice for experiments in chapter 4.

Prof Mark Wilson provided *Foxp3*$^{YFP/Cre}$ *R26R*^{FP635} *Il4*^{GFP} mice for stability experiments in chapter 3.

Prof Elaine Bignell and Dr Margherita Bertuzzi provided technical help with *A. fumigatus* culture methods.

Dr Meta Roestenberg, Dr Marijke Langenberg, Dr Jan Pieter Koopman, Dr Jacqueline Janse, Dr Marie-Astrid Hoogerwerf and Prof Maria Yazdanbaksch organised the controlled human infection *S. mansoni* infections in chapter 5.

Prof Alison Elliott, Dr Harriett Mpairwe, Irene Nambuya and the clinic team at UVRI organised participant recruitment, sampling and aided in study design and sample processing for the endemic human *S. mansoni* infection study in chapter 5.

All members of the MacDonald lab at some point helped with experimental work in this thesis, particularly with respect to mouse monitoring, tissue collection and processing.

Dr Matt Edwards, Dr Semra Kitchen and Dr Alexander Phythian-Adams at GSK were all involved in the planning of the project.

Dr Peter Cook was involved in the planning of all thesis chapters, established the *A. fumigatus* repeat exposure model, and optimised *Mgl2*^{DTr} depletion.

Prof Andrew MacDonald was involved in the planning of all thesis chapters.

Chapter 1

General introduction

Chapter 1 – General introduction

1.1. Asthma and schistosomiasis

The work in this thesis directly addresses two diseases which affect the lungs, asthma and schistosomiasis, with increased understanding of both urgently needed for rational design of long-lasting therapeutics.

Asthma

Asthma is characterized as chronic inflammation in the lower respiratory tract, causing recurrent acute exacerbations (asthma attacks), which are episodes of coughing, wheezing, breathlessness and chest tightness (National Asthma Education and Prevention Program, 2007). It is estimated that at least 339 million people have asthma worldwide, causing 420,000 annual deaths (Global Asthma Network, 2018). Taking into account years lived at less than full health, and those lost due to premature mortality, it is possible to infer that asthma results in the loss of 22 million disability adjusted life years worldwide per year (Murray *et al.*, 2012). The burden of asthma is set to increase, with cases of asthma rising year on year, especially in lower to middle income countries undergoing urbanisation (Lundbäck *et al.*, 2016). The preferred front-line therapy of daily inhaled corticosteroids has multiple anti-inflammatory effects, mediated via epigenetic control of activated inflammatory genes (Barnes, 2010). Inhaled corticosteroids are effective for the majority of asthmatics, but do not provide a long-term cure and upon discontinuation symptoms resume (Guilbert *et al.*, 2006; Barnes, 2010). In addition there are wide-ranging systemic side effects of long term corticosteroid use, including a reduction in bone density, as well as an increased risk of pneumonia and cataracts (Ye, He and D'Urzo, 2017). For the minority of asthmatics (5-10%) that have severe uncontrolled asthma heightened doses of corticosteroids are required, which still may not be sufficient to alleviate symptoms, and increase the risk of side effects (Wenzel and Busse, 2007; Chung *et al.*, 2014).

Many factors contribute to the occurrence of asthma. Concordance between monozygotic twins is about 53%, suggesting genetics plays a role in asthma occurrence but is not the only factor (Thomsen *et al.*, 2010). Environmental risk factors include diet, infection history and exposure to tobacco smoke (Beasley, Semprini and Mitchell, 2015; Stern, Pier and Litonjua, 2020). Allergen exposure is a key risk factor in asthma, with around half of asthmatics being atopic, defined as a heightened immune response to common allergens, specifically associated with a tendency to produce IgE antibodies (Pakkasela *et al.*, 2020). Not all asthmatics are atopic, and we are beginning to appreciate that this multifactorial disease has heterogenous pathogenesis, with different immunological foundations, known as endotypes (Kuruvilla, Lee and Lee, 2019).

It is also apparent that different allergens may provoke different immune responses, with sensitisation to fungi, such as *Aspergillus fumigatus* (*A.f*), associated with worse asthmatic symptoms and an increased risk of life threatening disease (Goh *et al.*, 2017; Medrek *et al.*,

2017). *A.f.* is ubiquitous in the environment, with a study in Manchester, United Kingdom, finding persistent levels of between 3-20 live spores per cubic metre (Alshareef and Robson, 2014). Sensitisation to *A.f.* amongst asthmatic patients has been reported at 28.5%- 52.5% (Maurya *et al.*, 2005; Agarwal *et al.*, 2011). The top level of this estimate is comparable to sensitisation with the allergen house dust mite (HDM), to which 48.8% of asthmatics are sensitised (de Vries *et al.*, 2005). Notably, ascertaining the frequency of sensitisation to fungal allergens is not simple, due to differing methods (skin prick test vs specific IgE), as well as cross reactivity between fungal allergens (Liang, Su and Jiang, 2006; Crameri *et al.*, 2009; O'Driscoll *et al.*, 2009).

Schistosomiasis

The other disease this thesis will address is schistosomiasis, worldwide more than 200 million people are infected with parasitic trematodes of the Schistosoma genus, including *S. mansoni*, *S. haematobium* and *S. japonicum* (Steinmann *et al.*, 2006). This makes schistosomiasis prevalence comparable to malaria, which affects 228 million people (World Health Organization, 2019). Schistosomiasis accounts for 3.3 million disability adjusted life years annually, and approximately 200,000 annual deaths (Murray *et al.*, 2012; Verjee, 2020). Schistosomiasis is a chronic disorder, which affects multiple organs depending upon the life cycle stage of the parasite. Schistosomes live on average 5-10 years in the human host, and overlapping exposures mean individuals could be infected for an entire lifetime (Warren *et al.*, 1974). A single dose of the drug praziquantel can clear schistosome infection, with a meta-analysis estimating the cure rate at 76.4% (Wegner, 1980; Zwang and Olliaro, 2017). It is possible this cure rate is an over-estimate, however, with a recent study utilising a more sensitive diagnostic method estimated the cure rate at only 18% with a single dose of praziquantel (Hoekstra *et al.*, 2020). Moreover, praziquantel does not provide resistance to reinfection, it has limited efficacy against immature stages of schistosomes, and, being the sole drug treatment, worries about the development of resistance exist (Gönnert and Andrews, 1977; Wegner, 1980; Wang, Wang and Liang, 2012).

The schistosome life cycle, detailed in Diagram 1.1, consists of sexual reproduction in its definite host (humans), producing eggs, which are passed in the urine (*S. haematobium*) or faeces (*S. mansoni and S. Japonicum*). These eggs hatch in water, releasing aquatic, free living, miracidia that infect snails, in which they develop into cercariae. During daylight cercariae rupture from snails into the water, where they can infect humans via the skin. After skin penetration, schistosomula enter the circulatory system, where they are swept to the lungs before reaching the hepatic portal system, where blood feeding begins. Sexual maturation and mating occur, with the paired parasites migrating to the mesenteric veins (*S. mansoni and S. Japonicum*) or venous plexus of the bladder (*S. haematobium*), where they lay eggs. Eggs pass into the intestinal lumen or bladder, where they are released into the environment (Pearce and Macdonald, 2002).

Symptoms of schistosomiasis can occur at multiple stages of infection. Acute schistosomiasis, or Katayama syndrome, occurs anytime 2 to 12 weeks post infection,

encompassing the period of larval migration and initial egg production. Symptoms of Katayama syndrome include fever, cough, myalgia, and headache (Ross *et al.*, 2007). This syndrome occurs in the majority of non-endemic individuals exposed to schistosomiasis, but is rare in endemic individuals, potentially due to exposure of endemic individuals to schistosome Ags and/or antibodies *in utero* modulating immune response upon subsequent exposure (Eloi-Santos *et al.*, 1989; Schwartz, Rozenman and Perelman, 2000; Jauréguiberry, Paris and Caumes, 2010). Chronic schistosomiasis, which can occur in endemic and non-endemic individuals, is characterised by organ specific pathology related to the deposition of schistosome eggs from patent adult worms. In *S. japonicum* or *S. mansoni* infections, intestinal and hepatosplenic symptoms, such as enlargement of the spleen or liver fibrosis, are most common, whereas urogenital symptoms occur with *S. haematobium* infection, reflecting the different egg deposition locations in both infections (McManus *et al.*, 2018).

The lack of a long-term treatment for schistosomiasis has led to calls for development of a schistosomiasis vaccine (Hotez *et al.*, 2019). The lung stage schistosomulum is thought to be a potential target for vaccination, as the lung was found to be the major site of larval attrition in early vaccine studies (Crabtree and Wilson, 1986). However, we currently have very little understanding of pulmonary immune responses to schistosomiasis, needed to inform and accelerate vaccine design, therefore necessitating increased basic immunological understanding.

1.2. Immune defences in the lungs

The immune system is required for defence against infectious pathogens, yet this defence must be carefully balanced in order to maintain physiological function, and not cause immune-mediated pathology. Lungs are the site of gaseous exchange, conducting 11,00-15,000 litres of air per day, with the 150 m^2 alveolar membrane the largest surface in the body with direct contact to the outside environment (Martin and Frevert, 2005; Rogan *et al.*, 2006). Maintaining gaseous exchange in the lungs is critical for organismal survival, and therefore the pulmonary environment can often impose regulatory responses, reducing the chance of immune-mediated pathology (Hussell and Bell, 2014).

Air reaches the lungs via the conducting airways, the trachea and bronchioles which are lined by ciliated and mucous secreting goblet cells, covered by a mucous layer. Inhaled particles become trapped in the mucous, and are wafted upwards by cilia at a rate of 5 mm/minute, to be swallowed (Yeates, Aspin and Levison, 1975). Lung epithelial cells are able to secrete a variety of defensive proteins into the epithelium, including α and β defensins, lysozyme and cathelicidin, which can kill or aggregate microbes, or neutralize their toxins (Fleming and Allison, 1922; Tecle, Tripathi and Hartshorn, 2010).

Although defences in the conducting airways reduce microbial load in the alveoli, some particles, particularly those less than 1 μM, including allergens, bacterial and fungal spores, can reach the alveolar spaces, necessitating alveolar defence mechanisms. In steady state, the main immune cell in the alveoli are alveolar macrophages, which constitutively

phagocytose foreign particles, including apoptotic cells (Parod and Brain, 1986). Alveolar macrophages are able to eliminate pathogenic bacteria without initiating inflammation, with directed migration and rapid phagocytosis preventing bacterial outgrowth and subsequent recruitment of inflammatory cell types (Neupane *et al.*, 2020). In addition, alveolar macrophages catabolise excess surfactant, which reduces surface tension in the alveoli (Trapnell, Whitsett and Nakata, 2003). The final barrier to infection is at the layer of the epithelia itself, with junctional proteins such as claudins making epithelial surfaces impermeable to microbes that do not have active invasion mechanisms (MJ, Sutherland and Bentley, 2014).

If the alveolar epithelium is breached, resident innate immune cells are the next line of defence in the lung parenchyma. The parenchyma contains a wider variety of immune cells, including interstitial macrophages, neutrophils, and DCs (Vieira Braga *et al.*, 2019). Circulating immune cells such as neutrophils can also be rapidly recruited to sites of inflammation in the lung, sequestering to the capillary bed and then emigrating into the lung parenchyma or alveoli (Doerschuk, 2000).

Specific immune responses to pathogens, as well as innocuous or self-Ag, are the domain of adaptive immunity. Ags drain from the lung tissue to the LNs, either freely or within DCs, where they are presented to the key cells of the adaptive immune system – T and B cells (Liao and von der Weid, 2015). In response to either tolerogenic or inflammatory signals, $CD4^+$ T cells are polarised towards a regulatory phenotype (Tregs) or a number of inflammatory phenotypes (Th1, Th2, Th17), and are able to migrate back to the tissue, releasing stereotypical cytokines upon re-encounter with their cognate Ag presented by multiple histocompatibility complex (MHCII) expressing cells, notably DCs (Zhu, Yamane and Paul, 2010).

Both human and murine systems have been used to understand pulmonary immune responses. Understanding lung immune responses in mice is simpler, with airway samples and whole lungs readily available, in line with current ethical standards. In comparison, human lung immunity is less advanced due to huge logistical challenges in obtaining samples. One way to tackle this issue is utilising resected lung tissue from uninvolved regions adjacent to tumour sites, this technique was recently used to produce a cell atlas of lung immune cells revealing immune cell subtypes in different lung regions (Travaglini *et al.*, 2020). Lung sampling from healthy individuals can also be performed, although the mechanism of sampling will lead to variable results. For instance, a recent study utilised single cell RNA sequencing (ssRNAseq) study of bronchial biopsies and bronchial brushes and compared these to tissue samples from autopsy (Vieira Braga *et al.*, 2019). In lower airway biopsies DCs constituted the major cell type, with macrophages as the next most frequent, in comparison to tissue samples in which macrophages and neutrophils dominated (Vieira Braga *et al.*, 2019). Airway measurements can also be obtained by sputum or bronchoalveolar lavage (BAL) sampling, again with variable outputs. Sputum samples are thought to be more a measure of mucosal responses, typically dominated by neutrophils,

whereas BAL samples are predominantly macrophages (Grootendorst *et al.*, 1997). Utilising both mouse models and human sampling provides opportunities to understand immune responses in lung disease.

Innate immune responses

If structural barriers are breached, innate immune responses are the first line of immune defence against pathogens. Innate responses are not specific to individual pathogens, but instead are initiated by more widely shared pathogen associated molecular patterns (PAMPs), such as the bacterial cell wall component lipopolysaccharide (LPS) or double stranded RNA in viruses (Janeway, 1989, 1992). PAMPs are recognised by pattern recognition receptors (PRRs), which include toll like receptors (TLRs) such as TLR4, responsible for recognising LPS (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999). PRRs can be expressed on multiple cell types, including macrophages and DCs, as well as in non-immune cells such as epithelial or endothelial cells (Iwasaki and Medzhitov, 2004). In addition to PAMPs, recognition of damage associated molecular patterns (DAMPs) allows the immune system to recognise sterile injury, as well as to evaluate the danger posed by an infectious pathogen (Bianchi, 2007). Endogenous molecules that signal tissue damage are known as alarmins, and may be released via non-apoptotic cell death or injury (Bianchi, 2007). Recognition of PAMPs or DAMPs can lead to the release of chemokines or cytokines by a wide variety of cell types, which are able to recruit or activate immune cells (Iwasaki and Medzhitov, 2004).

Recognition of pathogens or danger by the innate immune system is critical to stimulate adaptive responses (Janeway, 1989, 1992). As discussed in more depth later, DCs are able to direct this interaction, residing in the tissue where they take up Ag and express a broad range of PRRs. Integrating signals from the recognition of DAMPs or PAMPs, DCs move to a draining LN where they prime naïve T cells to drive stereotypical adaptive immune responses (Banchereau and Steinman, 1998).

Adaptive immune responses

Adaptive immune responses are the result of the development of Ag-specific lymphocytes, T cells and B cells. Specificity for Ags, commonly proteins or peptides, mean that targeted and memory responses can be developed. In addition to Ag specificity, $CD4^+$ T cells can be divided into different subsets, based upon the cytokines they express (Mosmann *et al.*, 1986). Initially two T cell subsets were discovered, named Th1 cells and Th2 cells, which expressed respectively interferon γ (IFNγ) or IL-4 (Mosmann *et al.*, 1986), however now a number of subsets are recognised, including Tregs and Th17 cells (Brunkow *et al.*, 2001; Korn *et al.*, 2009). By expression of stereotypical cytokines and chemokines Th subsets are able to cause characteristic changes, recruiting and directing the activation of other immune cells, thereby dictating “types” of immune responses. Recent studies have questioned the clear cut nature of this Th subset paradigm, suggesting the infectious agent might be a bigger driver in transcriptional variation in Th cells, at least when comparing Th1 and Th17 cells, than belonging to a particular Th subset (Kiner *et al.*, 2021).

Type-1 immune responses

Type-1 immune responses are directed by Th1 cells, a subset that is defined by expression of the transcription factor T-bet (Szabo *et al.*, 2000). As stated, Th1 cells express the characteristic cytokine IFNγ, as well as IL-2 and lymphotoxin (Mosmann *et al.*, 1986). Th1 cells are required to develop responses against intracellular bacteria, and viruses, as has been shown in $Tbet^{-/-}$ mice, which are unable to control *Leishmania major* infection (Szabo *et al.*, 2002), as well as many other infections (Pritchard, Kedl and Hunter, 2019). Th1 cells are able to activate macrophages, stimulating them to increase their rate of phagocytosis, and produce reactive oxygen species allowing them to kill intracellular pathogens (Johnston and Kitagawa, 1985; Szulc and Piasecki, 1988).

Type-2 immune responses

Development of Th2 cells is dependent upon expression of the transcription factor *Gata3* (Zheng and Flavell, 1997). Th2 cells express the cytokines IL-4, IL-5, and IL-13 (Mosmann *et al.*, 1986). Th2 cytokines can contribute to the recruitment of eosinophils, mast cells, basophils and type-2 innate lymphoid cells (ILC2s), as well as promoting alternative-activation of macrophages (Pulendran and Artis, 2012). Th2 cytokines can also induce a humoral response, with IL-4 stimulating B cells to produce IgE, which can activate the effector function of mast cells and basophils by cross linking surface FcεRI receptors (Coffman *et al.*, 1986).

The type-2 response is thought to have evolved in reaction to invasion by large, tissue damaging metazoan parasites (Allen and Sutherland, 2014). The logic behind these responses may be best represented in the intestines, where type-2 immunity induces smooth muscle cell contraction, mucous production and fibrosis, all helping to expel intestinal parasites, and fix the damage they cause (Zhao *et al.*, 2003; Hasnain *et al.*, 2011; Allen and Sutherland, 2014). Type-2 immune responses are also characteristic of allergic conditions such as asthma or food allergy, where they are proposed to be erroneous (Bacher and Scheffold, 2018). This idea allergy as an erroneous response has being challenged recently, with proposals that food allergy is part of a “quality control” system to avoid consumption of noxious substances (Florsheim *et al.*, 2021), and that type-2 responses in fungal allergy may protective against, more severe, invasive fungal infections (Porter *et al.*, 2011).

Type-17 immune responses

Type-17 immune responses are initiated by Th17 cells, which rely upon the transcription factor RORγt, required for their production of IL-17 (Ivanov *et al.*, 2006). Type-17 immune responses are characterised by the production of IL-17 and IL-22, as well as neutrophilia (Korn *et al.*, 2009). Although a family of IL-17 cytokines exists, one member of the IL-17 family, IL-17A, is commonly referred to as IL-17 (Korn *et al.*, 2009). Notably, other members of the IL-17 family have immunological roles, for instance IL-17E (also called IL-25) is an alarmin, produced by epithelial cells and implicated in the induction of AAI (Lambrecht and Hammad, 2015). The differentiation of Th17 cells requires a combination of the regulatory cytokine TGFβ and the pro-inflammatory cytokine IL-6 (Bettelli *et al.*, 2006; Veldhoen *et al.*, 2006). In the absence of IL-6, IL-21 can induce Th17 cells in concert with TGFβ (Korn *et al.*,

2007). In addition to Th17 cells, a number of other cell types are able to produce IL-17, including $CD8^+$ T cells, γδT cells, and natural killer T cells (Pichavant *et al.*, 2008; Hamada *et al.*, 2009; Murdoch and Lloyd, 2010). The relative contribution of each subset to the IL-17 response is not known and is likely to be context dependent.

IL-17 acts through the IL-17R, expressed on numerous cell types including lung epithelium and smooth muscle cells, with IL-17 signalling driving smooth muscle proliferation (Chang *et al.*, 2012). Upon binding of the IL-17R, epithelial and endothelial cells release neutrophil chemoattractants, including the chemokine IL-8 (McAllister *et al.*, 2005; Roussel *et al.*, 2010). Neutrophils can both respond to IL-17 and produce IL-17 in response to IL-6 and IL-23 signalling, and are critical for pathogen killing, via phagocytosis or release of antimicrobial factors (Taylor *et al.*, 2014). In addition, IL-17 has been shown to improve epithelial junction integrity, and increase production of antimicrobial peptides, all contributing to defence at epithelial surfaces (J. S. Lee *et al.*, 2015; Archer *et al.*, 2016). These characteristics make Th17 responses critical for immunity to a number of pathogens, including extracellular bacteria and fungi (Huang *et al.*, 2004).

Immune responses in asthma and AAI

Asthma, and AAI in mice, is driven by both innate and adaptive arms of the immune response. Allergic sensitisation is thought to begin at the airway epithelium, via exposure to allergens such as proteins in HDM, or pollen, or non-allergenic stimuli such as pollution (Cockcroft, 2018). These substances can damage epithelial cells, leading to release of alarmins such as IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) (Humphreys, Xu and Hepworth, 2008; Préfontaine, S and Foley, 2009). For instance, IL-33 and TSLP can be released in response epithelial TLR4 binding the fibrinogen cleavage products of allergen proteases (Hammad *et al.*, 2009; Millien *et al.*, 2013). Both TSLP and IL-33 are significantly associated with asthma genome wide association studies (GWAS), suggesting their pathological relevance (Hunninghake *et al.*, 2010; Moffatt, Gut and Demenais, 2010). IL-33, released upon damage to epithelial cells, can bind cells expressing the IL-33 receptor (ST2), including lung ILC2s, and ligation of this receptor mediates their production of type-2 cytokines such as IL-13 as part of the innate immune response (Neill *et al.*, 2010; Martin and Martin, 2016).

In addition to driving innate immune responses, alarmins may direct adaptive responses via interaction with DCs, which are able to migrate to the LN and prime Th responses, as discussed later. Allergic responses are dependent upon the generation of Th2 polarised $CD4^+$ T cells. This dogma was established in early studies, showing Th2 cell transfer in mice is sufficient to induce asthmatic pathology (Cohn *et al.*, 1997), and has been confirmed using CD4 antibody depletion to show Th2 cells are required for eosinophilic pathology in a chronic ovalbumin (OVA) allergy model (Doherty *et al.*, 2009). The critical importance of Th2 cells in driving asthmatic responses can be revealed by a study in which GATA3 messenger RNA was therapeutically inactivated in asthmatic patients, leading to a reduction in type-2 mediators and improving lung function following allergen challenge (Krug *et al.*, 2015).

Cross talk between the innate and adaptive immune response maintains the asthma disease phenotype. Mast cells, armed with IgE, are increased in the smooth muscle lining of asthmatic patients (Brightling *et al.*, 2002). In addition, eosinophil granule contents can directly cause bronchial hyper reactivity and amplify inflammatory cues via the release of toxic extracellular DNA traps (Coyle *et al.*, 1995; Dworski *et al.*, 2011). Basophils, on top of their role influencing Th2 priming, can enhance Th2 cell differentiation and survival, via production of IL-4 and acquisition of MHC-II molecules from DCs (Wakahara *et al.*, 2013; Miyake *et al.*, 2017).

Type-2 immune responses can also promote airway remodelling, a stereotypical, and often treatment-resistant, feature of asthma. The Th2 cytokines IL-4 and IL-13 can induce mucus hypersecretion in the airway (Temann *et al.*, 1997; Alimam *et al.*, 2000), as well as causing subepithelial fibrosis (Gour and Wills-Karp, 2015). The stereotypical type-2 antibody, IgE can stimulate the proliferation and constriction of airway smooth muscle cells (Roth *et al.*, 2015). The role of type-2 cytokines in driving airway remodelling means that biologic therapies that block type-2 cytokines may have the potential to reverse the remodelling phenotype. For example, the Th2 cytokine IL-5 has been shown to be required for subepithelial fibrosis in asthma, via promoting the development of eosinophils that express the pro-fibrotic factor TGFβ (Kopf *et al.*, 1996; Cho *et al.*, 2004), with therapeutic blocking of IL-5 reducing fibrosis in mild asthmatics (Flood-Page *et al.*, 2003).

Traditionally, asthma has been thought of a solely type-2 inflammatory disease, mediated by Th2 cells and eosinophils. However, we now understand that only about 50% of asthmatics belong to this type-2 high endotype (Woodruff *et al.*, 2009). Utilising unbiased clustering of sputum transcriptomes, recent work has revealed three asthma endotypes, characterised by differing expression of immune receptors, cytokines and metabolic pathways (Kuo *et al.*, 2017) One aspect of disease receiving increased focus is the type-17 immune response, classically characterised by IL-17 production and neutrophilia, with IL-17 levels elevated in severe asthma (Ricciardolo *et al.*, 2017; Hynes and Hinks, 2020). The influence of type-17 responses in asthma is still unclear, with both protective and pathogenic functions suggested, and studies showing a potential disconnect between IL-17 and neutrophilia, necessitating further research (Kuo *et al.*, 2017; Hynes and Hinks, 2020).

The relationship between type-2 and type-17 responses is a key area of current research, with one study showing IL-17 may be critical to initiate the type-2 inflammatory responses in nematode infection, via suppression of IFNγ (Ajendra *et al.*, 2020). There is evidence that a similar pathway may operates in AAI, with mice repeatedly challenged with a $2x10^6$ *A.f.* conidia shown to develop mixed type-2/type-17 inflammatory responses, with type-2 inflammation, most notably eosinophilia, reduced in IL-$17^{-/-}$ mice (Murdock *et al.*, 2012). The interrelation between type-2 and type-17 responses seen in mouse models contrasts with a human study of bronchial biopsies in asthma, in which Th2 and Th17 gene expression signatures were mutually exclusive, with no individual having a mixed Th2/Th17 gene phenotype (Choy *et al.*, 2015). Understanding how type-2 and type-17 responses interact

might be critical when thinking about treatment options for asthma, in which inhibiting both type-2 and type-17 responses are key therapeutic avenues (Poynter, 2016).

Type-17-dominated responses have been shown in asthma, and are commonly associated with an increase in IL-17 as well as neutrophilia (Hynes and Hinks, 2020). Neutrophilic asthma is associated with increased sputum IL-8 as well as neutrophil derived products such as neutrophil extracellular traps (NETs) when compared to healthy controls, with NET constituents correlated with asthma severity (Wright *et al.*, 2016). In mice IL-17A has been associated with enhanced smooth muscle contraction, with a reduction in IL-17 levels leading to reduced airway hyperresponsiveness (Kudo *et al.*, 2012; Chesné *et al.*, 2015). In addition, disruption in IL-6 signalling, that leads to a deficiency in Th17 cells, has been shown to reduce airway remodelling (Zhao, Lloyd and Noble, 2013). Human IL-17 gene polymorphisms have been associated with asthma (Kawaguchi *et al.*, 2006; Du *et al.*, 2016) and numerous small studies have reported a correlation between IL-17 levels and asthma severity (Bullens *et al.*, 2006; Ricciardolo *et al.*, 2017). Other studies have not replicated this finding, with no correlations observed between Th17 cell levels, and asthma severity (Hinks *et al.*, 2015). Murine models of asthma provide an opportunity to investigate the contribution of Th17 responses to AAI.

Murine models of AAI

Mice do not develop asthma. However, certain characteristics of human asthma can be provoked in murine models of AAI (Szelenyi, 2000), providing researchers the opportunity to dissect immunological mechanisms. Classical AAI models rely on peripheral sensitization with the model Ag chicken OVA, in combination with the Th2-promoting adjuvant alum. After a delay, to allow for generation of an adaptive response, mice are challenged with repeated exposure to OVA in the airways (Lloyd, 2007; Kumar, Herbert and Foster, 2008). As a result, mice develop a Th2 driven response, bronchial hyperreactivity and extreme eosinophilia (Farraj *et al.*, 2003). Alterations on this general model can be used to study specific aspects of the disease, for instance tolerogenic responses to allergens can be studied by exposure to OVA in the absence of adjuvant (Khare *et al.*, 2013). OVA/adjuvant models are readily dissectible with transgenic tools, such as OVA-specific transgenic mice, in which Ag-specific responses can be measured (Jacobsen *et al.*, 2008). Moreover, the separation of the sensitization and challenge phase allows for dissection of the role of immune mediators at these separate phases (Baru *et al.*, 2010, 2012). Despite these benefits, these models have been criticized for being unrepresentative of human disease (Lloyd, 2007). OVA is not an allergen in humans, and initial sensitisation to aeroallergens is very unlikely to occur in the peritoneal cavity, a common site of sensitisation in the OVA/alum model. Instead, allergic sensitisation is thought to occur in the airways, or even the skin, as suggested by the observed “atopic march”, with children progressing from dermatitis to asthma over a number of years (Beck and Leung, 2000). Finally, human asthma is a chronic disease, with characteristic airway remodelling not recapitulated in the acute OVA/alum model (Szelenyi, 2000), leading many investigators to switch to more representative models.

One strategy has been to utilise allergens that humans with asthma are sensitised to. For instance, HDM has been used in acute challenge models in a similar sensitization/challenge regime as stated above for OVA (Plantinga *et al.*, 2013). Extending the translatability of this kind of approach, HDM has been used in chronic models, with repeated intranasal HDM dosing over weeks/months, leading to bronchial hyperreactivity, type-2 inflammation, and airway remodelling, which persisted even after cessation of Ag administration (Johnson *et al.*, 2004). Although an advance, no mouse model can fully replicate human disease. For instance murine models often induce exaggerated airway eosinophilia, where these cells can comprise 40-80% of the total immune compartment, in contrast to the 1-5% generally seen in human asthmatics (Wardlaw *et al.*, 1988). Moreover, there is an urgent need to diversify our current murine models, to represent more than just the 50% of patients with type-2 asthma, as well as the divergent phenotypes of individuals exposed to different allergens (Woodruff *et al.*, 2009; Knutsen *et al.*, 2012).

Fungal allergens have been utilised in mouse models of AAI. For instance, a dose dependent mixed type-2/type-17 immune response has been reported after repeat intranasal exposure to 0.002-0.2x10^6 *A.f.* spores (Porter *et al.*, 2011). Quiescent *A.f.* conidia are not immunogenic, due to an outer hydrophobic layer of rodlet proteins which mask internal Ags from the immune system, and are mainly rapidly removed from the airways via phagocytosis by alveolar macrophages, as well as mucociliary clearance (Ibrahim-Granet *et al.*, 2003; Aimanianda *et al.*, 2009). However if spores escape these early defence mechanisms, germination can occur in 4-6 hours in the airways, revealing carbohydrate molecules such as β-glucan which is recognised by the innate immune system via dectin-1 receptors as a pathogen associated molecular pattern, stimulating immune activation (Brown and Gordon, 2001; Hohl *et al.*, 2005). Further work is needed to elucidate how *A.f.* induces allergic responses. Notably, as well as allergic disease, *A.f.* can cause fatal invasive pathology at high doses (5x10^6 spores intranasally) in mouse models (Porter *et al.*, 2011). Notably *A.f.* is not the only allergenic member of the aspergillus genus (Bowyer and Denning, 2007). For instance, *A. niger* is able to promote AAI in mice, dependent upon allergen proteinase activity (Porter *et al.*, 2009).

Immune responses in schistosomiasis

Unlike asthma, schistosomiasis is a multi-organ disease, which must be considered in the context of the complex life cycle of schistosomes in the human host, with time post infection a key indicator of stage of disease (Diagram 1.1). In both humans and murine models cercariae penetrate the skin and move to the blood vessels by 48-72 hours post infection (Wheater and Wilson, 1979; He, Chen and Ramaswamy, 2002). Cercarial penetration of the skin initiates both inflammatory and regulatory pathways, which can manifest as cercarial dermatitis in humans (Ross *et al.*, 2007). Inflammation in the skin peaks at 48-72 hours post infection, and consists of eosinophilic and neutrophilic infiltration (Incani and McLaren, 1984; Ward and McLaren, 1988) as well as release of pro-inflammatory cytokines including IL-6, IL-12 and IL-18 (Hogg *et al.*, 2003). In parallel with this inflammatory response, a regulatory immune response develops, with an increase in the cytokine IL-10 upon infection (Hogg *et al.*, 2003). If skin infections are repeated in mice, there is an enhancement of type-2 immune

responses, for instance increased expression of IL-4, as well as development of IL-10 dependent $CD4^+$ T cell hypo-responsiveness (Cook *et al.*, 2011; Prendergast *et al.*, 2015). A similar response has been observed in human *ex vivo* systems, with schistosome infection driving dermal DCs to produce both proinflammatory cytokines (IL-6), and the regulatory cytokine IL-10 (Winkel *et al.*, 2018).

After the skin, schistosomula migrate intravascularly to the lungs. Tracking schistosomula in the human host is not possible, meaning schistosome lung migration is less well understood. Our best estimates (as used for Diagram 1.1) derive from baboons, in which lung migration of *S. mansoni* peaks between day 5-9 post infection (Wilson *et al.*, 1990). It is not possible to predict whether infection will occur on exposure to water bodies with known cercarial contamination, and knowingly allowing individuals to be exposed in the field is ethically dubious. Practically, this means that identifying individuals infected with early larval stages, and assaying their immune responses, is almost impossible. However, infected individuals may present to a doctor with pulmonary symptoms of acute schistosomiasis (Katayama fever) from 2 to 12 weeks post infection, including cough, breathlessness and radiographically observed lung lesions (Schwartz, Rozenman and Perelman, 2000; Gobbi *et al.*, 2017). These symptoms can be accompanied by transient pulmonary eosinophilia (Schwartz, Rozenman and Perelman, 2000; Lucas *et al.*, 2012). It is not known whether lung migrating schistosomes drive Katayama syndrome, with timing of symptoms encompassing larval migration, maturation, and initial egg production. However, it is now known that egg production is not required, with Katayama syndrome occurring in individuals experimentally infected with single sex (male) schistosomes that do not produce eggs (Langenberg *et al.*, 2019). Experimental infections such as this have the potential to greatly increase our understanding of immune responses in schistosomiasis, allowing us to track immune responses throughout the course of infection.

Understanding of immune responses in lung migrating schistosomiasis is better understood in murine models. *S. mansoni* schistosomula first appear in the lungs at day 4 post infection, peaking at day 10-12, and steadily declining until approximately day 20, when they start to be detectable in the mesenteric vessels and liver (Wheater and Wilson, 1979; Wilson, Coulson and Dixon, 1986). Increased numbers of IL-4 expressing Th2 cells are observed in lung draining LNs during migration, at day 14 and 21 post infection (Redpath *et al.*, 2015). In addition, histological studies have shown inflammatory foci consisting of neutrophils, eosinophils, macrophages and lymphocytes during lung migration (Crabtree and Wilson, 1986). Similar histological observations of leukocyte, macrophage and eosinophil infiltration have been observed in murine *S. japonicum* infection, in addition to upregulation of wound healing and inflammatory genes, including *Retnla* (Resistin Like Molecule alpha, RELMα), commonly associated with type-2 polarization of innate immune cells (Burke *et al.*, 2011). Although superior to our currently limited understanding of the human situation, our understanding of murine immune responses to lung migrating schistosomula is not in line with our modern appreciation of the complexities of immune responses. For instance, we do not know how schistosome larvae influence the number or phenotype of myeloid cells in the lung.

Leaving the lungs, schistosomes then move to the liver, and then the mesenteric vessels, where they pair and release eggs which are swept to the intestines and liver where they cause granulomatous inflammation (Pearce and Macdonald, 2002). Eggs drive a robust systemic and local Th2 immune response in the host (Grzych *et al.*, 1991). The Th2 cytokine IL-13 induces characteristic structural changes surrounding the deposited eggs, as a potent inducer of gut and liver fibrosis (Fallon *et al.*, 2000). Despite sustained production of eggs, the Th2 response to egg Ags peaks between weeks 8-10 post infection, followed by $CD4^+$ T cell hyporesponsiveness and an increase in regulatory T and B cells (Jankovic *et al.*, 1998; Singh *et al.*, 2005; Taylor *et al.*, 2009; van der Vlugt *et al.*, 2012; Lundy and Lukacs, 2013).

During human patent schistosome infection lung symptoms can occur, despite the distal mesenteric or bladder location of adult worms. Most notably, pulmonary hypertension is a complication of hepatosplenic schistosomiasis, occurring in 6.3-13.5% of patients (Lapa *et al.*, 2009; Knafl *et al.*, 2020), with a five year survival rate of just 69% in untreated individuals (Fernandes *et al.*, 2018). The pathogenesis of pulmonary hypertension begins with liver fibrosis, leading to portal hypertension that can cause pulmonary hypertension, either directly or via the formation of porto-systemic shunts, abnormal veins connecting blood draining from the intestines directly to the heart, bypassing the liver, along which eggs can pass to the lungs (El-Gendi, 1979; Coulson and Wilson, 1989; Porres-Aguilar *et al.*, 2012). Translocated eggs cause a local inflammatory response, obstructing the pulmonary vasculature and contributing to hypertension (Shaw and Ghareeb, 1938; Pearce, 2005).

Pulmonary immune response in chronic schistosomiasis can be observed in mice, with egg deposition observed in lungs peaking at week 17 post infection (Crosby *et al.*, 2010). Granulomas, protective aggregations of immune cells, mainly macrophages, form around deposited eggs, and pulmonary expression of the Th2 cytokines IL-4 and IL-13 is increased, with IL-13 correlated to lung egg count (Crosby *et al.*, 2010, 2015). This inflammatory response is sufficient to drive vascular remodelling and hypertension, reflective of human disease (Crosby *et al.*, 2010, 2015). It is not known whether type-2 immune responses in the lung develop in the early stages of egg production, prior to egg translocation, in response to the dominant egg-driven systemic type-2 immune responses (Grzych *et al.*, 1991). Understanding this will be critical to understand the symptoms of acute schistosomiasis, which can occur at this early stage of egg production (Ross *et al.*, 2007). The proposal that an immune response taking place in the gut would affect the lung has been borne out by recent studies that have shown infection with a gut-resident helminth (*Heligmosomoides polygyrus)* was able to promote Th2 responses in the lung that were protective against the lung migrating stage of a different helminth (*Nippostrongylus brasiliensis*) (Filbey *et al.*, 2019). Our understanding of immune responses in schistosomiasis has been greatly aided by murine models. However, the relevance of such murine discoveries to human disease must always be addressed.

Murine models of schistosomiasis

Superficially, modelling human schistosomiasis in mice is easier, as mice can be infected with schistosoma species, with cercariae able to percutaneously infect the murine host, migrate, reach patency and release eggs (Pearce and Macdonald, 2002). There are differences, however. The scale and organ distribution of egg deposition in mice differs to humans, with much higher total egg deposition in mice per worm pair, focused in the liver in mice but more evenly distributed between the liver, colon and lungs in humans (Cheever, 1969). Specifically, 11-15% of total eggs were found in the lungs of humans with chronic schistosomiasis, whereas only 1% of total eggs were found in the lungs of mice at 48 weeks post infection (Cheever, 1969). The impact of schistosomiasis on the lungs, if lead solely by mouse models, may therefore be underestimated.

Choosing an appropriate infectious dose for murine studies also poses a challenge since, on a body weight basis, one worm pair in mice would relate to 5000 worm pairs in human (Fallon, 2000). For comparison, infection of human volunteers with 20-30 cercariae is sufficient to lead to diagnosable infection, and even symptoms of Katayama syndrome, in individuals experimentally infected with single sex (male) cercariae (Langenberg *et al.*, 2020). In contrast, murine models often utilise infectious doses of between 20-200 cercariae (Phythian-Adams *et al.*, 2010). Utilising these doses is often required to obtain a consistent phenotype, with variability inherent in larval infections, maturation and pairing, allowing for intervention studies that elucidate underlying mechanisms.

Finally, a criticism of murine models of schistosomiasis is their focus on primary infection, whereas natural infections are thought to follow a “trickle” model, with repeated exposure to low numbers of parasites via water contact (Freer *et al.*, 2018). Studies of intestinal helminths have revealed trickle infections to induce distinct immunological effects, in comparison to singular infection (Glover *et al.*, 2019). Trickle studies in *S. mansoni* infection have unique challenges, with liver fibrosis due to primary infection leading to the formation of porto-systemic shunts, and this altered vasculature reducing the ability of subsequent migrating larvae to lodge and mature in the vasculature surrounding the liver (Wilson, 1990). This is not thought to commonly occur in humans, and limits repeat infection studies to pre-patent stages (Cook *et al.*, 2011).

Immune regulation – the role of cytokines

Unregulated inflammatory responses to pulmonary pathogens, exemplified by the often fatal “cytokine storm” observed during COVID-19 infection, can lead to host death quicker than the pathogen itself (Cron, 2020). Therefore, it is critical to avoid inappropriate or excessive immune responses to self, or innocuous non-self-antigenic stimuli, that lead to autoimmune or allergic diseases such as asthma. A starting point for this is ensuring immune responses are not developed against self-Ags. Known as central and peripheral tolerance, this process is mediated in part by the actions of regulatory cells, including DCs and Tregs (Proietto *et al.*, 2008; Ohnmacht *et al.*, 2009). In addition, a number of cytokines have regulatory roles in the

lung, most notably TGFβ and IL-10 (Branchett and Lloyd, 2019; McEntee, Gunaltay and Travis, 2020).

The critical importance of TGFβ in regulating immune responses was revealed in TGFβ1$^{-/-}$ mice, which develop an inflammatory wasting syndrome, dying by weeks 3-4 post birth (Kulkarni *et al.*, 1993). TGFβ is required for the development of alveolar macrophages which are unable to mature neonatally in the absence of cell-intrinsic TGFβ (Branchett *et al.*, 2021). TGFβ is secreted in a complex with latency-associated peptide (LAP), preventing TGFβ binding to its receptor, as well as anchoring it to the extracellular matrix (Taipale *et al.*, 1994; Shi *et al.*, 2011). Removal of LAP is required for TGFβ activity, and can be realised by interaction with αv integrins, including αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 (Travis and Sheppard, 2014). Abundant latent TGFβ can be detected in multiple tissues, including the lung, with control of TGFβ activation thought to be critical for its physiological effects (Sheppard, 2006). TGFβ is a pleiotropic cytokine, critical for the induction of both Treg and Th17 responses, as well as fibrosis via the activation of fibroblasts to produce extracellular matrix (Border *et al.*, 1990; Chen *et al.*, 2003; Gutcher *et al.*, 2011; Meng, Nikolic-Paterson and Lan, 2016).

Another key regulatory cytokine is IL-10, initially discovered as a cytokine produced by Th2 cells that inhibited Th1 cytokine production (Fiorentino, Bond and Mosmann, 1989). IL-10 deficient mice suffer from a colitis like disease, dependent upon the presence of enteric bacteria (Kühn *et al.*, 1993). However, now it is known that IL-10 is able to be secreted from a large number of haematopoietic cells of both myeloid and lymphoid lineage, as revealed utilising transgenic reporter mice (Bouabe *et al.*, 2011). IL-10 is able to act on a number of hematopoietic cells via their expression of the IL-10R, with multiple effects (Berkman *et al.*, 1995; Kevin W Moore *et al.*, 2001). IL-10 was originally proposed to exert anti-inflammatory effects on via myeloid cells, reducing cytokine and chemokine production (Abrams *et al.*, 1991; Berkman *et al.*, 1995), but can also directly act upon T cells (Kamanaka *et al.*, 2011; Coomes *et al.*, 2016). During infection, IL-10 can be critical to protect against immune pathology, with inhibition of IL-10R signalling in influenza infection leading to a heightened Th1 response, and increased tissue injury (Sun *et al.*, 2009). In other infections, the regulatory effect of IL-10 may lead to pathogen survival. For instance, when IL-10 was transgenically upregulated, mice had a reduced ability to clear tuberculosis infection (Murray *et al.*, 1997).

Cytokine mediated immune regulation in asthma

TGFβ is critical for the regulation of AAI. TGFβ expression by CD11c expressing cells (macrophages and DCs) is required to suppress type-2 inflammation in a neonatal HDM model of AAI (Branchett *et al.*, 2021). Reduced TGFβ expression, in mice with a heterozygous TGFβ deletion leads to enhanced type-2 inflammation in an OVA/adjuvant model of AAI (Scherf, Burdach and Hansen, 2005). The importance of TGFβ in regulating responses in AAI was also shown via the transferral of engineered latent TGFβ producing CD4^{+} T cells, specific for the model Ag OVA, which are able to inhibit AAI responses (Hansen *et al.*, 2000). In the lung, TGFβ can be activated at steady state by αvβ6, expressed on airway epithelial cells,

maintaining a tolerogenic environment (Huang *et al.*, 1996). TGFβ activation by αvβ8 expressing DCs has been shown to be required for the development of Th17 responses in an OVA/alum model of AAI (Kudo *et al.*, 2012). TGFβ can also contribute to fibrosis in inflammatory conditions such as AAI, with one study finding a blocking antibody to αvβ8 was able to attenuate fibrotic responses to stimuli including allergens (Minagawa *et al.*, 2014).

IL-10 also plays a role in regulation of AAI, with IL-10$^{-/-}$ mice exhibiting increased type-2 cytokine production in an OVA/adjuvant model of AAI (Tournoy, Kips and Pauwels, 2000). Multiple sources of IL-10 have been shown to be critical during AAI, including T cells and DCs, with the relative contributions of each source still unclear (Akbari, DeKruyff and Umetsu, 2001; Campbell *et al.*, 2009; Branchett *et al.*, 2020).

Cytokine mediated immune regulation in schistosomiasis

TGFβ is critical in regulating immune and non-immune mediated aspects of schistosomiasis. TGFβ activation, via schistosome mediated upregulation of the extracellular matrix component thrombospondin-1, is required for the development of pulmonary hypertension in schistosome infection (Kumar, Herbert and Foster, 2008). In addition, exogenous TGFβ provided during schistosomula lung migration lead to an increase in Th17 cytokines in serum, with increased Th2/Th1 cytokines observed when an anti-TGFβ antibody is administered, suggesting that the balance of immune responses to migrating schistosomula is dependent upon TGFβ (Tallima *et al.*, 2009). This role for TGFβ may not extend to patent schistosomiasis however, as mice treated with anti-TGFβ during patent schistosomiasis did not show enhanced inflammation, or granulomatous pathology, except when depleted in concert with IL-10 (Herbert *et al.*, 2008).

In schistosomiasis IL-10 has been shown to reduce inflammatory pathology, at the expense of efficient pathogen killing. Early work on IL-10 in schistosomiasis revealed that in response to IFNγ macrophages upregulate production of nitric oxide, allowing them to kill schistosomula *in vitro,* with addition of IL-10 drastically reducing macrophage mediated schistosomula killing (Oswald *et al.*, 1992). This was followed by *in vivo* studies, demonstrating IL-10 downregulates both type-1 and type-2 responses in schistosome pulmonary granuloma models, following *i.v.* injection of *S. mansoni* eggs (Wynn *et al.*, 1997). A similar pattern is observed in patent *S. mansoni* infection, with IL-10$^{-/-}$ mice displaying increased expression if IL-5 and IFNγ, as well as reduced survival (Hoffmann, Cheever and Wynn, 2000). Both T cell derived and innate sources of IL-10 regulate Th2 responses in patent schistosomiasis, though T cell derived IL-10 is preferentially required for survival (Hesse *et al.*, 2004). IL-10 is able to work in concert with TGFβ to promote survival in schistosomiasis, and regulate Th1, Th2 and Th17 responses, with enhanced inflammation observed in mice deficient in both cytokines (Herbert *et al.*, 2008).

1.3. Tregs

In addition to regulatory cytokines, the idea of a regulatory T cell subset, Tregs, was proposed more than 40 years ago, but their existence was a source of strong debate until the early 2000's, when the lineage defining transcription factor Foxp3 was identified (Gershon and Kondo, 1970; Green and Webb, 1993; Brunkow *et al.*, 2001). Mutation of Foxp3 leads to a fatal lymphoproliferative disorder, in both mice and humans, and overexpression to a defect in effector T cells (Brunkow *et al.*, 2001; Levy-Lahad and Wildin, 2001; Kasprowicz *et al.*, 2003). In addition to Foxp3, Tregs can be identified by expression of the high affinity IL-2 receptor CD25, and are often identified as $CD25^+$ $CD127^-$ in humans, the addition of CD127 allowing researchers to isolate viable Tregs with increased specificity, without the requirement to permeabilise the cells to assay Foxp3 expression (Almeida *et al.*, 2002; W. Liu *et al.*, 2006). Less well defined are "Tr1" cells, that do not express Foxp3, but are capable of suppression via similar mechanisms to Tregs, such as expression of the regulatory cytokine IL-10, the co-inhibitory receptor CTLA4, or direct killing of myeloid cells by pore-forming perforin (Zeng *et al.*, 2015).

Treg development – thymic and peripheral

In vivo Tregs can be divided in to thymic and peripheral subsets (tTregs and pTregs). The first evidence for a regulatory population of tTregs, was that thymectomy of mice and rats leads to autoimmune inflammation, reverted by transfer of $CD4^+$ T cells. (Sakaguchi, Takahashi and Nishizuka, 1982; Fowell and Mason, 1993). Thymic Tregs preferentially accumulate in non-lymphoid tissue, promoting tolerance to self-Ags, reliant upon *Aire*, a transcription factor expressed in medullary thymic epithelial cells that allows for expression of non-thymic self-Ags in the thymus (Malchow *et al.*, 2016). Further evidence for the self-Ag specificity of tTregs is that they do not develop in T cell receptor (TCR) transgenic mice specific for non-self-Ag (Itoh *et al.*, 1999). Utilising mice with a $Nur77^{GFP}$ reporter, expressed upon TCR signalling, has suggested that Tregs receive stronger TCR signals than conventional T cells during thymic development (Moran *et al.*, 2011). In addition to signalling through the TCR, development of tTregs requires costimulation, and IL-2 signalling (Papiernik *et al.*, 1998; Salomon *et al.*, 2000)

Not all self-Ags are expressed in the thymus, and some may arise at different stages in development, in addition thymic tolerance provides no mechanism by which innocuous non-self-Ags can be recognised. To overcome these issues, Tregs are able to be induced in the periphery, named peripheral Tregs (pTregs). The requirement for peripheral Tregs was shown by Treg depletion causing inflammatory bowel disease-like pathology, attributed to excessive immune responses to commensal bacteria (Singh *et al.*, 2001). Evidence for pTreg development was shown by transfer of $CD4^+CD25^-$ T cells into lymphopenic mice, which were able to develop into suppressive $CD4^+CD25^+$ Tregs (Curotto de Lafaille *et al.*, 2004). As discussed in greater detail later, DCs are able to promote Treg development from TCR transgenic T cells specific for exogenous Ag, in the absence of inflammatory signals (Apostolou and Von Boehmer, 2004; Kretschmer *et al.*, 2005). Mice deficient in *CNS1*, a

response element in the *Foxp3* locus have reduced pTreg differentiation, and develop aberrant type-2 immune responses at mucosal sites, again supporting the critical role for pTregs in promoting tolerance to innocuous non-self antigens (Zheng *et al.*, 2010; Josefowicz *et al.*, 2012). There are no reliable markers to differentiate thymic and peripherally derived Tregs, with several studies now showing that the proposed markers Helios and Neuropilin-1 are not specific, complicating understanding of the differential roles of these cells (Szurek *et al.*, 2015; Thornton and Shevach, 2019).

Expression of the Treg-defining transcription factor Foxp3 can be induced by TGFβ, IL-2, and TCR stimulation (Chen *et al.*, 2003). Foxp3 expression, though not sufficient alone to induce the full range of Treg associated epigenetic changes, has been shown to be necessary for Treg differentiation and suppressive function (Hori, Nomura and Sakaguchi, 2003; Hill *et al.*, 2007). Other genes such as *Gata3, Lef1, Satb1, Eos* and *Irf4* work with Foxp3 to establish Treg characteristics (Li and Zheng, 2015). Notably, although multiple transcription factors control the Treg expression profile removal of any one of these factors, except Foxp3, does not affect Treg gene expression, suggestive of compensatory loops in place (Fu *et al.*, 2012). Once induced, Treg homeostasis is maintained by signalling through CD25 (Almeida *et al.*, 2002). The critical role of IL-2 in Treg homeostasis was revealed by experiments with IL-$2^{-/-}$ mice, that develop ulcerative colitis like pathology (Sadlack *et al.*, 1993). IL-2 from effector T cells and, to a lesser extent, DCs, has been shown to contribute to Treg development and homeostasis (Owen *et al.*, 2018). During inflammation the importance of DC produced IL-2 may increase, with one study showing that the alarmin IL-33 was able to stimulate DC production of IL-2, leading to Treg expansion (Matta *et al.*, 2014).

Treg functional mechanisms

Tregs are able to suppress inflammation via a contact dependent and independent mechanisms, as shown in Diagram 1.2. In terms of contact dependent mechanisms, Tregs can directly kill DCs, natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells via expression of the pore forming protein perforin, and the protease granzyme b, shown *in vitro* and in *in vivo* tumour models (Gondek *et al.*, 2005; Cao *et al.*, 2007; Boissonnas *et al.*, 2010). Tregs may also directly suppress Ag presenting cells, contacting DCs more frequently than $Foxp3^-$ $CD4^+$ T cells (Tang *et al.*, 2006). In addition, Tregs can suppress DCs via a CTLA4 dependent mechanism, with DCs interacting with Tregs increasing their expression of Indoleamine-pyrrole 2,3-dioxygenase (IDO), leading an increase in regulatory tryptophan metabolites (Fallarino *et al.*, 2003; Oderup *et al.*, 2006). Tregs also express Lymphocyte-Activation Gene 3 (LAG-3), which is able to bind MHC-II molecules, suppressing DC maturation (Buisson and Triebel, 2005; Liang *et al.*, 2008). Finally, one study has linked decreased ability of Tregs deficient for casein kinase 2 to control lung Th2 responses to an inability to suppress the expansion of a particular DC subset (Ulges *et al.*, 2015). Tregs are able to suppress numerous cell types, for instance culture of Tregs with macrophages and neutrophils leads to a reduction in pro-inflammatory, and increase in regulatory cytokine production (Tiemessen *et al.*, 2007; Lewkowicz *et al.*, 2013)

Tregs may also suppress inflammation via non-contact mediated mechanisms such as the production of IL-10, with Treg produced IL-10 shown to be required to prevent colitis (Asseman *et al.*, 1999). Another regulatory cytokine that contributes to the function of Tregs is IL-35, required for suppression of effector T cells *in vitro* and in colitis models (Collison *et al.*, 2007). Tregs are able to suppress via hydroxyprostaglandin dehydrogenase mediated metabolism of the eicosanoid PGE_2 into 15-keto PGE_2, required for *in vivo* suppression in a colitis model (Schmidleithner *et al.*, 2019). In addition Tregs are able to inhibit CD4 T cells via the expression of cell surface CD39 and CD73, enzymes capable of generating adenosine, which is required for the suppression of T cell proliferation *in vivo* and *in vitro* (Deaglio *et al.*, 2007). Finally, Tregs are able to suppress effector T cells by acting as a sink for IL-2, via expression of high levels of CD25 depriving effector T cells and leading to either a halt in proliferation, or apoptosis (Duthoit *et al.*, 2005; Pandiyan *et al.*, 2007).

In addition to preventing excessive or inappropriate immune responses, Tregs may reside in tissues, where they have a homeostatic role (Panduro, Benoist and Mathis, 2016). This was first shown in visceral adipose tissue, in which Treg depletion provoked metabolic abnormalities including insulin resistance (Feuerer *et al.*, 2009). Whether Tregs in the lung have comparable homeostatic functions is unclear. However, cells with characteristic tissue Treg phenotypes, for instance expression of the IL-33R ST2 alongside KLRG1, have been observed in the lung (Delacher *et al.*, 2017). Further, it has been shown that Tregs express the amphiregulin (AREG), a cytokine that is dispensable for suppressor functions, but promotes lung repair and the return to homeostasis post influenza infection (Arpaia *et al.*, 2015). The ability of Tregs to suppress immune responses, and help return organs to health, has led to the investigation of Tregs as a therapeutic target.

Treg stability

Therapies which aim to expand or transfer Tregs to control excessive or inappropriate immune responses rely on those Tregs not becoming pro-inflammatory cells. Treg instability can be defined as the loss of Foxp3 expression, suppressive function, or the gain of inflammatory function (Sawant and Vignali, 2014). The first studies to show Treg instability transferred $Foxp3^+$ Tregs into a lymphopenic environment, where they lost expression of Foxp3 (Duarte *et al.*, 2009). These studies were controversial, however, with a lymphopenic environment not representative of most natural physiological or pathological states and questions about possible transfer of contaminant $Foxp3^-$ cells.

Treg stability was therefore next questioned in immune-competent mice, utilising fate reporters, in which expression of Foxp3 leads to sustained expression of a reporter protein, even if Foxp3 transcription is halted. Utilising this technique, one study found that between 15-30% of cells in peripheral tissue that had expressed Foxp3 at some point in time were now Foxp3 negative (Zhou *et al.*, 2009). A subsequent study, using a pulse-labelling methodology to label all Tregs in a period of time, suggested that over 95% of the cells that had expressed Foxp3 were still expressing Foxp3 five months later (Rubtsov *et al.*, 2010). A potential explanation for this discrepancy is the possible presence of transient Foxp3 expression in

cells that are not true Tregs, which would be labelled at a much lower frequency in the pulse-labelling approach adopted by Rubtsov et al (Li and Zheng, 2015).

Whilst the aforementioned studies primarily focus upon steady state, Treg instability may be enhanced during inflammation, such as in a model of experimental autoimmune encephalomyelitis (EAE) where Tregs specific for auto-Ag downregulate Foxp3 expression at a rate higher than polyclonal Tregs, attributed to local IL-2 depletion around auto-Ag specific cells (Bailey-Bucktrout *et al.*, 2013). In addition, Tregs were shown to be unstable in a model of helminth infection, with a proportion of Tregs converting to Th2 cells by an IL-4 dependent mechanism (Pelly *et al.*, 2017).

Tregs may become unstable by gaining effector functions without losing Foxp3 expression, as was shown in a tumour model, in which neuropilin-1 deficient Tregs express IFNγ, and are able to contribute to tumour regression (Overacre-Delgoffe *et al.*, 2017). Further, in a model of IL-33 induced AAI Tregs were shown to upregulate expression of the stereotypical Th2 transcription factor GATA3, express the cytokines IL-5 and IL-13, and have reduced suppressive capacity (Chen *et al.*, 2017).

A number of factors have been shown to be crucial for maintaining Foxp3 expression, or suppressive function in Tregs. Treg lineage identity is maintained by inheritance of the demethylated CNS2 locus, with Foxp3 expression in *Cns2*$^{-/-}$ Tregs lost upon cell proliferation (Feng *et al.*, 2014). A number of proteins work together to maintain the stability of Tregs, one of these being USP21 which, when deleted, leads to a reduced number of Tregs, with decreased suppressive ability at steady state (Li *et al.*, 2016). Supporting a role of IL-4 in driving Treg instability, in mice with a mutated IL-4R, Tregs became unstable during OVA/alum driven AAI, converting to Th17 cells (Massoud *et al.*, 2016). The IL-4R mutation chosen for this study mimicked a naturally occurring *IL4R* variant, associated with asthma in human populations (Massoud *et al.*, 2016). Another study found that Tregs deficient in Wiskott-Aldrich Syndrome Protein are not able to suppress Th2 cytokine production in a model of food allergy, gaining a Th2-like phenotype with increased GATA3 expression (Lexmond *et al.*, 2016). The role of GATA3 in Tregs is complicated, whilst a number of studies have used it's expression to support proposals of Treg instability, as evidence of a Th2-like phenotype (Lexmond *et al.*, 2016; Chen *et al.*, 2017), other studies have found Treg GATA3 expression to be required for suppressive function, particularly in inflammation, with *Gata3* deficient Tregs gaining a Th17-like phenotype (Wang, Su and Wan, 2011; Wohlfert *et al.*, 2011). Certain factors are required specifically for inhibition of Th2 responses by Tregs, for instance Treg specific depletion of the transcription factor *Irf4* lead to a selective failure to control Th2 but not Th1 cytokine production at steady state (Zheng *et al.*, 2009). Two separate studies showed Treg specific depletion of, respectively, the ubiquitin ligase *Itch*, and the transcription factor *Bcl6*, lead to an inability to control Th2-mediated pathology, and Treg expression of GATA3 and Th2 cytokines both at steady state and during AAI (Sawant *et al.*, 2012; Jin *et al.*, 2013; Ulges *et al.*, 2015). The above studies provide insight into the factors that are required to maintain Treg stability, and may be have particular relevance in certain

situations – for instance in patients Wiskott-Aldrich Syndrome, or with asthma associated IL-4R polymorphisms – but do not support the proposal of Treg instability being common in non-mutant mice (Lexmond *et al.*, 2016; Massoud *et al.*, 2016).

Interaction with other cell types may contribute to Treg stability. For instance Treg conversion to Th17 cells was observed in mice lacking autophagy in their DCs (Niven *et al.*, 2019). In addition the incubation of Tregs with DCs matured in the presence of IL-33 leads to Tregs losing Foxp3 expression beginning to produce the Th17 cytokine IL-17 (Park, Jung and Kim, 2020). $CD8^+$ T cells have also been shown to be critical to maintain Treg function, via interaction of MHCI on Tregs with CD8 (Joetham *et al.*, 2007). Supporting the importance of this interaction, transfer of Tregs into $CD8^{-/-}$ mice enhanced AAI responses in an OVA/alum model, dependent upon Treg production Th2 and Th17 cytokines (Joetham *et al.*, 2017).

There is some evidence that Treg instability can be seen in human conditions. In asthma, IFNγ and IL-17 producing Tregs have been observed in peripheral blood, and are correlated with asthma severity (Xin *et al.*, 2018). Another study saw a similar effect, with increased Treg expression of Th2 cytokines, and reduced expression of the regulatory cytokine IL-10 in asthmatics compared to healthy controls (Chen *et al.*, 2018). Understanding Treg stability in humans presents an increased challenge, with transgenic fate mapping of Tregs not possible, and therefore studies on Treg instability reliant upon phenotyping, as well as *in vitro* models (Deknuydt *et al.*, 2009).

Tregs in AAI

Tregs have a key role in maintaining tolerance to innocuous Ags, and thereby preventing the development of allergy. Inhalation of allergens such as OVA in the absence of adjuvant induces tolerance via the generation of pTregs (Ostroukhova *et al.*, 2004). In addition in mice where CNS1, a cis-regulatory element on the FoxP3 gene critical for pTreg induction, is deleted asthma-like pathology is observed, even in the absence of allergen provocation (Josefowicz *et al.*, 2012).

If tolerance fails, Tregs may still play a role in dampening ongoing AAI. Tregs can be expanded during AAI, and one mechanism by which this can occur is via mast cells, activated by IL-33, releasing IL-2 that is able to drive Treg proliferation (Morita *et al.*, 2015). The requirement for Treg mediated suppression of AAI was first shown in a murine model of HDM driven AAI, with Tregs depleted by anti-CD25 treatment leading to an increase in bronchial hyperreactivity and airway eosinophilia, as well as upregulation of DC costimulatory markers (Lewkowich *et al.*, 2005). This study was followed up by work utilizing transgenic mice that express the human diphtheria toxin receptor (DTr) under the control of the *Foxp3* promoter, in these mice administration of diphtheria toxin (DTx) allows inducible and specific depletion of Tregs (Lahl *et al.*, 2007). In these studies Treg depletion during the sensitization, but not challenge phase, of an OVA/adjuvant driven model of AAI leads to enhanced Th2 inflammation, potentially suggesting Tregs are more critical in early stages of inflammation (Baru *et al.*, 2010, 2012).

There is interest in using Tregs therapeutically in asthma and allergic diseases, supported by a study which showed that adoptive transfer of induced Tregs prior to allergen challenge reduces AAI (Xu *et al.*, 2012). The potential for Tregs to be therapeutically relevant in AAI was supported by further work, finding that adoptive transfer of Tregs was sufficient to reverse established airway hyperresponsiveness, inflammatory cell influx and airway remodelling (McGee and Agrawal, 2009).

Tregs in schistosomiasis

Like in AAI, Tregs have been shown to be required to suppress type-2 inflammation following helminth infection (Blankenhaus *et al.*, 2011; Sawant *et al.*, 2014). In addition, Tregs may be critical in schistosome infection, first indicated in work showing the *S. mansoni* product lysophosphatidylserine induces tolerogenic DCs capable of enhancing Treg differentiation *in vitro*, dependent on DC-specific TLR2 expression (van der Kleij *et al.*, 2002; Layland *et al.*, 2007). This work was furthered by studies *in vivo,* showing subcutaneous injection of *S. mansoni* eggs promotes the development of both Th2 and Treg responses, with Treg depletion leading to enhanced Th2 and Th1 responses (Taylor, Mohrs and Pearce, 2006).

The role of Tregs in natural schistosome infection differs dependent on the developmental stage of the parasite. Repeat skin infection by *S. mansoni* is able to drive a regulatory phenotype, however this was not associated with Treg expansion, or a change in Treg phenotype, suggesting Tregs are not critical for regulating this stage of infection (Cook *et al.*, 2011; Prendergast *et al.*, 2015). In lung migrating *S. mansoni* infection, no clear Treg expansion is evident in LNs draining lung in response to lung migrating larvae (Redpath *et al.*, 2015). There is increased evidence for the importance of Treg function during chronic patent infection, where increased Treg frequencies are observed, and transferred Tregs able to decrease granuloma size (Hesse *et al.*, 2004; Turner *et al.*, 2011). Treg expansion in chronic schistosomiasis was independent of IL-10 and TGFβ, with an increase in splenic Tregs observed in patent schistosomiasis when these cytokines were depleted (Herbert *et al.*, 2008). Depletion of Tregs during patent infection increased both Th1 and Th2 cytokine production (Baumgart *et al.*, 2006; Taylor, Mohrs and Pearce, 2006). When Tregs are depleted two weeks post infection during murine *S. japonicum* infection there is an increase in worm burden, granuloma size and IFNγ production (Tang *et al.*, 2014). The function of Tregs induced in patent schistosome infection has been highlighted by studies showing that OVA/alum induced AAI is suppressed during patent schistosome infection, with protection ablated by Treg depletion, leading investigators to question if they can utilise schistosome induced Tregs to treat allergic diseases (Layland *et al.*, 2013).

Expansion of Tregs during schistosome infection is not solely observed in murine models, with increased systemic Treg frequencies observed in human *S. mansoni* and *S. haematobium* infection, decreasing following curative praziquantel treatment (Watanabe *et al.*, 2007; van der Vlugt *et al.*, 2012). Further work is needed to understand the function of Tregs during human schistosomiasis.

1.4. Dendritic cells

DCs are a specialized cell type with a superior ability to sample and process Ags from non-lymphoid tissues and migrate to draining LNs to activate naïve T cells. They have a key role in directing the immune response, integrating environmental signals and responding by appropriately directing polarisation of naïve T cells (Steinman and Cohn, 1973; Banchereau and Steinman, 1998).

The mononuclear phagocyte system

The mononuclear phagocyte system encompasses macrophages, monocytes and DCs (van Furth, Raeburn and van Zwet, 1979). This grouping is somewhat historical, with tissue macrophages and DCs originally thought to solely derive from blood monocytes (Chomarat *et al.*, 2000). We now know this is not always true, for instance in the absence of inflammation mouse lung macrophages are thought to have both consist of embryonically derived alveolar macrophages (Guilliams *et al.*, 2013), as well as monocyte derived interstitial macrophages (Svedberg *et al.*, 2019). In humans alveolar macrophages have been shown to be recruited from monocyte precursors post lung transplant, highlighting contrasts between human and mouse (Byrne *et al.*, 2020). Monocytes, DCs and macrophages have been shown to derive from separate haematopoietic progenitor cells in the bone marrow (as shown in Diagram 1.3) (Naik *et al.*, 2007; Hettinger *et al.*, 2013).

Confusion between members of the mononuclear phagocyte system is compounded by overlapping expression of “characteristic” markers utilised in flow cytometry or in transgenic models. For instance, murine macrophages may express “DC markers” MHCII or CD11c, and DCs may express “macrophage markers” such as CD64 and F4/80 (Tamoutounour *et al.*, 2012; Schlitzer *et al.*, 2015; Scott *et al.*, 2015). Utilisation of ssRNAseq to evaluate cell types, the development of universal nomenclature as well as more specific markers are beginning to clear up this confusion (Guilliams *et al.*, 2014, 2016), allowing appreciation of cell-type specific functions.

DCs in inflammation

To provoke an inflammatory response, DCs follow a well-defined set of steps. Immature cDCs constantly survey peripheral tissues, continuously sampling Ags from their environment (Diagram 1.4). This was recently shown using microscopy in the lung, where DCs have been shown to extend dendrites through the epithelium, allowing sampling of airway Ag (Thornton *et al.*, 2012). Sampling can occur by a variety of mechanisms, including macropinocytosis, phagocytosis or endocytosis, with DCs able to take up approximately their own volume in Ag containing fluid per hour (Reis e Sousa, Stahl and Austyn, 1993; Sallusto *et al.*, 1995; Norbury *et al.*, 1997; Platt *et al.*, 2010; Hoffmann *et al.*, 2012). DCs express broad range of PRRs, allowing recognition of stereotypical PAMPs or DAMPs characteristic of the pathogenic stimuli (Janeway, 1989; Heng *et al.*, 2008; Dalod *et al.*, 2014). Ligation of PRRs on DCs acts as a maturation signal, inducing a transient increase in Ag uptake, followed by a rapid shutdown of this function (Trombetta *et al.*, 2003; West *et al.*, 2004). Maturation of DCs can also be driven by signals of an ongoing immune responses, for instance inflammatory mediators such

as TNF, or ligation of CD40 by CD40L on activated T cells (Caux *et al.*, 1994; Sallusto and Lanzavecchi, 1994).

Upon maturation, DCs upregulate receptors that allow them to migrate to the LN, and prime naïve T cells (Diagram 1.4). Maturation of DCs drives the upregulation of the chemokine receptor CCR7 (Dieu *et al.*, 1998). The CCR7 ligand CCL21 is expressed by endothelial cells of afferent lymphatics, as well as stromal cells in the T cell zone of the LNs, guiding DCs from the tissue (Gunn *et al.*, 1998; Ngo, Tang and Cyster, 1998; Luther *et al.*, 2000; Stein *et al.*, 2000; Warnock *et al.*, 2000). In order for DCs to make this migration their locomotive activity is increased, with the actin cytoskeleton rearranged to allow enhanced motility (Shutt *et al.*, 2000; Verdijk *et al.*, 2004). Migratory DCs in the LN have been shown to have higher MHCII expression, allowing them to be distinguished from LN resident DCs (Idoyaga *et al.*, 2013). Once in the T cell zone of the LN DCs can prime naïve T cells, which requires three signals. Signal one is the binding of T cells via TCR receptors to their cognate Ag MHC complexes on DCs, whereas signal two is costimulation, the binding of molecules such as CD28 or CD40L on T cells to costimulatory molecules on DCs such as CD80, CD86, CD40 and inducible T cell costimulator ligand (ICOSL) (Lenschow, Walunas and Bluestone, 1996; Reis E Sousa, 2006). Upregulation of costimulatory molecules on DCs is driven by exposure to maturation signals, such as PRR ligation, ensuring T cell priming only occurs in situations of danger to the host (Kaisho and Akira, 2005). Signal three relates to molecules or receptors expressed by DCs that drive T cells towards an effector phenotype, for instance DC release of IL-12 is required for Th1 cell induction, and IL-6 and TGFβ for Th17 induction (Macatonia *et al.*, 1995; Bettelli *et al.*, 2006; Veldhoen *et al.*, 2006). The character of the Ag, and therefore which PRRs it engages is critical to determine the maturation events that occur in DCs, for instance the double-stranded RNA sensor TLR3 induces cross presentation, and induction of IL-12, the cytokine critical for Th1 induction (Jelinek *et al.*, 2011).

Once T cells are primed, DCs have a critical role in the restimulation of Th cells in the tissue (Diagram 1.4) (Bennett and Chakraverty, 2012). Although DCs have been shown to rapidly increase migratory behaviour to dLNs in viral infection or post skin irritation, baseline rates of DC migration return 2 days post exposure, suggesting a reduced role for migratory DC subsets after the initial phase of inflammation (Legge and Braciale, 2003; Tomura *et al.*, 2014). DCs remaining in the tissue are critical to licence T cell responses at the site of inflammation (Bennett and Chakraverty, 2012). Recruitment of primed Th2 cells back to the lung tissue can be promoted by expression of chemokines such as CCL17, produced by DCs in the lung (Medoff *et al.*, 2009). Moreover, post intranasal OVA/alum sensitisation and challenge an accumulation of DCs is observed in the lung tissue, with OVA-specific airway T cells in almost constant contact with DCs in the airways post challenge (Thornton *et al.*, 2012). This finding was replicated using a translationally relevant allergen (HDM), with HDM primed polyclonal $CD4^+$ T cells in contacting DCs in the lung epithelium (Veres *et al.*, 2017). $CD8^+$ T cells that have met their cognate Ag in the LN return to the tissue where they are expanded upon re-encounter with Ag (Sutherland *et al.*, 2017). Expression of MHCII and MHCI are not unique to DCs in tissues, and the extent to which DCs are specifically required to present Ag

and licence cognate T cells in tissues is not clear. Moreover specific roles of DCs in the tissue vs LN are difficult to unpick, as targeting one site and not the other for interventions is a challenge, and even when this is possible the constant low-level migration of DCs from tissues to LNs mean that these sites are functionally linked even in the absence of inflammation (Legge and Braciale, 2003; Tomura *et al.*, 2014). Whilst it has been shown that DCs isolated from the tissue and draining LN are both able to prime $CD4^+$ and $CD8^+$ T cell responses, it is not clear if this function is critical in tissue DCs, or merely a sign that these DCs are about to migrate to the LN, a site that is specialised to facilitate interactions between T cells and DCs (Bosteels *et al.*, 2020).

DCs in tolerance

Perhaps unexpectedly due to their role in promoting inflammation, congenic depletion of DCs provokes autoimmune pathology, highlighting an opposing role in driving regulatory responses (Ohnmacht *et al.*, 2009; Loschko, Schreiber, *et al.*, 2016). Central tolerance occurs in the thymus, where self-Ags are presented on both resident medullary DCs and migratory DCs (Bonasio *et al.*, 2006; Hubert *et al.*, 2011). T cells that express high affinity for MHC self-Ag complexes are deleted (Kappler, Roehm and Marrack, 1987), or alternatively thymic DCs can induce generation of tTregs from T cells reactive to self-Ag (Proietto *et al.*, 2008; Guerri *et al.*, 2013). DCs are also critical for peripheral tolerance, with immature DCs, constantly taking up self-Ag in the periphery and migrating to the lymphoid organs to present to T cells, proposed to be crucial mediators of this (Steinman and Nussenzweig, 2002). Immature DCs have not upregulated costimulatory molecules in response to PAMPs or DAMPs, leading to T cells encountering their cognate Ag presented on DCs (signal 1) in the absence of costimulatory molecules (signal 2), which can lead to T cell deletion (Hawiger *et al.*, 2001). In addition, uptake of apoptotic cells via DCs can promote tolerance, interfering with DC maturation by inhibiting TLR signalling, and therefore leading to the presentation of Ags on immature DCs, driving T cell anergy or Treg induction (Gallucci, Lolkema and Matzinger, 1999; Lu and Lemke, 2001; Rothlin *et al.*, 2007). Anergy is a long-term state of T cell hyporesponsiveness to stimulation via their cognate Ag, even in the presence of costimulatory molecules (Schwartz, 2003).

The dogma that immature DCs are solely responsible for promoting T cell tolerance has been questioned (Steinman and Nussenzweig, 2002). Maturation via TLR signalling may be required for DCs to gain tolerogenic capacity, for instance TLR2-mediated recognition of the fungal PAMP Zymosan by splenic DCs leads upregulation of IL-10 expression, and increase in production of retinoic acid (RA), both regulatory molecules that promote Treg induction (Manicassamy *et al.*, 2009). Moreover steady state maturation of DCs can be induced by physical disruption of DC clusters, otherwise maintained by E-cadherin interactions, leading to mature DCs that express increased levels of the maturation markers CD86 and CCR7 with reduced inflammatory cytokine expression, and increased IL-10 expression when compared to classically (LPS) matured DCs (Jiang *et al.*, 2007). Finally, in cancer models DCs have a clear role in promoting T cell anergy, via expression of the co-inhibitory ligand PDL1, which,

when eliminated leads to an expansion of $CD8^+$ T cells, able to eliminate tumour cells (Oh *et al.*, 2020; Peng *et al.*, 2020).

Whilst the "maturity" of tolerogenic DCs is up for debate, they have been repeatedly shown to be important for the induction of peripheral tolerance via promoting the induction, suppressive function, or proliferation of Tregs (detailed in Diagram 1.5). DCs can support Tregs in the periphery by expression or secretion of tolerogenic molecules. For instance, uptake of apoptotic cells by DCs can result in their secretion of TGFβ, a key factor in Treg induction (Kushwah *et al.*, 2010). Numerous studies have shown the requirement for TGFβ activity for Treg induction *in vivo* or *in vitro*. However, it is less clear whether DC specific secretion is required, or if TGFβ from other sources is sufficient (Ghiringhelli *et al.*, 2005; Coombes *et al.*, 2007; Matsumura *et al.*, 2007; Ko *et al.*, 2010). Additionally, the regulatory cytokine IL-10 has been shown to be required for maintenance of Foxp3 expression, though not Treg induction, in a model of colitis (Murai *et al.*, 2009). Moreover, overexpression of IL-10 in DCs was able to drive increased Treg induction in a model of AAI (Henry *et al.*, 2008). Finally, secretion of the alarmin IL-33 from DCs was required for Treg expansion during intestinal helminth infection, although the mechanism of IL-33 action was not determined (Hung *et al.*, 2020).

DCs may provoke tolerance and Treg induction via the expression of enzymes that catalyse the activation or production of tolerogenic substances. For instance DCs can express integrins that lead to the activation of latent TGFβ, such as αvβ8 (Travis *et al.*, 2007; Worthington *et al.*, 2011, 2013). In addition, DCs can express retinaldehyde dehydrogenase 2 (RALDH2), an enzyme that metabolises retinal (a vitamin A derivative) into RA, which can serve as a cofactor to induce Treg induction in the spleen at steady state or post TLR2 ligation (Manicassamy *et al.*, 2009). RA can suppress *ex vivo* splenic or mLN DC mediated Th17 induction, and lead to Treg induction and expression of CD103, a tissue residency marker on T cells (Benson *et al.*, 2007; Mucida *et al.*, 2007; Elias *et al.*, 2008). Although the aforementioned studies focus on intestinal or splenic DCs, RA has also been shown to be critical in driving lung Treg induction, with RALDH2 upregulated in lung DCs in a model of OVA inhalational tolerance, with DCs required for OVA-specific Treg expansion (Khare *et al.*, 2013). In addition, RA has been shown to be required for alveolar macrophage mediated Treg induction at steady state and in inflammatory and tolerogenic models (Coleman *et al.*, 2013). Finally, DCs can express the enzyme IDO, which catalyses tryptophan degradation to kynurenine. In certain situations DC IDO expression is dependent on ligation of the aryl hydrocarbon receptor (AHR), by ligands including kynurenine itself (Nguyen *et al.*, 2010). Kynurenine is then able to activate the AHR on naïve T cells, with its expression required for Treg induction in response to TGFβ or following coculture with pDCs (Mezrich *et al.*, 2010; Yeste *et al.*, 2016). It is tempting to suggest that these mechanisms arose to allow fine control over the regulatory effects of DCs, with control of the availability of the regulatory precursor (latent TGFβ, tryptophan, retinal), the activating enzyme on DCs, and the presence of the receptor on T cells all critical for Treg induction.

DCs also contribute to Treg induction via cell surface interactions (Diagram 1.5). Ligation of PD1 on naïve T cells by the coinhibitory ligand PDL1 on *ex vivo* splenic DCs was required for Treg induction in combination with TGFβ (Wang *et al.*, 2008; Francisco *et al.*, 2009). Moreover PDL1 ligation enhanced Treg suppressive ability (Francisco *et al.*, 2009). Mice with genetic deletion of PDL1, or antibody mediated PDL1 antagonism, have reduced Treg numbers in a variety of models, including during tumorigenesis, post naïve cell infusion into T and B cell deficient (RAG) mice, and during EAE (Wang *et al.*, 2008; Francisco *et al.*, 2009; Yogev *et al.*, 2012; Chen *et al.*, 2014). During EAE, DCs were identified as the PDL-1 expressing cell type, required for Treg induction (Yogev *et al.*, 2012). Mechanistically, PD1 ligation by PDL1 is thought to antagonise the mTOR signalling pathway, contributing to Treg induction (Francisco *et al.*, 2009). Converging at the same mechanism, splenic DCs signalling through the coinhibitory receptor B and T lymphocyte attenuator (BTLA) to the ligand herpesvirus entry mediator (HVEM) in naïve T cells increases T cell CD5 expression, with downstream antagonism of mTOR signalling, increased Treg induction, and tolerance against an EAE inducing Ag (Jones *et al.*, 2016). The costimulatory molecule OX40L, expressed on DCs, has a more complex role in Treg expansion, reducing Treg induction from naïve T cells, but enhancing proliferation of existing OX40 expressing Tregs by a TCR independent pathway (So and Croft, 2007; Vu *et al.*, 2007; Bhattacharya *et al.*, 2011; Kumar *et al.*, 2017). Finally, interactions between ICOSL, a costimulatory ligand on DCs, with ICOS on Tregs is required for optimal Treg suppressive capacity in *in vitro* assays with bone marrow derived DCs (BMDCs) (Akbari *et al.*, 2002). Certain developmentally phenotypically distinct DC subsets may play particular roles in the aforementioned tolerogenic and inflammatory functions of DCs.

Murine DC development and classification

DCs are heterogenous, with a number of different subsets that differ developmentally and functionally, as shown in Diagram 1.3 (Durai and Murphy, 2016). Development of DCs occurs in the bone marrow from hematopoietic stem cells, with committed progenitors moving to the blood and mature subsets in tissue and lymphoid organs. In comparison to other cells of the myeloid lineage, for instance tissue macrophages which can survive for months to years, DCs have a short half-life, surviving for only 1-9 days after arriving at lymphoid organs (Kamath *et al.*, 2002; Murphy *et al.*, 2008).

Development of cDCs is shown in Diagram 1.3, and begins with restriction to the common myeloid and then common DC progenitors (CMP and CDP) (Naik *et al.*, 2007; Onai *et al.*, 2007). CDPs then differentiate into pre-cDC1s and pre-cDC2s. The transcription factors *Batf3* and *Irf8* are required for cDC1 development, in order to express these transcription factors, at the CDP stage the transcription factor *Nfil3* mediates the transition to committed *Zeb2*^{lo} and *Id2*^{hi} CDPs which are the earliest cDC1 progenitors (Schiavoni *et al.*, 2002; Tamura *et al.*, 2005; Hildner *et al.*, 2008; Grajales-Reyes *et al.*, 2015; Bagadia *et al.*, 2019). In contrast to cDC1s, cDC2s are a heterogenous population, often appearing as multiple clusters in ssRNAseq experiments (Brown *et al.*, 2019; Bosteels *et al.*, 2020; Maier *et al.*, 2020). Although consensus has not yet been reached on this issue, two subsets of cDC2s may be

distinguished based upon their dependency on the transcription factors KLF4 or Notch2 for development (Lewis *et al.*, 2011; Satpathy *et al.*, 2013; Tussiwand *et al.*, 2015). The development of different cDC2 subsets may be tissue dependent, with inflammatory cDC2s (inf cDC2s) recently defined in the lung, and CD11b^{lo} vs CD11b^{hi} cDC2 populations in the skin (Ochiai *et al.*, 2014; Connor *et al.*, 2017)

In contrast to cDCs, pDCs are thought to develop from either lymphoid or myeloid progenitors (Manz *et al.*, 2001; D'Amico and Wu, 2003; Sathe *et al.*, 2013, 2014). Development of pDCs is dependent upon the transcription factors TCF4 and ZEB2, with depletion of either impairing development of pDCs but not cDC1s (Reizis, 2019). It has been proposed that the function of pDCs may differ depending upon whether they are derived from myeloid or lymphoid progenitors, with only myeloid derived pDCs able to process and present Ag (Rodrigues *et al.*, 2018).

Finally, it has been proposed that monocytes are able to differentiate into DCs, known as moDCs (Jakubzick, Randolph and Henson, 2017). The development of these cells differs significantly from cDCs, deriving from monocyte progenitors, but they fulfil the functional ability of DCs to migrate to LNs and prime T cell responses (Langlet *et al.*, 2012; Plantinga *et al.*, 2013). To assert that moDCs exist, and have DC-like functions it is first imperative to accurately identify moDCs as monocyte derived, a status that has been defined by expression of the marker CD64, as well as dependency on the chemokine CCR2 (Plantinga *et al.*, 2013; Min *et al.*, 2018). Recent work has indicated that, in some situations, cDCs, identified based on CD26 expression, can be CCR2 dependent, and upregulate CD64, suggesting such cells may have been erroneously identified as moDCs in previous studies (Kamphorst *et al.*, 2010; Bosteels *et al.*, 2020).

Identification of DCs has historically proved a challenge, with the overlap of DC and macrophage function, growth factor reliance, and surface marker expression even leading some to question the existence of DCs as a distinct cell type (Hume, 2008). However, the past decade has resolved many of these questions with key discoveries in the ontogeny of DCs, for instance the definition of the pre-DC restricted transcription factor Zbtb46, and concerted efforts to standardize surface marker based DC identification and subsetting (Satpathy *et al.*, 2012; Guilliams *et al.*, 2016). Utilization of transgenic mice allowing specific depletion of DCs, or alterations of gene expression in DCs, has been key to our understanding of their role *in vivo*. An early example of this was development of *Cd11c*^{DTr} murine models, which allowed specific depletion of all CD11c^{hi} cells (Jung *et al.*, 2002; Hochweller *et al.*, 2008). Notably these models are not DC specific, with depletion of some macrophages also reported (Jung *et al.*, 2002; Hochweller *et al.*, 2008). To gain increased specificity, a number of transgenic mouse lines based upon *Zbtb46* have been developed, allowing specific depletion of cDCs (Meredith *et al.*, 2012; Loschko, Rieke, *et al.*, 2016).

Subset-specific murine DC marker expression is summarised in Diagram 1.6. Identification of murine pDCs can be achieved via B220, SiglecH and PDCA1 positivity (Blasius *et al.*, 2006; Zhang *et al.*, 2006). A number of markers have been proposed to identify murine cDC1s

in lymphoid and non-lymphoid tissue, including CD24, CD103 and XCR1, with XCR1 proposed to consistently demarcate DCs in all murine and human tissues (Guilliams *et al.*, 2016). In murine tissue, cDC2s can be distinguished by expression of CD11b and CD172a (also known as SIRPα) (Guilliams *et al.*, 2016). The cDC2 compartment is heterogenous, with differential expression of a number of markers proposed to identify cDC2 subsets, for instance MGL2 in the lung and skin, CD11b in the skin, CD24 in the lung and ESAM in the spleen and intestines (Lewis *et al.*, 2011; Kumamoto *et al.*, 2013; Satpathy *et al.*, 2013; Ochiai *et al.*, 2014; Ainsua-Enrich *et al.*, 2019). ssRNAseq studies have the potential to provide clarity in this, with unbiased clustering algorithms revealing transcriptional similarities and differences between cells, allowing the delineation of transcriptionally similar clusters, as shown in Diagram 1.5 (Han *et al.*, 2018; Schaum *et al.*, 2018). Utilising flow cytometry and ssRNAseq, the canonical Th1 transcription factor T-bet has been proposed to be a marker of two different cDC2 subsets across tissues, with T-bet$^+$ cDC2s functionally distinct from Tbet$^-$ DCs (Brown *et al.*, 2019). During inflammation an inf cDC2 subset has been shown in the lung, expressing markers characteristic of cDCs and monocytes (Bosteels *et al.*, 2020)

Multiple studies have also reported CCR7$^+$ DCs as a potential new DC subset (Bosteels *et al.*, 2020; Maier *et al.*, 2020). Due to the role of CCR7 in mediating DC migration to the LN, CCR7$^+$ DCs have been termed migratory DCs, with one study finding two populations of migratory DCs with either cDC1-like or cDC2-like features (see Diagram 1.6) (Bosteels *et al.*, 2020). Another study has found these cDCs to have both cDC1 and cDC2-like features, for instance expression of CD11b and XCR1, and proposed they represent one subset (Maier *et al.*, 2020). The authors highlighted that this subset was enriched in both maturation markers (costimulatory markers such as CD40), as well as regulatory markers (PDL1 and RALDH2), a transcriptional phenotype which they replicated via exposing a BMDC line to apoptotic cells, which were able to induce both Treg and Th1 responses *in vitro* (Maier *et al.*, 2020). Further work is needed to understand the development and function of CCR7$^+$ DCs.

Human DC development

Human DCs have been proposed to arise from common lymphoid progenitors (CLPs) (Chicha, Jarrossay and Manz, 2004; Doulatov *et al.*, 2010), granulocyte-monocyte-DC progenitors which further specialise into monocyte-DC progenitors, and then CDPs (J. Lee *et al.*, 2015), or cDC1s and pDCs can develop directly from a multipotent lymphoid progenitor (Helft *et al.*, 2017). A proposed explanation for this complexity is that human DCs do not, in comparison to mouse DCs, arise through a series of lineage committed progenitors, but instead are able to arise from a number of developmental intermediates (Jardine and Haniffa, 2020)

Heterogeneity in human DCs is being uncovered, led by a number of ground-breaking ssRNAseq studies (Villani *et al.*, 2017; Dutertre *et al.*, 2019). These studies reveal clear overlap in murine and human DC systems, identifying cDC1s, cDC2s and pDCs, with cDC2s in human systems also displaying heterogeneity, shown in Diagram 1.7 (Dutertre *et al.*, 2019). Like murine DCs, human DCs can be defined by expression of ZBTB46 (See *et al.*, 2017).

Human cDC1s have expression of a number of lineage defining transcription factors shared with mice, including IRF8, BATF3 (Robbins *et al.*, 2008; Meredith *et al.*, 2012; See *et al.*, 2017). Markers to identify human DCs are shown in Diagram 1.7, and can be identified as HLA-DR$^+$ CD11c$^+$, with cDC1s further delineated as XCR1$^+$ CADM1$^+$ CD26$^+$ CD141$^+$ Clec9a$^+$ BDCA3$^+$ IRF8$^+$, and cDC2s as CD172a$^+$CD1c$^+$ IRF4$^+$ (Bachem *et al.*, 2010; Guilliams *et al.*, 2016; Anderson *et al.*, 2021). Clear delineation of DC subsets is a pre-requisite to understand subset specific functional differences, as discussed below.

A key line of research is trying to understand how DC subsets in mice and human functionally overlap, with a recent study showing transcriptional overlap of macrophage, monocyte and DC populations in lymphoid and non-lymphoid sites between human and mice (Leach *et al.*, 2020). Notably in the aforementioned study overlap of top marker genes was only up to 23%, suggesting functional differences in human and murine DCs are likely to be present (Leach *et al.*, 2020). Despite this our understanding of human DC function has been greatly aided by murine models, which are able to be transgenically manipulated, allowing for mechanistic studies.

Transgenic models to understand murine DC function

In order to study functions of cDC1s, a number of depletion models have been developed. An early model for depletion of cDC1s was the *Ly75^{DTr}* mouse, which targets DCs that express CD205, mainly cDC1s (Fukaya *et al.*, 2012). However, as CD205 is expressed on germinal centre B cells and all LN migratory cDCs the specificity of this model is questionable (Victora *et al.*, 2010; Idoyaga *et al.*, 2013). More specific depletion of cDC1s may be achieved by use of a *Clec9a^{DTr}* strain, although this also depletes a proportion of pDCs (Piva *et al.*, 2012). Specific depletion can be achieved via use of an *Xcr1^{DTr}* strain, or by utilizing the known dependency of cDC1s on the transcription factor BATF3, with *Batf3$^{-/-}$* mice generally exhibiting long term and specific depletion of cDC1s (Hildner *et al.*, 2008; Yamazaki *et al.*, 2013). In certain situations cDC1s may be reconstituted in *Batf3$^{-/-}$* mice, by a compensatory developmental pathway involving cytokine induced by IFNγ and IL-12 (Tussiwand *et al.*, 2012).

Depletion of cDC2s in transgenic models has proven a greater challenge than cDC1s, perhaps reflective of their heterogeneity. Depletion of a subset of cDC2s is possible by utilising the *Mgl2^{DTr}* mouse model, specifically allowing for depletion of MGL2 expressing cDC2s, as well as other MGL2 expressing cell subsets, notably some macrophages (Kumamoto *et al.*, 2013, 2016). Depletion of cDC2s is also possible utilising transcription factor deficient mice. *Klf4$^{-/-}$* and *Notch2$^{-/-}$* mice lack specific cDC2 subsets, in line with their roles in cDC2 development (Lewis *et al.*, 2011; Satpathy *et al.*, 2013; Tussiwand *et al.*, 2015). Mice lacking the transcription factor IRF4 also have reduced numbers of cDC2s (Persson *et al.*, 2013). However, although this transcription factor is characteristic of cDC2 populations, it is not fundamentally required for their development, with cDC2s still present, though functionally impaired and unable to migrate to draining LNs (Bajaña *et al.*, 2012, 2016).

Depletion of moDCs specifically is not currently possible, and as mentioned earlier their existence is questioned (Bosteels *et al.*, 2020). There are a number of models available to deplete monocyte/macrophage lineage cells, however due to their developmental interconnectedness depleting one cell type without the other is a challenge (Hua *et al.*, 2018). *Ccr2*$^{-/-}$ mice can be used to study tissue functions of monocyte derived cells, including moDCs, as in the absence of CCR2 monocytes have a reduced ability to leave the bone marrow (Boring *et al.*, 1997). The depletion of monocytes in peripheral tissues is not complete in *Ccr2*$^{-/-}$ mice, for instance in the skin other chemokine receptors able to compensate for the loss of CCR2, with full monocyte depletion in peripheral tissues only possible in mice with deficient in multiple chemokines (Dyer *et al.*, 2019).

Development of pDCs is dependent upon expression of transcription factors Zeb2 and TCF4 (Reizis, 2019), and depletion of pDCs is possible *in vivo* via use of *SiglecH*^{DTr} or *Bdca2*^{DTr} transgenic mice, allowing for dissection of their function (Swiecki *et al.*, 2010; Takagi *et al.*, 2011). In addition, mice that have specific deficiency in the transcription factors *Tcf4* or *Zeb2* in CD11c^{+} cells allows for pDC deficiency, although in the case of *Zeb2* cDC2s are also altered (Cisse *et al.*, 2008; Scott *et al.*, 2016). Utilisation of these models has revealed the function of DC subsets.

Inflammatory and tolerogenic functions of DC subsets

cDC1

Functionally, cDC1s are specialized for priming CD8 T cells, via cross presentation of extracellular Ags on MHCI (Den Haan, Lehar and Bevan, 2000; Dudziak *et al.*, 2007). This function is particularly important for defence against intracellular pathogens, viral or bacterial, allowing cDC1s to present Ags without themselves being infected. One way this is achieved is by expression of endocytic receptors for damaged or necrotic cells, for instance CLEC9a which binds F-actin exposed on damaged cells, allowing cDC1s to take up Ags from dying infected cells and present these Ags to CD8^{+} T cells (Zhang *et al.*, 2012; Hanč *et al.*, 2015). In addition, cDC1s have been shown to preferentially induce Th1 cells, via high level endogenous expression and secretion of IL-12 (C.-H. Liu *et al.*, 2006; Mashayekhi *et al.*, 2011; Everts *et al.*, 2016). Finally, cDC1s may play a role in antagonizing the type-2 immune response, with steady state IL-12 production antagonizing Th2 cell induction, leading to exacerbated type-2 responses in *Batf3*$^{-/-}$ mice (Everts *et al.*, 2016).

In addition to having the ability to promote type-1 inflammation, cDC1s have been shown in some settings to have tolerogenic roles. In the mLN at steady state, cDC1s are able to activate latent TGFβ, via expression of αvβ8 integrin, to a greater extent than cDC2s, leading to increased Treg induction (Païdassi *et al.*, 2011; Worthington *et al.*, 2011). Intestinal and lung cDC1s have increased RALDH2 expression, compared to cDC1s, lending a greater ability to produce RA which promotes Treg induction (Coombes *et al.*, 2007; Khare *et al.*, 2013). Superior Treg induction capability, in comparison to cDC2s, has been shown in steady state splenic DCs also, via increased expression of TGFβ (Yamazaki *et al.*, 2008). Splenic

cDC1s have also been shown to be required for development of Tregs in a model of tolerance to self-Ag, via expression of the coinhibitory molecule BTLA (Jones et al., 2016).

Like mice, human cDC1s have a superior ability to cross present Ags, when compared to $CD1c^+$ cDC2s and $CD16^+$ DCs (Bachem *et al.*, 2010), although the enrichment of cross presentation genes in cDC1s, compared to other DC subsets, is not as convincing as that observed in mouse (Carpentier *et al.*, 2016). Cross presentation by human cDC1s is also supported by their unique expression of CLEC9A (Schreibelt *et al.*, 2012). Coupled with the ability of human cDC2s to activate Th1 cells, discussed below, this suggests that the two key functional features of murine cDC1s – cross presentation and Th1 induction – are not as restricted to the cDC1 lineage as they are in mice (Nizzoli *et al.*, 2013; Sittig *et al.*, 2016). At steady state sorted blood $CD141^+$ cDC1s are able to induce T cell expression of the Th2 associated transcription factor GATA3, this was reduced in responses to activation of viral associated TLRs (TLR3 and TLR7/8) however, suggesting human cDC1s tailor their Th subset promotion to the inflammatory environment (Sittig *et al.*, 2016).

cDC2s

cDC2s have been proposed to be functionally specialised for the induction of Th2 responses. Utilising a $MGL2^{DTr}$ depletion model, a subset of cDC2s expressing the lectin MGL2 were shown to be required for induction of Th2 responses in the skin in response to immunisation with OVA plus the Th2 promoting adjuvant alum (Kumamoto *et al.*, 2013). This subset of cDC2s is thought to be IRF4 dependent and, when depleted in $Cd11c^{cre}$ $Irf4^{fl/fl}$ (CD11cΔIRF4) mice, Th2 responses are not induced in response to helminth or allergen challenge (Gao *et al.*, 2013; Persson *et al.*, 2013; Williams *et al.*, 2013). The ability of cDC2s to preferentially induce Th2 responses may be enhanced by their preferential uptake of helminth derived Ags (Blecher-Gonen *et al.*, 2019). In addition, cDC2s have been shown to be required for the induction of Th17 responses, with depletion of intestinal cDC2s resulting in fewer Th17 cells at steady state (Lewis *et al.*, 2011; Schlitzer *et al.*, 2013). Moreover, cDC2s are required for promoting Th17 responses during inflammation, as shown by cDC2 deficiency utilising in CD11cΔIRF4 mice during infection with a large dose of $2x10^7$ *A.f* conidia, sufficient to cause invasive disease, and in an OVA/adjuvant model of intestinal inflammation (Persson *et al.*, 2013; Schlitzer *et al.*, 2013). The phenotype in CD11cΔIRF4 has been proposed to be explained by IRF4 dependent LN migration in cDC2s, with IRF4 deficient DCs equally able to drive Th2 responses when injected directly into the LN (Mayer *et al.*, 2017). It should be noted that the functions of DC subsets may overlap, with cDC2s able to promote proliferation of $CD8^+$ T cells in viral infection, though to a lesser extent than cDC1s, as well as promoting increased IFNγ production from $CD4^+$ T cells (Bosteels *et al.*, 2020).

Like cDC1s, cDC2s in some settings are also able to contribute to peripheral tolerance via supporting Treg responses. Cutaneous LN DCs expressing Aldehyde dehydrogenase (ALDH), thought to derive from the cDC2 population, have enhanced Treg induction potential when compared to DCs without ALDH activity (Guilliams *et al.*, 2010). Moreover, oral and submandibular LN cDC2s at steady state have superior Treg induction capability, with

increased ALDH activity and TGFβ production in comparison to cDC1s (Tanaka *et al.*, 2017). Induction of Tregs in the mLN in response to commensal Ags has been shown to be independent of cDC1s, but requires *Notch2* dependent cDC2s (Nutsch *et al.*, 2016). DCs may also expand Tregs by stimulating proliferation of existing Tregs, and splenic cDC2s have been shown to preferentially drive Treg proliferation *ex vivo* by a cell contact dependent mechanism (Zou *et al.*, 2010). Finally, in a skin allergic OVA/adjuvant model, IRF4 dependent cDC2s were shown to be required for Treg expansion (Vander Lugt *et al.*, 2017).

In humans cDC2s can be identified as CD172a$^+$ CD1c$^+$, with separation of this cell type from monocytic cells possible by exclusion of CD88$^+$ and CD14$^+$ cells (Dutertre *et al.*, 2019; Leach *et al.*, 2020). Individuals who lack cDC2s due to a mutation in the *SPPL2A* gene mount impaired Th1 responses to mycobacteria, suggesting that this subset may have increased relevance to type-1 responses in human disease (Kong *et al.*, 2018). As in mice, cDC2 populations in humans appear to be heterogenous, with two subsets identified, referred to as DC2 and DC3 (Villani *et al.*, 2017). CD5$^-$ DC2s have a reduced ability to induce Th2 and Th17 priming, when compared to DC3s (Dutertre *et al.*, 2019). CD5$^+$ DC3s also have also been shown to have increased migratory ability when compared to CD5$^-$ DC2s (Yin *et al.*, 2017). In comparison to mice, human CD1c$^+$ cDC2s may have increased flexibility with respect to the type of immune responses they promote, being able to induce Th1, Th2, Th17 and CD8$^+$ T cells (Nizzoli *et al.*, 2013; Di Blasio *et al.*, 2016; Sittig *et al.*, 2016). Specifically, human cDC2s can secrete higher levels of IL-12 than BDCA3$^+$ cDC1s, and prime CD8$^+$ T cell responses (Nizzoli *et al.*, 2013). Further work has supported this proposition with CD1c$^+$ cDC2s able to release IL-12, as well as promote Th1 differentiation of CD4+ T cells *in vitro*, in this latter work comparisons with cDC1s were not performed, perhaps due to the relative rarity of this subset (Bourdely *et al.*, 2020). The capacity of cDC2s to promote Th1 induction *in vitro* is supported by ligation of viral associated TLRs (Sittig *et al.*, 2016).

pDCs

In comparison to cDCs, pDCs do not have dendrites, instead having a plasma-cell like morphology, and are able to rapidly induce type I IFNs in response to viruses (Cella *et al.*, 1999; Siegal *et al.*, 1999). The role of pDCs as Ag presenting cells is controversial. Although they have been shown to be able to present peptide Ags, they lack Ag processing machinery, limiting their ability to present Ags derived from proteins (Siegal *et al.*, 1999; Irla *et al.*, 2010). A recent study has shown that pDCs are able to induce both type I IFN expression, and T cell activation during viral infection, but that these occurred sequentially in different splenic locations, highlighting temporal and spatial instruction of DC function (Abbas *et al.*, 2020).

Numerous studies have implicated pDCs in provoking tolerance via supporting Treg induction. In the lung, pDCs have been implicated in the induction of Treg-mediated tolerogenic responses, with ablation of pDCs diminishing the generation of OVA-specific iTreg responses *in vivo* (de Heer *et al.*, 2004; Oriss *et al.*, 2005; Takagi *et al.*, 2011). The method of pDC depletion is critical, with some studies of pDC mediated tolerance reliant upon depletion with Gr1 targeted antibodies, which are unlikely to be specific as Gr1 is found on

numerous cell types, including neutrophils and monocyte lineage cells (de Heer *et al.*, 2004; Gabrilovich, 2017; Boivin *et al.*, 2020). Further evidence for the importance of pDCs in provoking lung Tregs is that adoptive transfer of $CD8\alpha^+$ pDCs pulsed with OVA prior to OVA/adjuvant sensitisation and challenge prevented the development of AAI, whilst increasing Ag specific Treg numbers. In addition, pDCs were able to induce Ag specific Tregs *in vitro* dependent upon TGFβ and RA (Lombardi *et al.*, 2012). The tolerogenic properties of pDCs are not lung-restricted, with pDCs shown to produce TGFβ, and be required for Treg induction in pancreatic LNs in a model of viral infection induced type-1 diabetes (Diana *et al.*, 2011). Finally, two studies have shown human pDCs to have tolerogenic properties, able to induce Tregs *in vitro*, in a mechanism dependent on pDC expression of IDO, and subsequent tryptophan metabolites or via ICOSL signalling (Ito *et al.*, 2007; Chen *et al.*, 2008). The reduced ability of pDCs to act as Ag presenting cells, priming naïve T cells with protein Ags, suggests that much of the tolerogenic function of pDCs can be attributed to secretion of cytokines, rather than cognate interactions.

DCs in type-2 inflammation

DCs are required to induce type-2 inflammatory responses, as has been shown in helminth models utilising DC depletion with $Cd11c^{DTr}$ mice (Phythian-Adams *et al.*, 2010; Smith *et al.*, 2012). Complementary studies proved DCs are sufficient to provoke type-2 responses *in vivo*, with adoptive transfer of DCs isolated from helminth (*Nippostrongylus brasiliensis*) infection able to drive dermal Th2 responses (Connor *et al.*, 2014). However, the mechanism(s) by which DCs are able to skew naïve T cells to a Th2 phenotype has been a subject of rigorous debate for some years and remain unresolved. The clear role of a DC-produced cytokine in the polarization of naïve T cells to Th2 cells has not been demonstrated, in contrast to the role of DC IL-12 in Th1 polarisation (Macatonia, Hosken and Litton, 1995). The location of priming may influence the ability of DCs to prime Th2 responses, with Th2 priming during helminth (*Heligmosomoides polygyrus*) infection shown to occur outside of the T cell zone, and in contrast to Th1 priming, be uniquely dependent on DC interaction with B cells (León *et al.*, 2012). A key source of confusion is that DCs have not been shown to produce significant levels of IL-4, required for Th2 induction, with $Il4^{-/-}$ DCs able to drive Th2 responses (Jankovic *et al.*, 1999; MacDonald and Pearce, 2002). Moreover, DCs exposed to Th2-inducing stimuli such as schistosome egg Ag (SEA) display minimal transcriptional changes and do not express high levels of pro-inflammatory cytokines (MacDonald *et al.*, 2001). This has led to the idea that Th2 polarisation may be a default process, resultant from low costimulatory and T cell receptor (TCR) signals (Wang *et al.*, 1994; Schnare *et al.*, 2001). Opponents of this theory would point to the discovery of cell surface molecule and transcription factors that define these Th2-polarising DCs. For example, in response to SEA, the DC cell surface molecule OX40L acted as a costimulatory signal triggering optimal Th2 cytokine production (Jenkins *et al.*, 2007). Recently, it has been shown that in type-2 inflammatory situations (allergy and schistosome infection) the transcription factor Mbd2 is required for optimal Th2 induction (Cook *et al.*, 2015).

DCs in AAI

DCs play a key role in both inducing and maintaining an allergic response. CD11c depletion models have shown DCs are required for the characteristic features of AAI (bronchial hyperresponsiveness, eosinophilia, goblet hyperplasia) in OVA-driven murine models. Moreover transfer of DCs pre-loaded with Ag is sufficient to induce AAI in pre-sensitised mice (van Rijt *et al.*, 2005). DCs have been shown to carry Ag to LNs during AAI (Thornton *et al.*, 2012). In an ongoing allergic response DCs in airway adjacent regions maintain contact with activated T cells, leading to T cell signalling and potentially maintaining the allergic response (Thornton *et al.*, 2012).

Recognition of allergens can occur via engagement of pattern recognition receptors, for instance Dectin-1 can recognise β-glucans in HDM, leading to migration of lung cDC2s to the LN and Th2 development (Ito *et al.*, 2017). In addition, the main allergen in HDM, Der p 2, has been shown to signal via TLR4, with this signalling critical to drive allergic responses (Trompette *et al.*, 2009). A number of epithelial derived factors, produced from the epithelium in responses to allergens, such as TSLP IL-25 and IL-33 are able to promote DC maturation, and migration to the LN (Ito *et al.*, 2005; Schmitz *et al.*, 2005; Allakhverdi *et al.*, 2007; Kaiko *et al.*, 2010; Gregory *et al.*, 2013; Claudio *et al.*, 2019).

There is evidence for a division of labour between DC subsets, with cDC2s, but not $CD103^+$ cDC1s found to be sufficient to sensitise mice to HDM, leading to AAI on HDM challenge (Plantinga *et al.*, 2013). Correspondingly, using specific depletion models cDC2s, but not cDC1s were required for HDM sensitization at low dose (Plantinga *et al.*, 2013). In comparison, at high doses of HDM, infcDC2s (which express CD64 and were previously erroneously identified as moDCs), were the main cells required for Ag uptake, with both cDC2 subsets able to promote AAI (Plantinga *et al.*, 2013). cDC2s, but not cDC1s, isolated from skin draining LNs post cutaneous HDM transfer were shown to be sufficient to drive AAI following intradermal transfer (Deckers *et al.*, 2017). These findings have been supported by work investigating the role of KLF4 and IRF4 dependent cDC2s in dust mite allergies, with these cDC2s required for development of AAI (Zhou *et al.*, 2014; Tussiwand *et al.*, 2015). The role of cDC2s in promoting responses to airway allergens may also be connected to their location in the airways, with cDC2s shown to be the dominant DC subtype in the tracheal mucosa post HDM challenge (Veres *et al.*, 2017).

DCs in schistosomiasis

Exposure of DCs to schistosome Ags induces Th2 inducing properties in DCs. Immunising mice with schistosome egg Ag promotes Th2 responses, which have been shown to require *Klf4* dependent cDC2s (Tussiwand *et al.*, 2015). DCs exposed to schistosome egg Ag (SEA) are able to drive Th2 responses when transferred *in vivo*, a phenotype dependent upon the expression of type-1 interferon (Webb *et al.*, 2017). A key factor in SEA that conditions DCs for Th2 priming is omega-1, a secreted ribonuclease that is required for eggs to drive Th2 responses via DCs (Everts *et al.*, 2009; Ittiprasert *et al.*, 2018).

In schistosome infection, the exact function of DCs depends on the tissue migratory stage of the parasite. In the skin, DCs take up larval Ag and migrate to the draining LN to prime $CD4^+$ T cells (Paveley *et al.*, 2009). This can be modulated by parasite products that direct DCs towards a Th2 inducing phenotype that, upon multiple infections, becomes increasingly suppressive (Stéphanie, Curwen and Mountford, 2008; Cook *et al.*, 2011). The role of DCs during lung migrating schistosomiasis has not been studied.

In the liver cDCs, and not pDCs, are required to induce Th2 responses to *S. mansoni* infection (Phythian-Adams *et al.*, 2010; Lundie *et al.*, 2016). In response to patent *S. mansoni* infection, hepatic DCs upregulate costimulatory markers CD86, CD40 and OX40L, the latter being required for optimal development of the Th2 response (Jenkins and Mountford, 2005; Lundie *et al.*, 2016). Transferred SEA-pulsed DCs are also able to polarise cells *in vivo* to a classical IL-4, IL-5, IL-13 producing phenotype (MacDonald *et al.*, 2001). Exposure of human DCs *in vitro* to *S. mansoni* SEA induces a type-2/tolerogenic phenotype, with SEA reducing inflammatory cytokine release by toll-like receptor (TLR)-stimulated DCs, and inducing Th2 polarisation (Thomas *et al.*, 2003; Liempt *et al.*, 2007).

1.5. Thesis hypotheses

As detailed prior, DCs have been shown to be critical to direct pulmonary immune responses, with subset-specific roles for the instruction of T cell inflammatory and regulatory responses. In this thesis we focus on the development of regulatory responses in type-2 inflammation, specifically the role of Tregs during ongoing AAI, and how particular subsets of DCs may contribute to their expansion. In the final section of this thesis, we move to a helminth (*S. mansoni*) model of pulmonary inflammation, elucidating type-2 immune responses in the murine system and translating this work into human infection. The three main hypotheses in this thesis are:

Tregs are critical for regulation of *A.f.* driven AAI but may have altered suppressive capacity, or even show signs of inflammation during AAI.

1. How does the location and number of Tregs change in fungal AAI?
2. Do Tregs modify expression of functional markers, including IL-10, during fungal AAI?
3. Are Tregs required to suppress immune responses and inflammation in fungal AAI?
4. Do Tregs display instability during fungal AAI?

Treg expansion during AAI is dependent upon interaction a particular subset of DCs.

1. How does the DC compartment change during fungal AAI?
2. Which DC subset – pDCs, moDCs, cDC1s or cDC2s – is required for Treg expansion during fungal AAI?
3. What is the mechanism of DC Treg expansion during fungal AAI? Is there a role for DC activation of TGFβ via αVβ8?

Immune responses in pulmonary *S. mansoni* infection differ between lung migratory and patent phases, in both human and mouse infection, with critical roles for DCs.

1. How does pulmonary inflammation change during lung migrating and patent schistosomiasis?
2. How does pulmonary inflammation differ between murine and human infection?
3. What is the role of DCs in schistosome mediated lung inflammation?

1.6. Diagrams

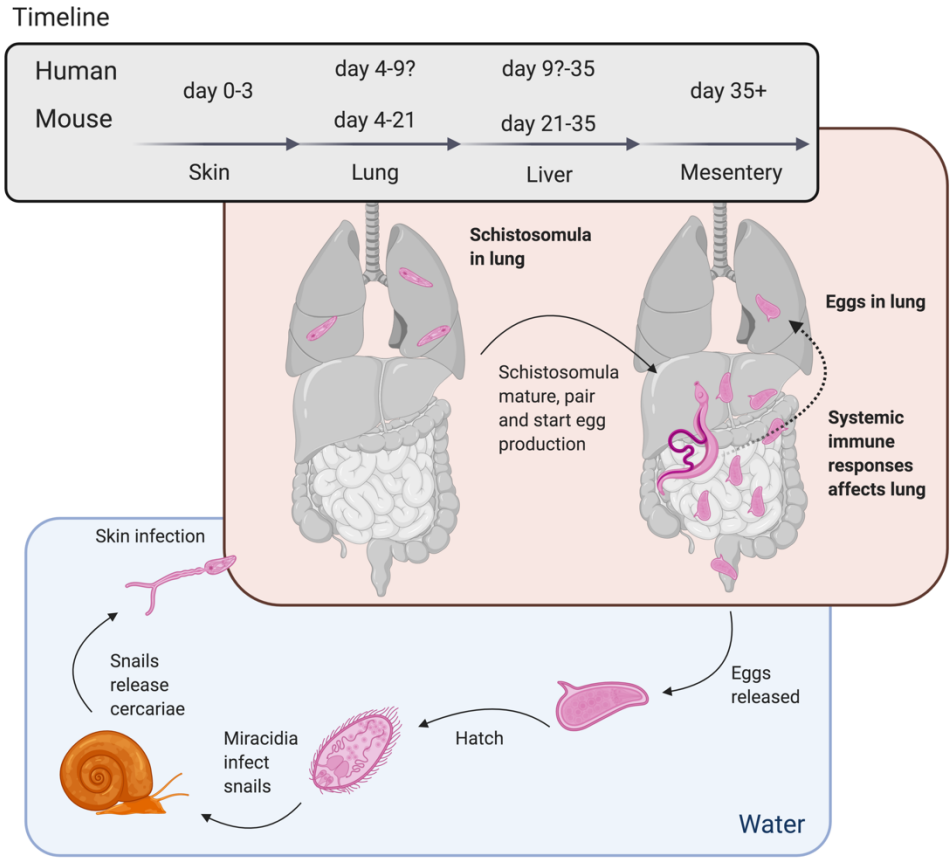


Diagram 1.1. *S. mansoni* infection life cycle and timeline
S. mansoni has a complex multi-host life cycle, with eggs released into water hatching into miracidia, which infect snails, where they replicate asexually, prior to release into the water as free living cercariae. Cercariae infect the human host via the skin, migrating in the bloodstream though the lungs, to the liver where they pair, migrate to the mesenteric vessels and release eggs. Eggs transit across the intervening tissues to be released in faeces into water, completing the cycle. A timescale for infection in humans and mice is shown (for lung and liver stages in human, as estimated from baboon models).
Created with BioRender.com

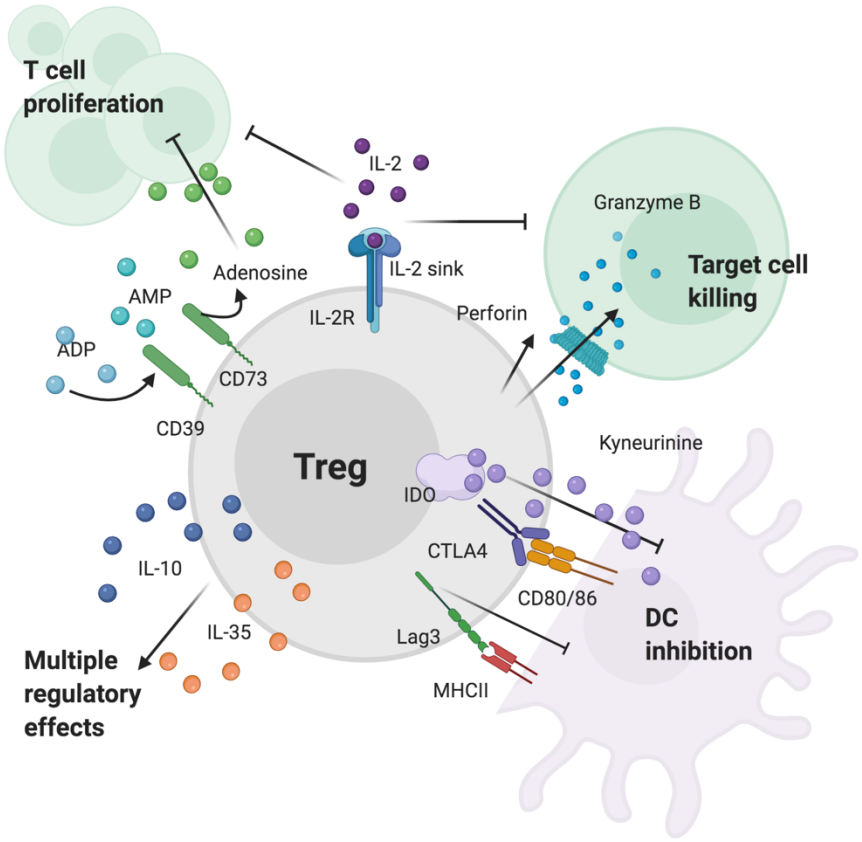


Diagram 1.2. Mechanisms of Treg suppression
This diagram details mechanisms by which Tregs are able to regulate the immune response, via cell-contact mediated interactions of expression or activation of tolerogenic factors.
Created with BioRender.com

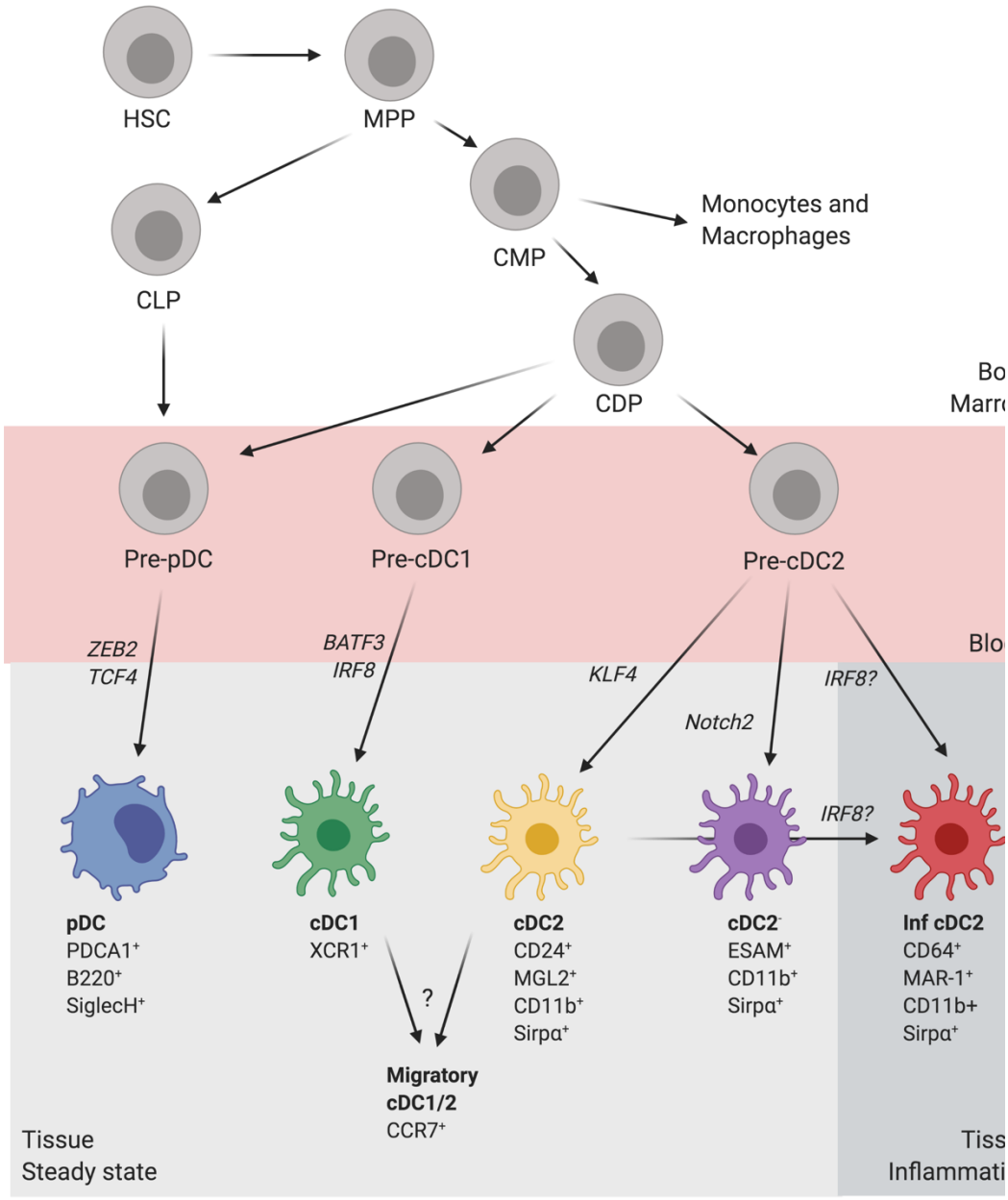


Diagram 1.3. Murine DC ontogeny
This diagram details the development of murine DCs from hematopoietic stem cell (HSC) progenitors, in the bone marrow, blood, and tissue. Key subset determining transcription factors are shown in italics, with subset defining surface markers shown below.
Updated from (Sichien *et al.*, 2017)
Created with BioRender.com

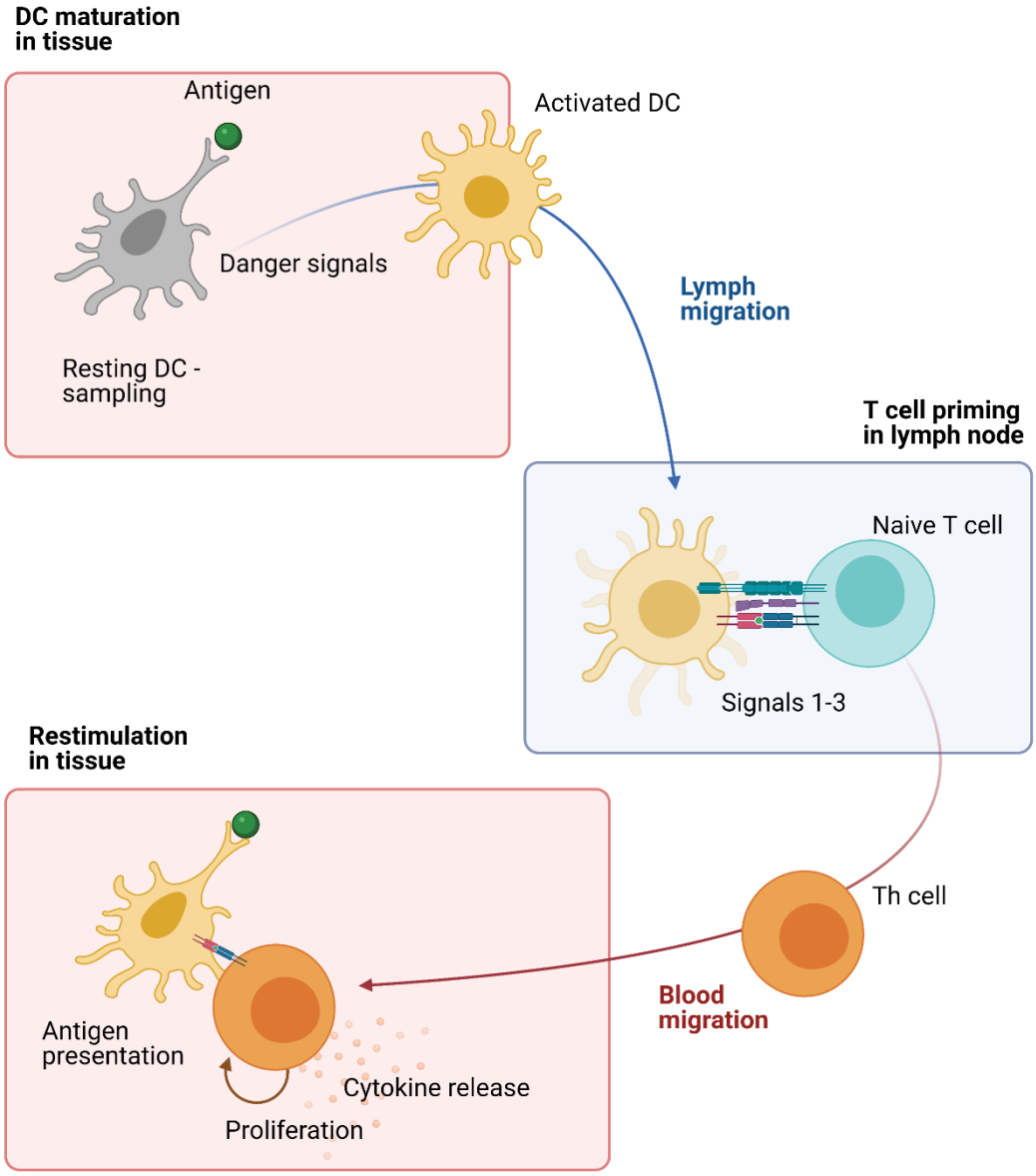


Diagram 1.4. DC T cell interactions initiating tissue inflammation
This diagram details how DCs take up antigen in the tissue, mature and migrate to the lymph node where they prime T cells. Th cells then migrate via the blood to the tissue, where they interact with DCs presenting cognate antigen, leading to Th cell cytokine release and proliferation.
Adapted from (Cabeza-Cabrerizo *et al.*, 2021)
Created with BioRender.com

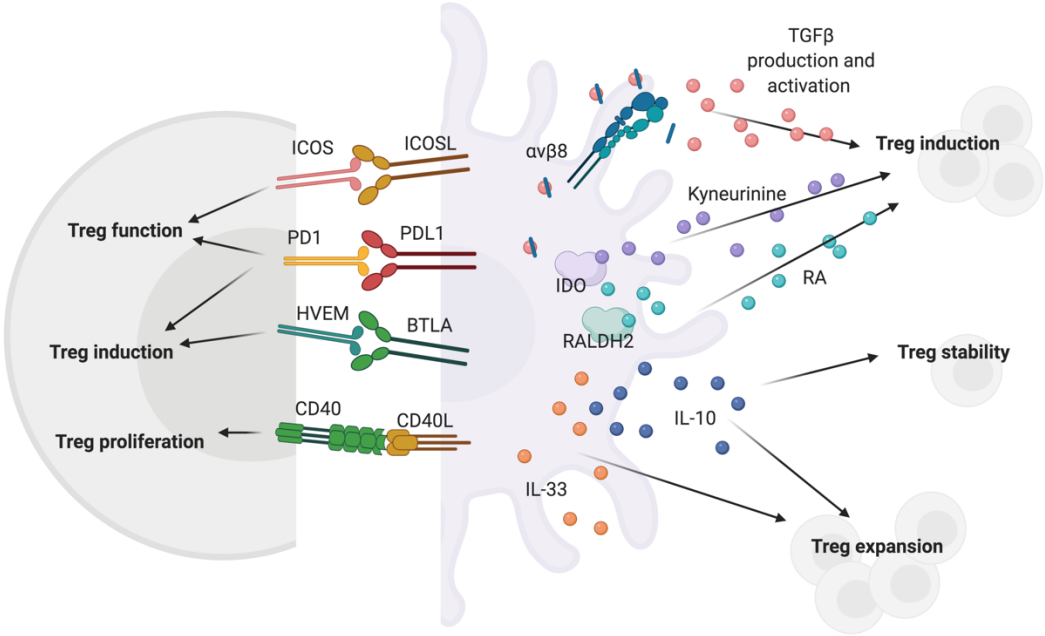
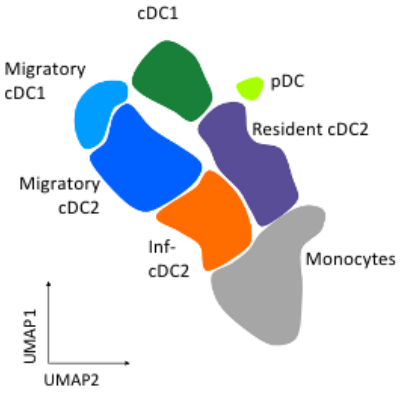
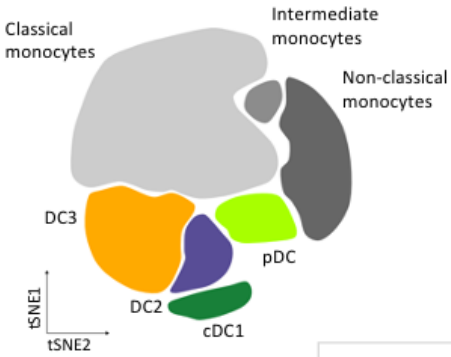


Diagram 1.5. DC/Treg interaction mechanisms
This diagram details how DCs can interact with T cells via cell contact mediated, and soluble factors to induce Treg induction, stability, proliferation, or enhance Treg suppressive function. When studies have not differentiated between Treg induction and proliferation, the term Treg expansion is used.
Created with BioRender.com



	DCs						
	cDC1		cDC2				
	Migratory	Resident	Migratory	Resident	Inf-cDC2	pDC	Monocytes
CCR7	+	-	+	-	-	-	-
CD26	+	+	+	+	+	?	-
CD64	-	-	-	-	-	-	+
IRF8	+	+	-	-	-	-	-
CD103	-	+	-	-	-	-	-
XCR1	-	+	-	-	-	-	-
CLEC9A	-	+	-	-	-	-	-
CADM1	-	+	-	-	-	-	-
CD24	-	+	-	-	-	-	-
MGL2	-	-	-	+	-	-	-
CD172	-	-	+	+	+	-	+
IRF4	-	-	-	+	-	-	-
MAR-1	-	-	-	-	+	-	+
SIGLEC-H	-	-	-	-	-	+	-
PDCA1	-	-	-	-	-	+	-
CCR2	-	-	-	-	-	-	+
CSF1R	-	-	-	-	-	-	+

Diagram 1.6. Mouse lung DC subset markers
This diagram details markers ascribed to mouse DC subsets. Relationships between cell subsets are shown in the upper left plot, adaption of a Uniform Manifold Approximation and Projection (UMAP) plot from the ssRNAseq analysis reported in (Bosteels *et al.*, 2020). Characteristic markers are compiled from the aforementioned study, as well as (Guilliams *et al.*, 2016; Brown *et al.*, 2019; Maier *et al.*, 2020).



	DCs				Monocytes		
		cDC2					
	cDC1	DC3	DC2	pDC	Classical	Intermediate	Non-classical
HLA-DR	+	+	+	+	+	+	+
CD11c	+	+	+	-	+	+	+
CD89	-	-		-	+	+	-
CD88	-	-		-	+	+	+
CD14	-	+	-	-	+	+	-
CD163	-	+	-	-	+	+/-	-
CD5	-	-	+	-	-	-	-
CD16	-	-		-	-	+	+
IRF8	+	-	-	+	-	?	?
CD141	+	-	-	-	-	-	-
XCR1	+	-		-	-	-	-
CLEC9A	+	-	-	-	-	-	-
CADM1	+	-		-	-	-	-
Fcεr1α	-	+		+	-	-	-
CD301	-	+		-	-	+	+
CD1C	-	+	+	-	-	-	-
CD172	-	+	+	-	+	-	-
IRF4	-	+		?	-	?	?
CD64	-	+	-	-	+	+	+
CD303	-	-	-	+	-	-	-
CD123	-	-	-	+	-	-	-

Diagram 1.7. Human DC subset markers
This diagram details markers ascribed to human DC subsets. Relationships between cell subsets are shown in the upper left plot, adaption of a tSNE plot from the ssRNAseq analysis reported in (Dutertre *et al.*, 2019). Characteristic markers are compiled from the aforementioned study, as well as (Guilliams *et al.*, 2016; Villani *et al.*, 2017; Collin and Bigley, 2018; Mair and Liechti, 2020)

Chapter 2

Materials and Methods

Chapter 2 – Materials and Methods

2.1. Mice strains and *in vivo* models

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals

Strains and maintenance

C57BL/6 (purchased from Envigo), *Il4*^{GFP}*Foxp3*$^{YFP/Cre}$*R26r*^{FP635} (Mohrs *et al.*, 2005; Rubtsov *et al.*, 2008; Coomes *et al.*, 2015; Pelly *et al.*, 2017), *VertX IL-10*^{GFP} (Madan *et al.*, 2009), *DEREG* (Lahl *et al.*, 2007), *Ccr2*$^{-/-}$ (Boring *et al.*, 1997), *Bdca2*^{DTr}(Swiecki *et al.*, 2010), *Batf3*$^{-/-}$ (Hildner *et al.*, 2008), *Mgl2*^{DTr} (Kumamoto *et al.*, 2013), *Itgax*^{cre}*Itgb8*^{fl} (referred to as Cd11cΔαVβ8) (Caton, Smith-Raska and Reizis, 2007; Travis *et al.*, 2007), *Itgax*^{cre}*Irf4*^{fl} (referred to as Cd11cΔIRF4) (Caton, Smith-Raska and Reizis, 2007; Persson *et al.*, 2013) mice were used in this study. All mice were on a C57BL/6 background and maintained under specific pathogen free (SPF) conditions at the University of Manchester. All procedures were carried out according to the United Kingdom (UK) Home Office Animals in Scientific Procedures Act 1986 (ASPA 1986) and under license. Euthanasia of mice occurred via exposure to a rising concentration of carbon dioxide (CO_2), a schedule 1 method (ASPA 1986). Male or female mice aged 6-21 weeks were used for analysis, with no impact of age or gender evident in experimental outcomes.

Aspergillus fumigatus AAI model

To model fungal allergy, mice were exposed to repeat low doses of *A.f.* spores, in a model adapted from Porter *et al.* (Porter *et al.*, 2011). *A.f.* spores (CEA10 strain) were prepared as follows. *A.f.* spores were streaked on to solid aspergillus culture media (Pontecorvo *et al.*, 1953) and incubated at 37°C for 72 hours. Sterile water was added to distilled flasks, and shaken to resuspend, spores were then filtered through Miracloth (Calbiochem) to isolate conidia. Collected spores were washed in sterile water (3x 1200 xg, 5 minute spins), and frozen at -80°C in phosphate buffered saline (PBS) 0.05% Tween (Sigma).

0.4 x10^6 *A.f.* conidia were administrated *i.n.* in 30-40µl PBS 0.05% Tween 3x per week – on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. Off days between dosing allowed spore clearance to occur (P.C. Cook, unpublished data). Mice were euthanized at day 12 or 19 post first *A.f.* dose.

DEREG (Lahl *et al.*, 2007) mice were utilised to deplete Treg cell populations in this model. Mice were dosed *i.p.* with 500ng DTx on d12, d13, d15 and d17 to deplete Foxp3 expressing cells (Lahl and Sparwasser, 2011). To assess requirements for MGL2$^+$ cells in this model, we utilised MGL2^{DTr} (Kumamoto *et al.*, 2013) mice. These were dosed *i.p.* with 500 ng DTx on d13 and d16 to deplete MGL2 expressing cells (Kumamoto *et al.*, 2013). Finally, to assess the role of pDCs during *A.f.* driven AAI BDCA2^{DTr} (Swiecki *et al.*, 2010) mice were used. Mice

were dosed *i.p.* with 4-8 ng/g DTx to deplete pDCs (Lundie *et al.*, 2016). The higher (8 ng/g) dose was trialled to see if it improved depletion, as no difference was observed, both experiments were included.

Schistosoma mansoni infection model

Biomphalaria glabrata snails, exposed to *S. mansoni* miracidia (NMRI strain) were acquired from Biodefense and Emerging Infections research resources repository, or from Karl Hoffmann (Aberystwyth University). Snails were maintained in aquaria until patency, 4-5 weeks post exposure to *S. mansoni* miracidia, with a regular 12 hour light 12 hour dark cycle. Approximately 24 hours before infection lights were turned off in the room containing the snails. On the day of infection patent snails were moved to a beaker containing 5-10 ml of aquarium water and exposed to a warm light source for 50 minutes, to induce shedding of cercariae. After 50 minutes water containing cercariae was poured into a 50 ml falcon, snails returned to aquaria, and an aliquot of cercarial water stained with a 1:1 dilution of Lugol's iodine solution (Sigma) and counted under a dissection microscope. Cercariae were diluted to the required concentration for the stated infection dose.

Prior to infection, mice were anaesthetised with medetomidine (domitor) and ketamine (vetalar) *i.p.*, with 0.25 ml domitor (1 mg/ml stock) and 0.19 ml vetalar (100 mg/ml stock) diluted in 2.6 ml PBS, injected at 0.01 ml/g for female mice, and 0.0066 ml/g for male mice. Once anaesthesia was achieved, the abdomen of the mice was shaved, dampened with water, and a 1 cm steel ring taped in place on the shaved region. 200 µl of water containing 180 cercariae was placed into the ring, and an extra 200 µl of clean aquaria water was added to ensure the full surface area of the ring for infection. Percutaneous invasion of cercariae occurred over a 30 minute period, with mice on heat mats to maintain body temperature. After 30 minutes the steel ring and cercarial water was removed, and animals were revived with a *s.c.* injection of 100 µl of an atipamezole (antisedan 5 mg/ml stock) solution - 0.04 ml atipamezole and 0.96 ml PBS. Animals were placed in a heated incubator and monitored closely whilst recovering from anaesthetic. Mice were euthanised at day 21 or day 49 post infection for immunological outputs.

2.2. Murine cell isolation

BAL

BAL was collected by injecting 500 µl lung buffer (2% fetal calf serum (FCS) and 2 mM ethylenediaminetetraacetic acid PBS (all from Sigma)) into the trachea via a cannula, and then withdrawn into the syringe. Note all FCS used was pre-inactivated by placing at 58˚ C for 50 minutes, to denature any components of the complement cascade. A total of four washes were performed, with the first wash kept separate from the subsequent three washes. Collected BAL wash was spun at 400 xg for 5 minutes. Supernatant from the first wash was saved for subsequent cytokine analysis at -80˚ C. Cell pellets from all washes were combined, resuspended in 200 µl lung buffer, counted, and then taken on to flow cytometry staining.

Lung tissue

Lung tissue was diced with a razor blade and digested in 1ml Hanks H9269 (Sigma) plus 1.6U Liberase TL (Roche) and 160U DNAse (Sigma) for 40 minutes at 37˚ C, with 150 rpm shaking. Digestion was stopped by the addition of 10 ml ice cold lung buffer, and then lungs pushed through a 70 µm cell strainer (Fisher Scientific) with 20 ml of lung buffer. Cells were spun for 500xg for 6 minutes. Cell pellets were then resuspended in 3 ml RBC lysis buffer (Sigma) for 4 minutes, before lysis was stopped with 10 ml of lung buffer. Cells were then spun at 500 xg for 3 minutes, resuspended in lung buffer, counted, and taken on to flow cytometry staining or PMA/ionomycin stimulation.

Lung draining LN

Lung draining (mediastinal) lymph nodes (lung dLN) were processed in two separate ways, depending on if myeloid cells were assayed. In experiments where lymphocytes were isolated lung dLNs were crushed between gauze to release cells, which were washed in lung buffer, spun at 500 xg for 5 minutes, resuspended in lung buffer, counted and taken on to downstream assays. In experiments where myeloid cells were assayed lung dLNs were digested in 500 µl Hanks H9269 (Sigma) plus 1U/ml Liberase TL (Sigma) and 80 U/ml DNAse (Sigma) for 30 minutes at 37˚ C, with 150 rpm shaking. Digestion was stopped by the addition of 5 ml ice cold lung buffer, and then lung dLNs were pushed through a 70 µm cell strainer (Fisher Scientific), with 20 ml of lung buffer. Cells were then spun at 500 xg for 3 minutes, resuspended in lung buffer, counted, and then taken on to downstream assays.

2.3. Murine *in vitro* assays

Treg suppression assay

BMDC cell culture for Treg suppression assay: To establish Treg suppression assays, BMDCs were generated by culture of bone marrow cells with granulocyte macrophage colony stimulating factor (GM-CSF) for ten days. This was achieved by removing the femur and tibia of C57BL/6 mice. These were sterilised in 70% ethanol, rinsed in PBS, and ends removed with a razor blade. PBS was flushed through bones with a 23 gauge needle and syringe. Cells were spun at 300xg for 5 minutes, counted, and resuspended to 1 $x10^7$ cells/ml. 2 $x10^6$ cells were added dropwise to the centre of a petri dish (Fisher) containing 10 ml of pre-warmed DC medium. DC medium is RPMI-1640 with 1% Pen/Strep (Gibco, final concentration 100 U/ml Penicillin & 100 µg/ml Streptomycin), 10% FCS (Sigma), 1% L-Glutamine (Gibco), and 20ng/ml GM-CSF (Invitrogen). 10 ml of DC media was added gently on d3, on d6 and d8 9ml of DC media was removed and replaced with 10 ml of fresh DC media, taking care not to disturb the central semi-adherent cells. DCs were harvested on d10, spun down at 300 xg for 5minutes and resuspended.

Lung and lung dLN cell isolation for Treg suppression assay: Lungs and lung dLNs were taken from $Il4^{GFP}Foxp3^{YFP/Cre}R26R^{FP635}$ (Mohrs *et al.*, 2005; Rubtsov *et al.*, 2008; Coomes *et al.*, 2015; Pelly *et al.*, 2017) mice on d19 of *A.f.* driven AAI, or PBS treated control mice. Lung tissue was diced with a razor blade and digested in 1 ml Hanks H9269 (Sigma) plus 1.6U

Liberase TL (Roche) and 160U DNAse (Sigma) for 30 minutes at 37˚ C, with 150rpm shaking. Digestion was stopped by the addition of 10 ml ice cold lung buffer, and then lungs pushed through a 70µm cell strainer (Fisher Scientific) with 20 ml of lung buffer. Cells were spun for 500xg for 6 minutes and the pellet resuspended in 3 ml RBC lysis buffer (Sigma) for 4 minutes, before lysis was stopped with 10 ml of lung buffer. Lung dLNs were crushed between gauze to release cells, washed in RPMI 1640 (Sigma). Lung and lung dLN cells were then spun at 500 xg for 5 minutes prior to MACs enrichment.

MACs enrichment: a cocktail of biotinylated antibodies encompassing myeloid cells, B cells, RBCs, neutrophils, macrophages, NK cells and CD8 T cells - CD11c, CD19, Ter119, Ly6G, NK1.1, MerTK, CD8 – was added to lung and lung dLN cells and incubated for 5 minutes at 4˚C. Anti-Biotin MicroBeads (Miltenyi Biotec) were added and incubated for 10 minutes at 4˚C. LS Columns (Miltenyi Biotec), were prepared by addition of 3 ml ice cold 2% lung buffer, prior to addition of lung and lung dLN cells. To ensure all unlabelled cells passed through the column, it was then flushed with 6 ml ice cold lung buffer. Unlabelled cells that passed through the column, enriched for $CD4^+$ T cells, were then spun down at 500xg for 5 minutes, and taken on to antibody staining.

Antibody staining for Treg suppression assay: cells were incubated in 50 µl FcR block plus antibodies – TCRβ e780, CD4 AF700, CD45 BV510 (see Table 2.1) – and incubated for 30 minutes at 4˚C. 10ml of lung buffer was added and cells spun down at 500xg for 5 minutes then resuspended in 500µl lung buffer.

Sorting for Treg suppression assay: Immediately prior to sorting 0.25µg/ml DAPI was added to identify dead cells. $CD4^+$ $Foxp3^-$ T cells, $CD4^+$ $Foxp3^+$ Tregs, and $CD4^+$ $Foxp3^+$ $IL\text{-}4^+$ Tregs were sorted via fluorescence assisted cell sorting (FACs) on a BD FACS Aria Fusion at 45 psi using an 85 µm nozzle as per the sort strategy depicted in Fig. 2.6. Purity of sorted populations was assessed by re-sorting cells, with purity shown in Fig. 2.6.

Treg suppression assay: $CD4^+$ $Foxp3^-$ T cells were spun down at 500 xg for 5 minutes, and 1 ml cell trace violet (Invitrogen) added as per manufacturers protocol. $CD4^+$ $Foxp3^-$ T cells were incubated at 37˚C for 20 minutes, before staining was stopped by the addition of 10 ml Complete RPMI (RPMI 1640 (Sigma), 1% Pen/Strep (Gibco, final concentration 100 U/ml Penicillin & 100µg/ml Streptomycin), 10% FCS (Sigma), 1% L-Glutamine (Gibco), 50 µM β-mercaptoethanol (Sigma)). $CD4^+$ $Foxp3^-$ T cells, $CD4^+$ $Foxp3^+$ Tregs, $CD4^+$ $Foxp3^+$ $IL\text{-}4^+$ Tregs, and BMDCs were then spun at 500 xg for 5 minutes, before resuspending in complete RPMI. 1µg/ml soluble anti-CD3, 600 BMDCs and 10,000 $CD4^+$ $Foxp3^-$ T cells, were added to each well, and a serial dilution of $CD4^+$ $Foxp3^+$ Tregs or $CD4^+$ $Foxp3^+$ $IL\text{-}4^+$ Tregs were added, from a 1:1 ratio with $CD4^+$ $Foxp3^-$ T cells, to an 8:1 ratio. All wells had a final volume of 200 µl. Cells were incubated for four days at 37°C in a humidified environment of 5% CO2, prior to flow cytometry staining.

Treg induction assay

Lung, lung dLN and spleen cell isolation for Treg induction assay: Lungs and lung dLNs were taken from C57BL/6 mice (Envigo) on d19 of *A.f.* driven AAI. Lung tissue was diced with a razor blade and digested in 1 ml Hanks H9269 (Sigma) plus 1.6U Liberase TL (Roche) and 160U DNAse (Sigma) for 30 minutes at 37˚ C, with 150rpm shaking. Lung dLNs were digested in 500 µl Hanks H9269 (Sigma) plus 1U/ml Liberase TL (Roche) and 80U/ml DNAse (Sigma) for 30 minutes at 37˚ C, with 150 rpm shaking. Digestion was stopped by the addition of ice-cold lung buffer, lungs, and lung dLNs were then combined and pushed through 70µm cell strainers (Fisher Scientific), with 20 ml of lung buffer.

Spleens were taken from naïve Il4^{GFP}Foxp3$^{YFP/Cre}$R26R^{FP635} (Mohrs *et al.*, 2005; Rubtsov *et al.*, 2008; Coomes *et al.*, 2015; Pelly *et al.*, 2017) mice, crushed between gauze, and spun at 500xg for 5 minutes. Cells were resuspended in 2 ml of RBC lysis buffer (Sigma) and incubated at room temperature (RT) for 3 minutes. 10 ml RPMI 1640 (Sigma) was then added to stop lysis. All cells were then spun at 500xg for 5 minutes prior to enrichment.

DC optiprep enrichment for Treg induction assay: Lung and lung dLN cells were resuspended in 3 ml Hanks H9394 (Sigma)/15% Optiprep (Stemcell), on to which lung buffer/11.5% Optiprep (Sigma), and then 3 ml Hanks H9394 (Sigma) was layered. The gradient was centrifuged at 600xg for 15 minutes at RT with no brake. Enriched DCs were isolated from the interface below the top Hanks H9394 (Sigma) layer, added to 10 ml lung buffer and spun at 500xg for 5 minutes.

T cell enrichment for Treg induction assay: A cocktail of biotinylated antibodies – Ly6G, NK1.1, CD19, Ter119, CD11c, CD8 – were added to RBC lysed splenocytes, and incubated at 4°C for 20 minutes. 10 ml lung buffer was added, and cells spun at 500xg for 5 minutes, before being resuspended in 500 µl pre-washed Dynabeads Biotin Binder (Invitrogen) in 4.5 ml lung buffer and incubated at 4°C for 15 minutes with gentle rotation on a HulaMixer (Invitrogen). After 15 minutes, 5 ml more lung buffer was added, and cells placed in a DynaMag-15 (Invitrogen) magnet for 3 minutes at RT, in which time Dynabead labelled cells magnetically adhered to the outer sides of the 15 ml tube, allowing the suction of non-labelled cells, enriched for CD4$^+$ T cells, from the centre of the tube. 5 ml more lung buffer was added to the 15 ml falcon, and magnetic enrichment repeated once more. Enriched CD4$^+$ T cells were then spun down at 500 xg for 5 minutes prior to staining.

Antibody staining for Treg induction assay: cells were incubated in 50 µl FcR block (αCD16/CD32, 2.4G2 (Biolegend)) plus antibodies and incubated for 30 minutes at 4˚C. Antibodies added to enriched CD4$^+$ T cells: CD3 e780, TCRβ e780, CD4 AF700, CD45 BV510, BV785 CD44 (Table 2.1). Primary antibodies added to enriched DCs: CD45 BV510, CD11c BV605, MHCII AF700, Lineage (MerTK Bio, CD3 Bio, CD19 Bio, B220 Bio, NK1.1 Bio, Ly6G Bio, Ter119 Bio, SiglecF PE/CF594), XCR1 BV421, CD11b BV711, MGL2 PE (Table 2.1). 10 ml of lung buffer was added, and cells were spun down at 500xg for 5 minutes. Streptavidin PE/CF594 was then added to enriched DCs to label the Lineage$^+$ cells and

incubated for 15 minutes at 4˚C. 10 ml of lung buffer was then added, and cells spun down at 500 xg for 5 minutes. Cells were resuspended in 500 µl lung buffer prior to sorting.

Sorting for Treg induction assay: Immediately prior to sorting 0.25 µg/ml DAPI was added to identify dead cells. $CD4^+$ $Foxp3^-$ T cells, as well as DC subsets were FACS-sorted on a BD FACS Influx at 20 psi using a 100 µm nozzle as per the sort strategy depicted in Figs. 2.7&8 Cell populations were resorted to test purity, shown in Figs. 2.7&8.

Treg induction assay: Sorted DC subsets, and $Foxp3^-$ T cells were spun down at 300 xg for 5 minutes. 2,500 DCs, and 50,000 $CD4^+$ $Foxp3^-$ T cells were added per well, in addition to 0.06 µg/ml antiCD3 and 5 ng/ml IL-2 (Roche). To some wells 2 ng/ml recombinant TGFβ (Peprotech) was added.

2.4. Flow cytometry

PMA/Ionomycin stimulation

To assess cytokine secretion potential cells were stimulated for three hours at 37˚C with 20 ng/ml PMA (Sigma) and 1 µl/ml GolgiStop (BD) in X-vivo-15 (Lonza), supplemented with 1% L-glutamine (Gibco) and 0.1% β-mercaptoethanol (Sigma). Stimulation was carried out in a 96 well u bottom plate, at 400,000 cells per well in a 200 µl final volume.

Staining for flow cytometry

Surface staining: Post cell isolation, cells were plated at 1 $x10^6$ cells per well, in a 96 well v-bottom plate. For all wash steps, cells were centrifuged at 500 xg for 2 minutes. Incubation steps were performed in the dark to prevent fluorochrome bleaching, and at 4˚C unless otherwise stated. Post-plating, cells were washed twice in PBS to remove proteins in the supernatant and then 10 µl of a 1:2000 dilution of the amine reactive viability die ZombieUV or Zombie NIR (Biolegend) was added for 10 minutes at RT. 50 µl FcR block (Biolegend)) was added to cells and incubated for 10 minutes. Antibodies reactive to surface Ags were then added to cells in 50 µl of lung buffer, at the concentrations detailed in Table 2.1, for 30 minutes. Post staining cells were washed twice in lung buffer and then fixed with 1% paraformaldehyde (PFA) for 10 minutes at RT, cross linking molecules to allow for acquisition at a later stage. Cells were washed and resuspended in lung buffer prior to acquisition.

Intracellular and intranuclear staining: To detect intracellular or intranuclear Ags cells were fixed with 100 µl BD cytofix/cytoperm (BD) for 1 hour. Cells were then washed three times with 1X eBioscience permeabilization buffer (Thermofisher), and then antibodies to intracellular or intranuclear Ags added and incubated overnight. Cells were washed twice in the morning in 1X eBioscience permeabilization buffer (Thermofisher) to remove unbound antibody. Cells were washed and resuspended in lung buffer prior to acquisition. This protocol was chosen as it allowed for consistent staining of intranuclear Ags, without loss of signal of fluorescent reporter proteins (protocol provided by Prof David Withers).

Counting beads: for some experiments CountBright™ Absolute Counting Beads (Thermofisher) were used to calculate cell counts. 10-50 μl beads were added prior to running the samples on the flow cytometer, with beads identified by high side scatter (SSC), low forward scatter (FSC) properties during analysis. The absolute number a of beads added as well as bead events acquired was compared to the volume of cells added and cell events acquired to calculate absolute cell count, as per manufacturers protocol.

I.t. and *i.v.* anti-CD45 labelling

To understand the location of cells within the lungs, *i.v.* and *i.t.* labelling was performed, as previously published (Svedberg *et al.*, 2019). Cells in the blood were labelled by *i.v.* injection of 100 µl 15µg/ml antiCD45 APC (Biolegend), and mice euthanised 3 minutes later. A cannula was inserted into the trachea, and 250 µl 0.1 µg/ml antiCD45 PE (Biolegend) injected, inflating the lungs. Lungs were removed and immediately placed into a large volume (>20 ml) of DMEM media (Sigma), with the large volume diluting any unbound antibody to reduce binding to other lung compartments.

Flow cytometry strategies

Different gating strategies were used to identify lymphoid, myeloid and granulocytic cells in mouse and human samples. These are detailed in Figs 2.1-5, along with the rationale behind these strategies.

For human sputum staining a sentinel gating strategy was used, allowing for investigation of numerous cell types in one panel, to allow for the variable to low-cell number of sputum samples. Certain sentinel markers were chosen – CD3, CD14, CD16, CD45, CD11c, CD66b – to identify distinct cellular identities for instance T cells, monocytes, myeloid cells, granulocytes. In analysing the data these sentinel gates were used first, and then next more detailed phenotypic or subsetting markers were applied, for instance CD1c, CD8 and CD28. These secondary markers were sometimes on the same fluorophore, as shown on Table 2.2. As an example, the antibodies TCRβ and Siglec8 were both conjugated to the fluorophore BB700, however prior to utilising the fluorescence in this channel for identification of cell subsets CD3 was used to gate on T cells, or CD66b to gate on granulocytes. Utilising this technique allowed us to look at a wide variety of cell-specific markers with the 16 available fluorescent channels used.

Flow cytometry analysis

Post staining cells were acquired on a BD Fortessa or LSRII analyser, with FacsDiva (BD) software. Standardised photomultiplier voltages were optimised for greatest separation between positive and negative signals, without allowing the fluorescent signal to go beyond the linear detection range of the photomultiplier. These settings were stored within application settings of the software. This ensured consistent settings (taking account for slight machine fluctuations) were used for all samples collected via flow cytometry. The resultant flow cytometry data was analysed with Flowjo v9 or v10 (Tree Star).

2.5. Histology

Sample processing

Lungs were collected into 10% neutral buffered formalin (NBF) (Sigma) and left overnight to fix and then washed and stored in 70% ethanol. Lungs were then placed in tissue cassettes and processed in a Leica ASP 300 Tissue Processor (Leica Biosystems) to dehydrate. After overnight processing samples were embedded in paraffin wax, and 5 μm thick sections cut utilising a Leice RM2255 Rotary Microtome (Leica Biosystems) and mounted on Superfrost slides (Thermofisher).

For assessment of airway epithelium thickness, and overall pathology Haematoxylin and Eosin (H&E) staining was performed. Staining of slides with H&E was performed utilising a Leica autostainer (Leica).

For assessment of mucous producing goblet cell numbers AB-PAS (Alcian blue - periodic acid Schiff) staining was used. Slides were rehydrated using a Leica autostainer (Leica) and placed in distilled water for one minute. Slides were then stained with Alcian blue (Sigma) for 15 minutes, 1% periodic acid (Sigma) for 5 minutes, Schiff's reagent (Sigma) for 10 minutes, Papan haematoxylin (Sigma) and 5% acetic acid (Sigma) for 30 seconds. Following every staining step slides were washed in distilled water.

For assessment of collagen deposition Masson's trichrome staining was performed. Slides were rehydrated using a Leica autostainer (Leica). Slides were then placed in saturated aquarous picric acid (Sigma) for one hour, then stained with Weigart's iron hematoxylin (Sigma) for 10 minutes, then Biebrich scarlet-acid fuchsin (Sigma) for 10 minutes, then phosphotungstic/phosphmolybdic acid (Sigma) for 10 minutes, then 1% acetic acid (Sigma) for two minutes. Following every staining step slides were washed in distilled water.

Following staining all slides were then dehydrated and mounted on the Leica autostainer and Leica CV5030 coverslipper (Leica). A Panoramic250 slide scanner (3D Histec) was used to obtain images.

Image analysis

All analysis was performed utilising ImageJ (NIH). To assess airway inflammation in H&E stained samples images were taken from ten different areas in the lung, with five measurements of epithelial thickness taken per image. The average epithelial thickness was measured. To assess goblet cell hyperplasia airways between 100 μm – 300 μm were chosen from lung sections, and mucin secreting goblet cells (purple) were counted. Goblet cell counts were normalised to the diameter of the basement membrane. To quantify collagen deposition in lung tissue a Hue Saturation Brightness threshold on ImageJ was chosen to select only pule/blue pixels, representing stained collagen. These pixels were quantified and expressed as a percentage of the total lung pixels.

2.6. Human specific methods

The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.

Experimental human *S. mansoni* infection

Participant characteristics are shown in Table 5.1. Experimental human schistosome infections were performed at the Department of Parasitology, Leiden University Medical Centre (LUMC) by Dr Meta Roestenberg and colleagues, (ClinicalTrials.gov identifier:NCT02755324) as previously described (Langenberg *et al.*, 2020). Infections were performed by exposing volunteers to 20 male cercariae in water on the forearm for 30 minutes. Volunteers were monitored bi-weekly for adverse events. All volunteers were cured with praziquantel 12 weeks post exposure. As part of this larger study, induced sputum was taken from three individuals prior to infection, and then 11-14 days post infection. The study was reviewed and approved by the LUMC Institutional Medical Ethical Research Committee. Analysis of samples at the University of Manchester was reviewed and approved by the University of Manchester Research Ethics Committee (proportionate).

Endemic *S. mansoni* infection

Participant characteristics are shown in Table 5.2. This study was performed in collaboration with Dr Harriet Mpairwe at the MRC/LSHTM Uganda Research Unit. Participants aged 18-25 were recruited from a 2 km^2 area surrounding the Kigungu Landing site, Entebbe, Wakiso county, Uganda, shown in Fig. 2.9. Participants underwent pre-screening and schistosome infection was assayed by Kato Katz and circulating cathodic Ag (CCA) testing (Rapid Medical Diagnostics). Participants with previous history of pulmonary disease were excluded. After testing infection status participants were allocated into control or case groups. The study was reviewed and approved by the UVRI Research Ethics Committee, as well as the Uganda National Council for Science and Technology and the University of Manchester Research Ethics Committee.

Sputum induction and processing

Sputum induction was performed by the following procedure in experimental schistosome infection studies. Baseline forced expiratory volume (FEV) was measured prior to beginning, to ensure adequate lung function. Participants were administered with 200 μg Salbutamol prior to induction. 4.5% NaCl was administered to participants via a nebuliser to induce mucus secretions. Participants then attempted to expectorate sputum. The procedure (5 minute inhalation + expectoration) was repeated a total of three times, with an additional round if no sputum sample is produced. After each round of sputum induction FEV was measured, with the procedure stopping if a greater than 20% decrease in baseline FEV occurred. Sputum was placed on ice prior to processing. For endemic schistosome infection studies the procedure was performed almost identically, except administration of nebulised NaCl (4.5%) was administered orally for increasing intervals from 30 minutes to 4 minutes, up to a total time of 20 minutes. In all situation's sputum induction was halted if requested by the subject.

Sputum was processed by the following procedure at both sites. Sputum plugs were collected, and placed into a 15 ml falcon, weighed, and a 4X volume of 0.1% dithiothreitol (Sigma) added, and shaken for 15 minutes to reduce mucus viscosity, allowing for sample handling. An equal volume of PBS was then added, and sputum shaken for a further 2 minutes. Sputum was then poured through a 100 μm and then 40 μm strainer to obtain a single cell suspension. If unless the total sample was less than 1-2 ml straining was not performed so as to reduce sample loss. Samples were then spun at 700xg for 10 minutes at 4˚C to pellet cells. Sputum supernatant was frozen at -80 ˚C prior to analysis. Sputum pellet was resuspended in 1 ml 400KU/ml DNAse (Sigma) for 30 minutes at 37 ˚C. Cells were then spun at 600 xg for 6 minutes, and the pellet frozen down at -80 ˚C in 500 μl 50% FCS + 20% Dimethyl sulfoxide in RPMI (all Sigma). Sputum supernatants from endemic infection were filtered through 0.22 μm filters prior to analysis to remove microbial contamination.

Blood processing

Blood was drawn by venepuncture. 5 ml of blood was placed in BD Vacutainer Serum tubes (BD Biosciences) and left undisturbed at RT for 15-30 minutes to allow separation. Tubes were then spun at 1000 xg for 5 minutes at RT, with separated serum aliquoted and stored at -80˚C prior to analysis.

2.7. Soluble immune mediator analysis

ELISA

To assess soluble cytokines in sputum supernatant and BAL enzyme linked immunosorbent assays (ELISAs) were performed. Human CCL22, CCL17, IL-33, TSLP, IL-17E and YKL-40 and mouse Ym-1, Relmα, CCL17 and CCL22 were assayed by ELISA, utilising R&D DuoSet ELISA kits, according to manufacturer's instructions. To summarise, capture antibodies were diluted in PBS and added to 96 well flat bottom plates (Nunc) overnight at RT. Plates were washed four times with wash buffer (0.1% Tween 20 in PBS) and then blocked with 1% BSA in PBS at RT for a one hour. Plates were then washed four times with wash buffer, as before, prior to addition of samples, and a 15-point standard curve with blank, in duplicate for 2 hours at RT. Plates were washed four times in wash buffer as previously, and then 50 μL of Streptavidin-HRP added, and incubated for 20 minutes at RT. Plates were washed four times in wash buffer as previously, and then 100 μL of TMB (Life Technologies) added. Once a deep blue was achieved on the top standard, or colour change had ceased, 100 μl of 0.18M H_2SO_4 was added to stop the reaction. Plates were read at 450nm, with 570nm as the reference wavelength to correct for optical imperfections on the plate, using a Tecan Infinite M200 Pro plate reader (Tecan). ELISA data was analysed using Prism v9 (GraphPad), with a sigmoidal dose response curve fitted to log-transformed standards, utilised to interpolate samples.

Multiplex immunoassays (Luminex)

To assess multiple cytokines and chemokines in human serum and sputum supernatants, a 27plex BioRad BioPlex (BioRad) Luminex assay was performed. The assay was performed according to manufacturer's instructions, all washes in wash buffer. To summarise, 50 µl of antibody coupled magnetic beads were added to a 96 well clear bottomed plate, washed twice, and then 50 µl of standards, samples and controls added and incubated at 850 rpm for 1 hour at RT. The plate was then washed three times, and 50µl of streptavidin-PE added, and incubated at 850rpm for 10 minutes at RT. The plate was again washed three times, 125 µl of assay buffer added and the plate analysed on a Bio-Plex 200 reader (BioRad). Raw data was analysed on BioPlex Manager (Biolegend), and a weighted 5 parameter logistic regression curve fitted to standards to interpolate data. Analytes in which over two thirds of the samples had values extrapolated below the range of the standard curve were not included. Analytes in which less than two thirds of the samples were below the range of the standard curve were included, with samples below the range of the standard curve assigned the value of the lowest standard. By doing this we were able to retain the information provided by a below-range sample, whilst ensuring improper conclusions drawn from extrapolation below the standard range were not presented.

2.8. Statistical techniques

Statistical analysis for repeat murine experiments was performed using JMP Pro 12 (SAS). Mixed linear models were fitted, with experimental repeat as a random effect variable, to account for random effects of experimental day, and treatment, timepoint or genotype as the fixed effect variable. Significance was calculated utilising LSMeans Students T test (if only two groups compared) or Tukeys HSD test (if multiple groups were compared).

For analysis of human data Prism (GraphPad Software) v7-9 was used. For statistical analysis of cellular and cytokine responses in sputum from endemic individuals the D'Agostino-Pearson normality test was performed, and samples found to be non-normally distributed. Therefore a non-parametric test (Mann-Whitney) was used to compare differences between groups. For statistical analysis of cellular and cytokine responses in sputum from experimentally infected individuals it was not possible to perform a normality test, due to the low sample size, therefore normality was assumed, and T tests used to compare differences between groups.

2.9. Figures and tables

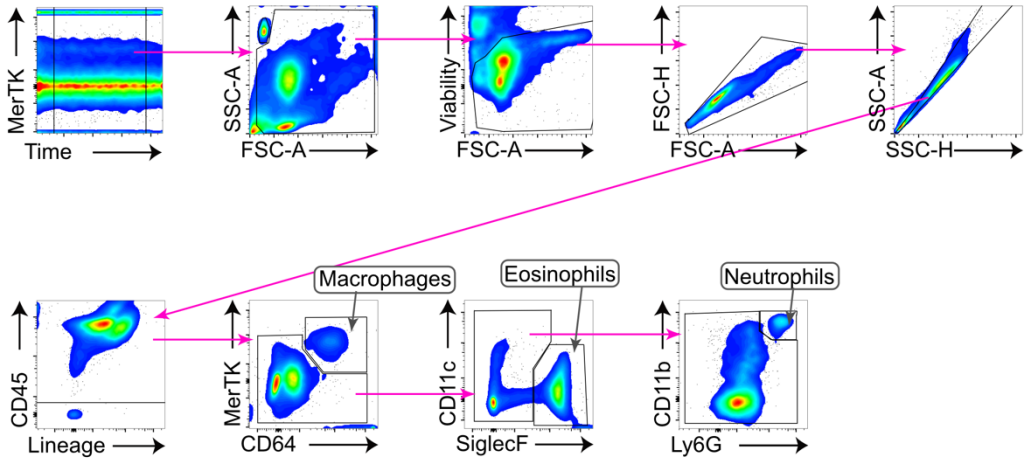


Fig. 2.1 Identifying BAL granulocytes by flow cytometry

Plots shown show BAL taken from *S. mansoni* infected mice. Gating schemes and cell populations are representative of all experiments, although the proportion of different cell types differed. Counting beads are shown as SSC-A^{high}, FSC-A^{low} cells in the second plot.

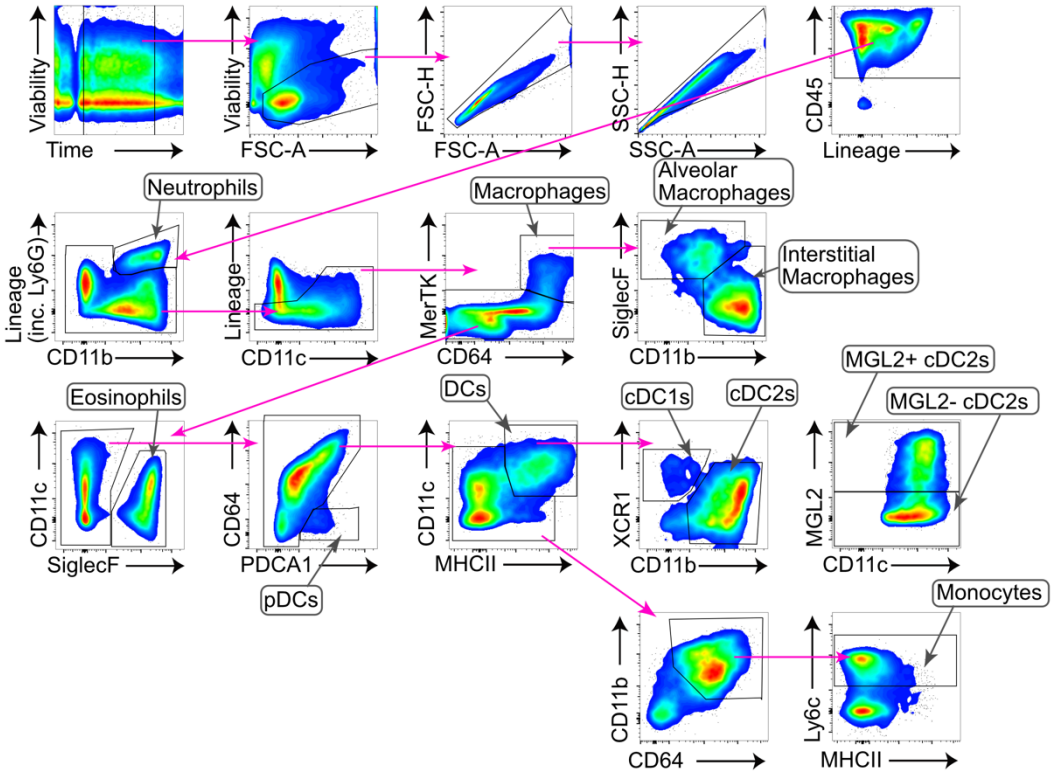


Fig. 2.2 Identifying Lung myeloid cells with flow cytometry

Plots shown show lung cells taken from d19 of *A.f.* driven AAI. Gating schemes and cell populations are representative of all experiments, although the proportion of different cell types differed.

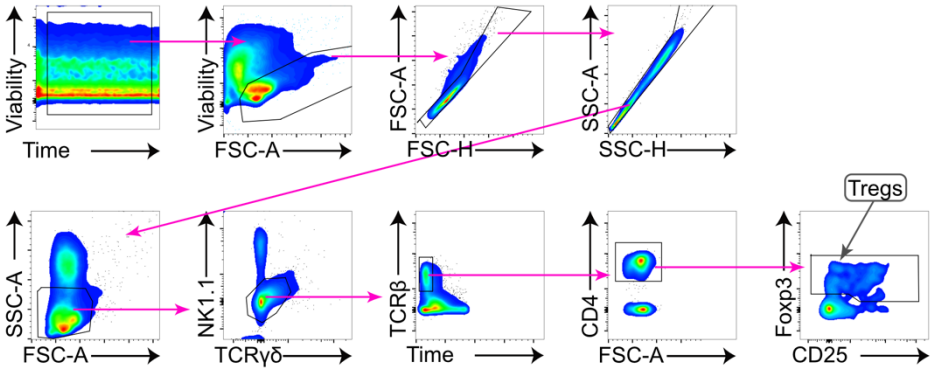


Fig. 2.3 Identifying Lung Tregs by flow cytometry

Plots shown show lung cells taken from naïve mice. Gating schemes and cell populations are representative of all experiments, although the proportion of different cell types differed.

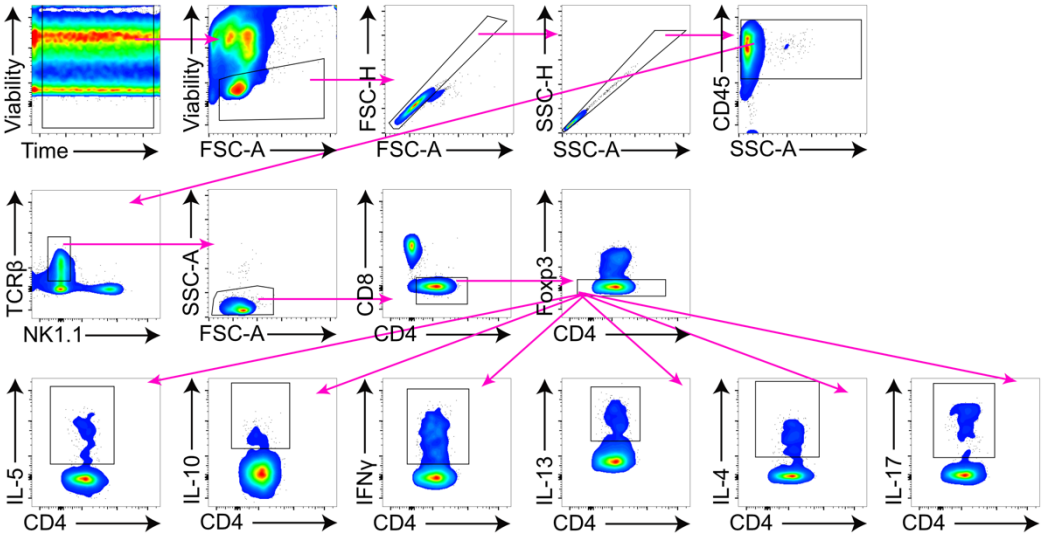


Fig. 2.4 Identifying lung cytokine secreting CD4⁺ T cells by flow cytometry

Plots shown show lung cells taken from d19 of *A.f.* driven AAI. Gating schemes and cell populations are representative of all experiments, although the proportion of different cell types differed.

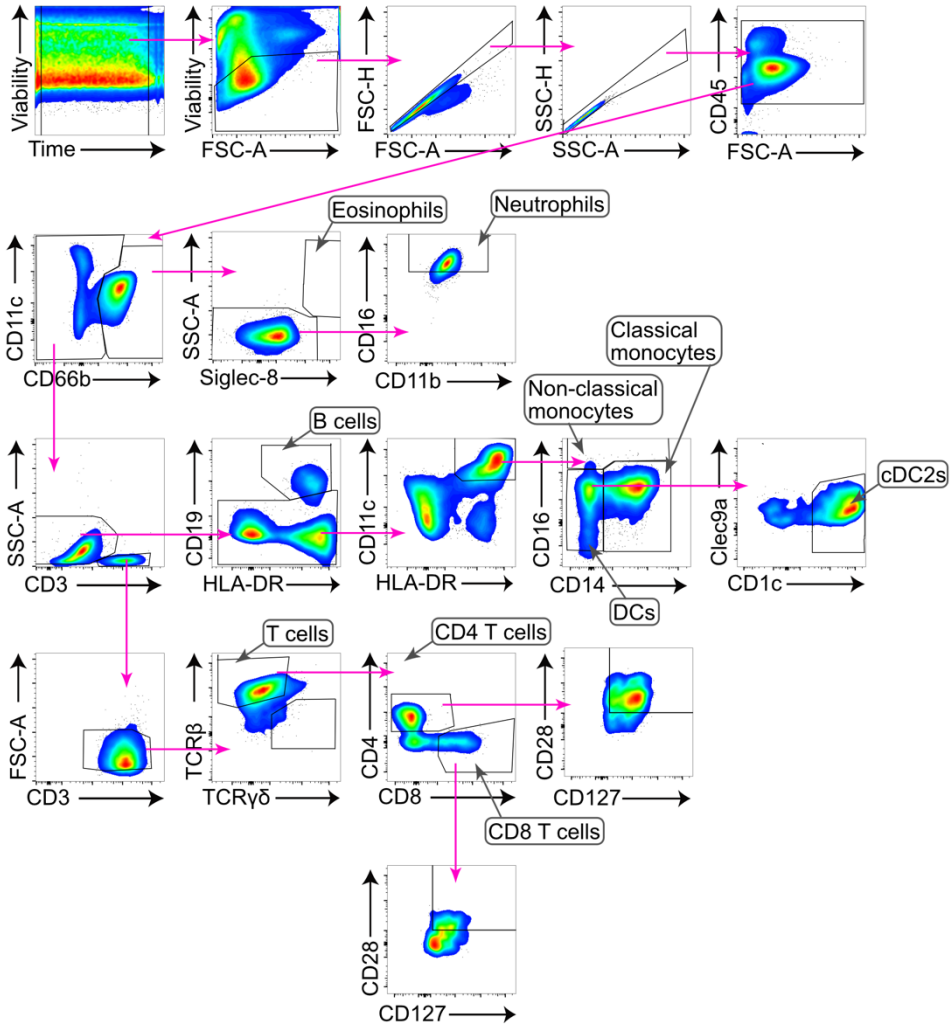


Fig. 2.5 Identifying sputum cell types with flow cytometry

Plots shown show sputum cells taken from sputum of an endemic individual infected with *S. mansoni*. Gating schemes and cell populations are representative of all individuals, although the proportion of different cell types differed between samples. Sentinel gating was used, with some fluorescent channels including two antibodies, as detailed in Table 2.

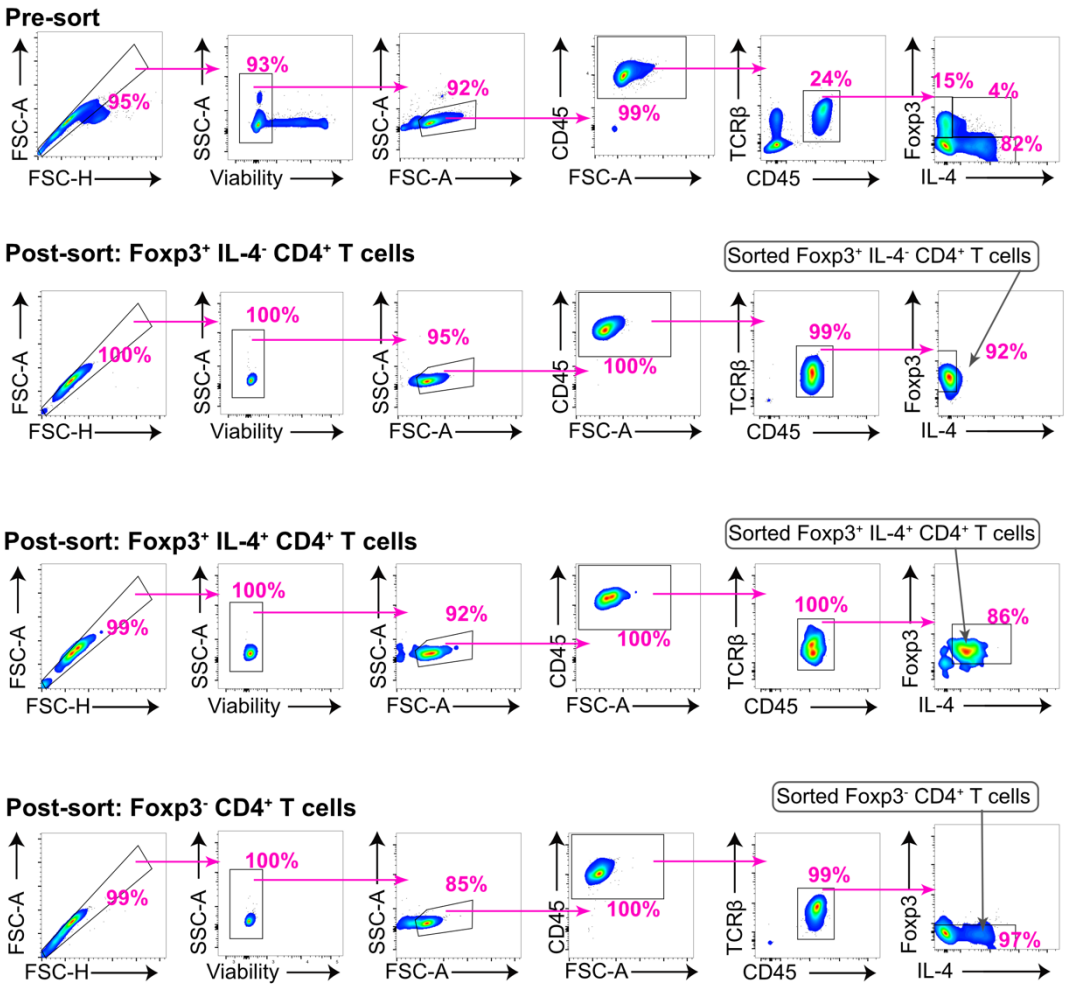


Fig. 2.6. Sorting strategy for Treg suppression assays

Enriched lung dLN T cells were stained as detailed above. Post sort populations were re-sorted, to analyse purity of cell populations.

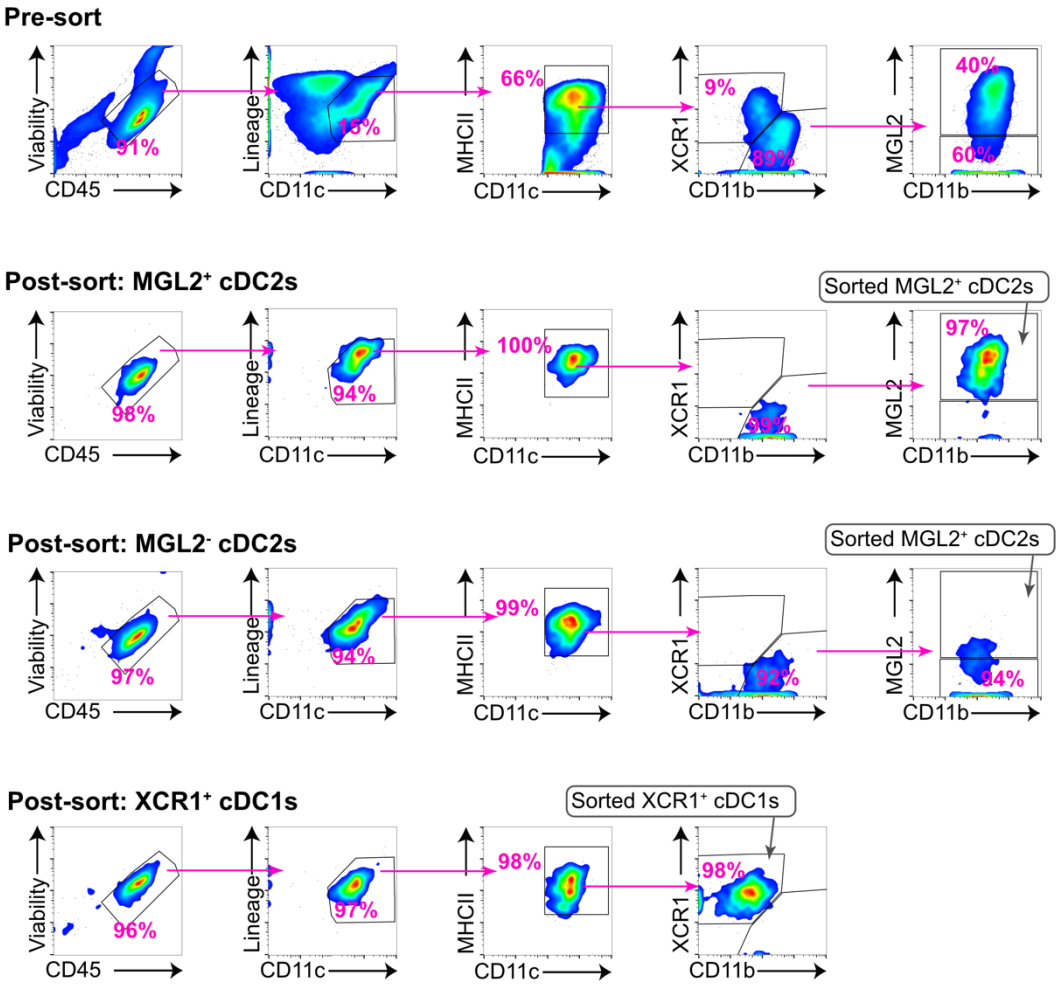


Fig. 2.7. DC Sorting strategy for Treg induction assays

Enriched DCs cells were stained as detailed above. Post sort populations were re-sorted, to analyse purity of cell populations.

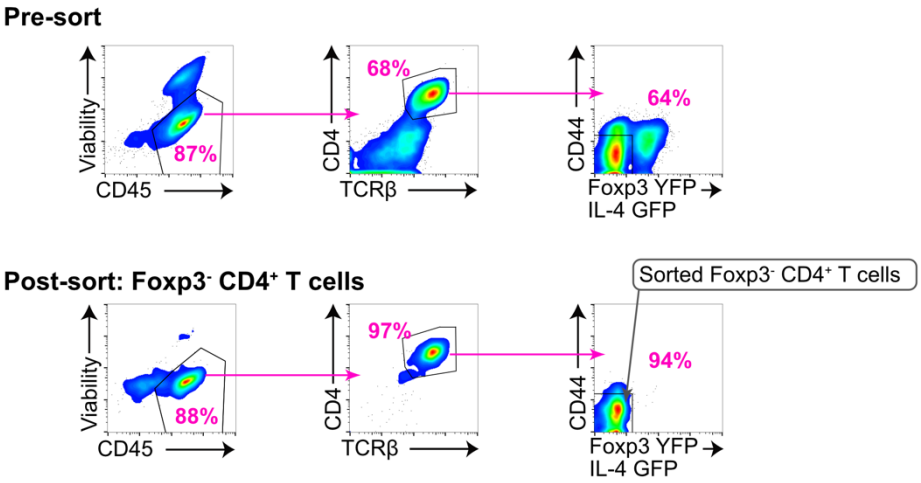


Fig. 2.8. Foxp3$^-$ T cell sorting strategy for Treg induction assays

Enriched T cells were stained as detailed above. Post sort populations were re-sorted, to analyse purity of cell populations.

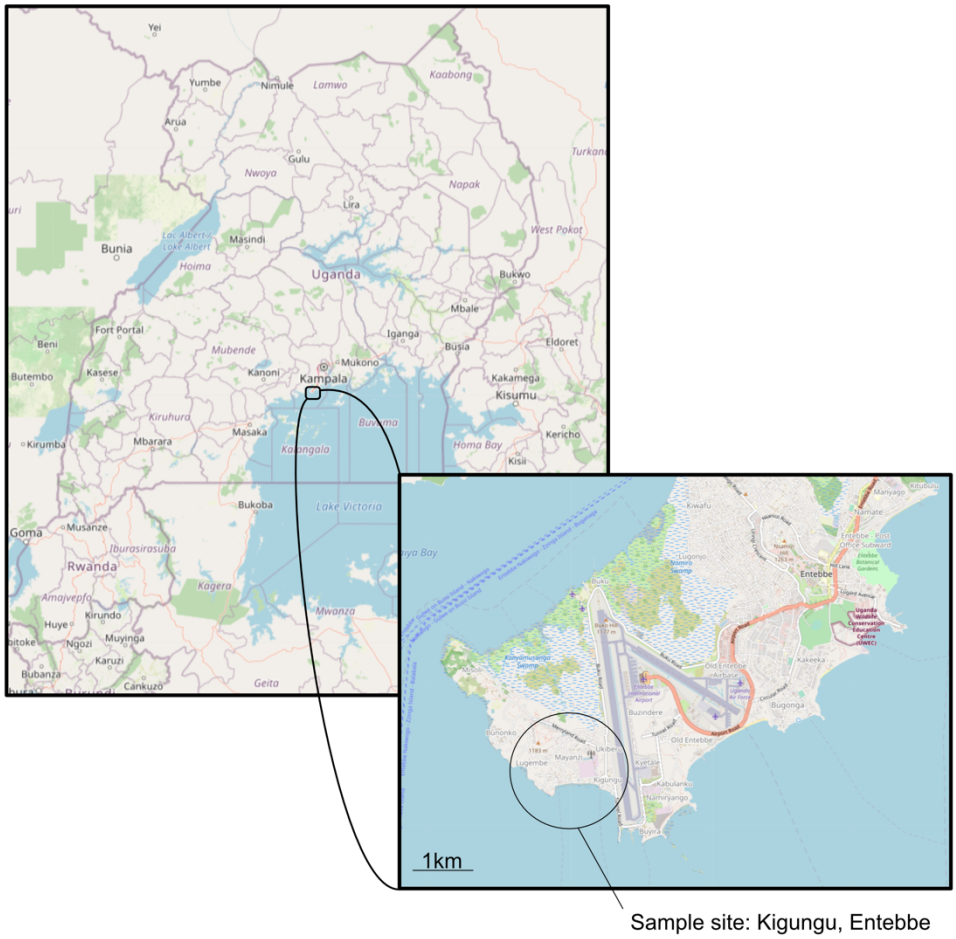


Fig. 2.9. Map of study area for endemic patent infection study

Participants in the study of endemic patent schistosome infection were recruited from Kigungu, Entebbe (lower right map), a region in the south of Uganda (upper right map), East Africa.

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Table 2.1 Mouse Flow Cytometry antibodies				
Ag	**Clone**	**Conjugate**	**Titration**	**Manufacturer**
Zombie UV	N/A	N/A	1:2000	BioLegend
B220	RA3-6B2	BV605	1:100	BioLegend
B220	RA3-6B2	Biotinylated	1:100	BioLegend
CD11b	M1/70	BV711	1:1000	BioLegend
CD11c	N418	BV605	1:600	BioLegend
CD11c	HL3	Biotinylated	1:100	BD Biosciences
CD19	eBio1D3 (1D3)	APC/e780	1:100	eBioscience
CD19	ID3	PE CF594	1:200	BD Biosciences
CD19	6D5	Biotinylated	1:100	BioLegend
CD16/32 (Fcblock)	2.4G2	Purified	1:200	BD Biosciences
CD25	PC61	BV650	1:100	BioLegend
CD3	17A2	APC/e780	1:100	eBioscience
CD3	17A2	Biotinylated	1:100	BioLegend
CD4	RM4-5	AF700	1:200	BioLegend
CD44	IM7	BV785	1:200	BioLegend
CD44	IM7	PE	1:200	BioLegend
CD45	30-F11	FITC	1:400	BioLegend
CD45	A20	BV785	1:500	BioLegend
CD45	30-F11	APC	1:600	BioLegend
CD45	30-F11	BV510	1:600	BioLegend
CD64	X54-5171	PE	1:100	BioLegend
CD69	HI.2F3.	PerCP Cy5.5	1:100	BioLegend
CD8	53-6.7	PerCP Cy5.5	1:200	BioLegend
CD8	53-6.7	PE Cy7	1:800	BioLegend
CD8	53-6.7	Biotinylated	1:100	BioLegend
Foxp3	FJK-16s	ef450	1:200	eBioscience
Foxp3	FJK-16s	APC	1:200	eBioscience
Gata3	TWAJ	ef660	1:200	eBioscience
IFNγ	XMG1.2	BV711	1:200	BioLegend
IL-10	JESS-16E3	BV605	1:200	BioLegend
IL-13	eBio13A	FITC	1:200	BioLegend
IL-13	eBio13A	APC	1:200	BioLegend
IL-17	TC11-18H10	PE Cy7	1:200	BioLegend
IL-4	11B11	PE CF594	1:200	BioLegend
IL-5	TRFK5	BV421	1:200	eBioscience
IL-5	TRFK5	PE	1:200	BioLegend
KLRG1	2F1/KLRG1	BV785	1:200	BioLegend
Ly6C	HK1.4	AF700	1:200	BioLegend
Ly6G	1A8	APC/Cy7	1:200	BioLegend
Ly6G	1A8	BV510	1:200	BioLegend
Ly6G	1A8	Biotinylated	1:200	BioLegend
MerTK	2Biol42	APC	1:100	BioLegend
MerTK	Polyclonal	Biotinylated	1:100	R&D Systems
MGL2	URA-1	PE Cy7	1:400	BioLegend
MGL2	URA-1	PE	1:100	BioLegend
MHCII	M5/114.15.2	PE Cy5	1:20000	BioLegend
MHCII	M5/114.15.2	AF700	1:100	BioLegend
NK1.1	PK136	APC	1:200	eBioscience
NK1.1	PK136	APC/e780	1:200	eBioscience
NK1.1	PK136	PE Cy5	1:200	BioLegend
NK1.1	PK136	Biotinylated	1:100	BioLegend
PD1	29F.1A12	BV605	1:200	BioLegend
PDCA-1	927	BV650	1:200	BioLegend

PDL2	TY25	PE	1:200	BioLegend
SiglecF	E50-2440	PE CF594	1:400	BD Biosciences
St2 (IL-33R)	RMST2-33	Biotinylated	1:300	eBioscience
Streptavidin	N/A	PE CF594	1:300-2000	BioLegend
TCR$\gamma\delta$	eBioGL3	PE Cy7	1:200	eBioscience
TCR$\gamma\delta$	eBioGL3	PerCP Cy5.5	1:100	BioLegend
TCR$\gamma\delta$	eBioGL3	PE	1:200	eBioscience
TCRβ	H57-597	APC/e780	1:100	eBioscience
Ter119	TER-119	APC/e780	1:200	eBioscience
Ter119	TER-119	Biotinylated	1:100	BioLegend
XCR1	2ET	BV510	1:400	BioLegend
XCR1	2ET	BV421	1:100	BioLegend

Table 2.2 Human Flow Cytometry antibodies				
Ag	**Clone**	**Conjugate**	**Titration**	**Manufacturer**
Zombie UV*	N/A	N/A	1:2000	BioLegend
Zombie NIR**	N/A	N/A	1:2000	BioLegend
CD3	UCHT1	BB515	1:100	BD Biosciences
CD8	RPA-T8	BB700	1:200	BD Biosciences
CD19	H1B19	BB700	1:200	BD Biosciences
CCR3*	5E8	APC	1:200	BioLegend
CD4	RPA-T4	AF700	1:200	BioLegend
CD11b	ICRF44	AF700	1:200	BioLegend
CD16*	3G8	APC/Fire750	1:200	BioLegend
CD28	CD28.2	BV421	1:200	BioLegend
CD1c	L161	BV421	1:200	BioLegend
CD45*	H130	BB488	1:200	BD Biosciences
TCRβ	IP26	BV605	1:50	BioLegend
Siglec8	837535	BV605	1:50	BD Biosciences
CD11c	BU15	BV650	1:200	BioLegend
TCRγδ	11F2	BV711	1:200	BD Biosciences
CD14*	M5E2	BV711	1:200	BioLegend
HLA-DR*	L243	BV785	1:100	BioLegend
CD127	A019DS	PE	1:200	BioLegend
Clec9a	8F9	PE	1:100	BioLegend
CD66b	G10FS	PE CF594	1:200	BioLegend
CD7	CD7-6B7	PE Cy5	1:200	BioLegend
Foxp3	236A/E7	PE Cy7	1:100	eBioscience
CD1a	HI149	PE Cy7	1:100	BioLegend
CD45**	HI30	BV785	1:100	BioLegend
HLA-DR**	L243	BV711	1:50	BioLegend
CD16**	3G8	BV510	1:200	BioLegend
CD14**	M5E2	APC	1:200	BioLegend

* Just utilised in analysis of experimental lung migratory infection
** Just utilised in analysis of endemic patent infection

Colours highlight markers for which two antibodies were used on the same channel, with cell population defining “sentinel markers” not coloured.

Chapter 3

The role of Tregs in *A.f.* driven AAI

Chapter 3 – The role of Tregs in *A.f.* driven AAI

3.1. Introduction

3.1.1 Introduction

Understanding how the immune response is regulated in allergic airway inflammation (AAI) is critical for intelligent design of therapeutics. Conventionally, murine research into AAI has utilised peripheral sensitisation with the model Ag OVA, plus an adjuvant (usually alum) followed by pulmonary challenge, this regime promotes a predominantly type-2 immune response (Lloyd, 2007). In reality, human asthma is a much more heterogenous condition however, with both Th17 and neutrophilic aspects of human disease (Kuo *et al.*, 2017). Moreover, allergic asthma can be triggered by sensitisation to a variety of allergens (Martorano and Erwin, 2018), including fungal allergens (Denning *et al.*, 2006). Fungal sensitisation is associated with increased asthma severity (Zureik *et al.*, 2002; Medrek *et al.*, 2017). It is critical therefore that *in vivo* models are used to help us understand the regulation of fungal AAI. In this work we have chosen to utilise a repeat *A.f.* exposure model, which drives a mixed Th2/Th17 immune response (Porter *et al.*, 2011). We have further characterised this model to ensure it demonstrates key features of AAI, such as airway remodelling. Regulatory T cells (Tregs), defined by expression of the transcription factor Foxp3, are critical for the regulation of immune responses both in inflammation and during homeostasis (Brunkow *et al.*, 2001). Both heathy and asthmatic individuals have circulating Tregs specific for airborne allergens (aeroallergens), including *A.f.*, however the ratio of aeroallergen specific Treg: T memory cells is reduced in allergic individuals, suggesting an escape from Treg-mediated suppression is important for loss of allergen tolerance (Bacher *et al.*, 2016). Therefore, there is much interest in understanding the role of Tregs in AAI, in order to develop appropriate therapies.

Experimental Treg depletion is a key strategy to understand the role of Tregs in AAI. Tregs express high levels of the IL-2 receptor (CD25), and therefore can be depleted with anti-CD25 antibodies, however this strategy also depletes activated T cells, which upregulate CD25 (Lewkowich *et al.*, 2005; Couper *et al.*, 2009). Therefore, transgenic approaches, targeting Foxp3 expressing cells have been developed (Lahl *et al.*, 2007). Specific depletion of $Foxp3^+$ Tregs in reductionist OVA/adjuvant models has been shown to exacerbate AAI (Baru *et al.*, 2010; Jang *et al.*, 2017). Timing of Treg depletion is critical, with Baru *et al.* showing Tregs dampen inflammation during the sensitisation but not the challenge phase of AAI (Baru *et al.*, 2010, 2012). Utilising anti-CD25 antibodies, Tregs have been depleted in an adjuvant-free aeroallergen (HDM) exposure model of AAI (Lewkowich *et al.*, 2005). Transgenic Foxp3-dependent Treg depletion has not been studied in repeat aeroallergen exposure models, which more faithfully represent the repeated exposure of asthmatics to ubiquitous airway allergens (Martorano and Erwin, 2018). Treg depletion has not been studied in fungal AAI, despite studies showing their importance in dampening Th2 responses in fungal (cryptococcal) lung infection (Schulze *et al.*, 2014). However, individuals with an absence or

dysfunction of Tregs, as a result of immunodysregulation, polyendocrinopathy, enteropathy, X- linked (IPEX) syndrome, experience severe allergy (Bacher and Scheffold, 2018). There is therefore an unmet need to assess the requirement of Tregs to dampen inflammation in relevant models of AAI.

Beyond showing a requirement for Tregs to dampen inflammation, it is critical to understand how Tregs are able to do this. Increasing the number of Tregs at the site of inflammation may allow enhanced Treg-mediated regulation. Tregs expand in lung tissue in models of type-2 inflammation, including fungal airway infection and non-fungal AAI (Baumgart *et al.*, 2006; Taylor, Mohrs and Pearce, 2006; Schulze *et al.*, 2014; Jang *et al.*, 2017). Murine models have shown reducing the number of Tregs exacerbates AAI, whereas increasing Treg number via adoptive Treg transfer dampens AAI (Lewkowich *et al.*, 2005; Baru *et al.*, 2010; Xu *et al.*, 2012; Wiesner *et al.*, 2016). Expansion of the human Treg pool, via adoptive Treg transfer, has not been tested in asthma, however it has been shown to reduce immune pathology in Crohn's and graft versus host disease (GVHD) (Di Ianni *et al.*, 2011; Desreumaux *et al.*, 2012; Arellano, Graber and Sentman, 2016). It is therefore critical to understand how the number of Tregs changes during AAI, as this may be relevant for the ability of Tregs to dampen inflammation.

Knowing the location of Treg expansion is critical to understand Treg function. This is because Tregs in the lung tissue or airways have more access to the site of inflammation, and therefore a likely greater input in regulating AAI than Tregs in the vasculature. $CCR4^{-/-}$ mice, in which Tregs are not able to migrate to and expand in the lung or airways during AAI, show enhanced type-2 inflammation during allergy, displaying the importance of Tregs being at the site of AAI to dampen inflammation (Faustino *et al.*, 2013). Previous efforts to characterise murine lung Tregs have neglected to account for Tregs in the lung vasculature, potentially due to the difficulty of distinguishing cells in vessels vs tissue (Anderson *et al.*, 2012). However, this distinction may be important, as recent studies have shown murine and human "tissue Tregs" to have distinct functional properties at steady state (De Simone *et al.*, 2016; Delacher *et al.*, 2017; Niedzielska *et al.*, 2018; Miragaia *et al.*, 2019). For example, when compared to lymphoid Tregs, at steady state murine "tissue Tregs" have enhanced expression of the cytokine IL-10 (Delacher *et al.*, 2017)

One of the main mechanisms by which Tregs exert their regulatory function is by secretion of IL-10. IL-10 is a regulatory cytokine, produced by a diverse range of cells, including Tregs (Fiorentino, Bond and Mosmann, 1989; Kevin W. Moore *et al.*, 2001; Rubtsov *et al.*, 2008). In HDM asthmatic children, AAI can be abrogated by repeat subcutaneous exposure to HDM, known as specific immunotherapy (IT), and this has been associated with an increase in blood $Foxp3^{+}IL\text{-}10^{+}$ cells, suggesting this cell type may help rebuild tolerance during IT (Wei *et al.*, 2010). In an OVA/alum murine model of airway inflammation, IL-10 expression by Tregs has been shown to be critical for regulation of the allergic response (Rubtsov *et al.*, 2008). To understand which cell types produce IL-10, transcriptional reporter mice such as the VertX $Il10^{GFP}$ strain have been used, which allow observation of IL-10 cytokine mRNA expression

in vivo (Madan *et al.*, 2009). T cells, including Tregs, and macrophages have been shown to express IL-10 during HDM driven AAI (Branchett *et al.*, 2020). However, it is not yet clear whether Treg IL-10 production can be observed during fungal AAI and, if so, what the dominant producer of IL-10 may be in fungal allergy settings. To further assess the functionality of Tregs, it is possible to look at expression of characteristic markers, such as surface proteins CD69 and PD1, which have been associated with Treg suppressive function and survival in a variety of situations – including allergy, colitis and during low-dose IL-2 therapy (McGee *et al.*, 2010; Asano *et al.*, 2017; Yu *et al.*, 2018).

Stability, namely maintenance of Foxp3 expression and suppressive ability without a gain in inflammatory effector activity, is key for effective Treg function (Overacre and Vignali, 2016). Some studies have suggested Tregs are a stable lineage, both in steady state and during Type-1 and autoimmune inflammation (Rubtsov *et al.*, 2010). In contrast, utilising a dual Th2 reporter (*Il4*^{GFP}) and Foxp3 fate reporter (*Foxp3*$^{YFP/Cre}$*R26R*^{FP635}) model, Pelly *et al.* have shown that, during helminth (*Heligmosomoides polygyrus*) induced type-2 inflammation, the number of ex-Tregs (that had lost Foxp3 expression) increased. Ex-Tregs expressed IL-4 and were sufficient to drive parasite expulsion (Pelly *et al.*, 2017). However, the stability of Treg Foxp3 transcription during AAI is not yet clear. Utilising a similar Foxp3 fate reporter in an OVA/alum model, one study found no increase in ex-Tregs in WT mice during AAI (Massoud *et al.*, 2016). The concern is that unstable Tregs may maintain Foxp3 expression, whilst gaining inflammatory effector activity. This has been shown in a model of IL-33 driven AAI, in which Foxp3^{+} Tregs produced Th2 cytokines and contributed to the allergic response (Chen *et al.*, 2017). Additionally, Tregs expressing inflammatory cytokines (IL-17 and IFNγ) have been observed to be increased in asthmatic patients, correlating with disease severity (Xin *et al.*, 2018). The stability of Tregs during AAI is therefore still unclear, and this knowledge is critical to enable informed development of Treg therapeutics.

There is an unmet need to understand Treg function in AAI, particularly fungal AAI. In this chapter we utilised a translatable model of *A.f.* induced AAI to address this. We identified expansion of lung tissue Tregs expressing the regulatory cytokine IL-10, and activation markers CD69 and PD1 during fungal AAI. Importantly, depletion of Tregs resulted in more severe type-2 inflammation. We found no evidence for instability of Foxp3 expression by Tregs during AAI. However, a small proportion of Tregs showed potential signs of dysfunction, with increased IL-4 expression. Finally, we utilised *in vitro* assays to better understand the intrinsic suppressive capability of Tregs during fungal AAI, finding that IL-4 expression was associated with reduced suppressive function. Together this shows for the first time, the critical role of Tregs in fungal AAI, which may be relevant for designing therapeutics.

3.1.2 Key questions

The hypothesis underlying the work in this chapter was: Tregs are critical for regulation of *A.f.* driven AAI, but that over the course of inflammation Treg phenotype changes, showing signs of instability.

My specific Aims relating to this hypothesis were to use a relevant model of fungal AAI to ask:

1. How does the location and number of Tregs change in fungal AAI?
2. Do Tregs modify expression of functional markers, including IL-10, during fungal AAI?
3. Are Tregs required to suppress immune responses and inflammation in fungal AAI?
4. Do Tregs display instability during fungal AAI?

3.2. Results

3.2.1 Repeat exposure to live *A.f.* spores induces a mixed Th2/Th17 AAI response, and airway remodelling

Before investigating the role of Tregs, we first defined general features of AAI in a previously described model of fungal (*A.f.*) exposure (Porter *et al.*, 2011). Mice were dosed *i.n.* with *A.f.* spores or PBS, and tissues harvested at d12 or d19 post initial dose (Porter *et al.*, 2011). As expected, repeat *A.f.* exposure led to a significant increase in expression of IL-13 and IL-17 by lung $CD4^+$ $Foxp3^-$ T cells at d12 and d19, compared to PBS treated mice (Fig. 3.1A). This was seen both as a percent of $CD4^+$ $Foxp3^-$ T cells, as well as by cell number (Fig. 3.1.A). The proportion of cells expressing IL-17 increased significantly between d12 and d19, whereas there was no corresponding increase in IL-13 cells between d12 and d19, potentially suggesting the Th2 response had plateaued at d12 whereas the Th17 response continued to increase. Other Th2 cytokines (IL-4 and IL-5) followed a similar trajectory to IL-13, whereas the Th1 cytokine IFNγ did not increase (data not shown), all cytokines were measured using PMA/ionomycin stimulation of lung cell isolates. Although similar cytokine expression patterns have been seen previously (Porter *et al.*, 2011), this extends this work by identifying $CD4^+$ T cells as the source of these cytokines. One of the consequences of type-2 and type-17 inflammatory responses during AAI is influxes of eosinophils (type-2) and/or neutrophils (type-17) to the airways of patients, characteristic of asthmatic patient endotypes (Wood *et al.*, 2012; Peters *et al.*, 2014). Repeat *A.f.* exposure led to a significant increase in BAL eosinophils ($SiglecF^+$ $MerTK^-$) at d12 and d19, and neutrophils ($Ly6G^+$ $CD11b^+$) at d12, comparing to PBS treated mice. This was seen both as a percent of total BAL leukocytes ($CD45^+$), as well as by cell number (Fig. 3.1.B). Airway eosinophils were more dominant than neutrophils at d12 and d19 of *A.f.* driven AAI (Fig. 3.1.B). Together, this shows an overall mixed type-2/type-17 immune response to *A.f.*, with an early wave of neutrophilia overtaken by a more dominant eosinophilic airway phenotype.

In addition to inflammation, human AAI is characterised by extensive changes in lung architecture and pathology. Therefore, we next assessed *A.f.* driven airway remodelling, this time extending fungal exposure up to d26, to be able to assess tissue changes more characteristic of chronic AAI, such as fibrosis. First, lungs were stained with H&E and airway epithelial thickness measured, a readout that correlates with human asthma severity (Cohen *et al.*, 2007). Compared to PBS treated mice, airway epithelial thickness increased from d12, and remained significantly increased up to d26 of *A.f.* induced AAI (Fig. 3.2.A). Next, we used AB-PAS staining to measure an increase in airway goblet cell hyperplasia, which is observed in asthmatic patients, and contributes to excess airway mucus and therefore luminal occlusion (Kuyper *et al.*, 2003). Goblet cell hyperplasia followed similar kinetics to epithelial changes, significantly increased from d19 and remaining elevated up to d26 (Fig. 3.2.B). Finally, we used Masson's trichrome staining to quantify lung fibrosis, which is observed in asthmatics and correlates with disease severity (Chetta *et al.*, 1997). Fibrosis was mainly observed around medium/large airways and blood vessels, with the proportion of lung tissue stained for collagen increasing significantly by d26 (Fig. 3.2.C). Overall, this shows that *A.f.* induced

AAI consists of a mixed type-2/type-17 immune response and airway remodelling, with epithelial thickening, goblet cell hyperplasia and fibrosis all reminiscent of features of human asthma.

3.2.2. Lung Tregs expand during *A.f.* induced AAI

Having established the basic features of the live spore exposure *A.f.* model of AAI, we moved on to investigate the role of Tregs in this system. To begin, we assessed the changes in Treg number and frequency at the site of AAI. The number of Tregs increased in the lung, lung dLN and BAL during *A.f.* driven AAI, at both d12 and d19, compared to PBS mice (Fig 3.3.A-C). There was also an increase in Tregs as a proportion of $CD4^+$ T cells at d19 in the lung, but not in the lung dLN or BAL (Fig 3.3.A-C).

As we observed both proportional and numerical increases in Tregs in the lung, we next investigated Treg changes more closely in this tissue. Recently published work has demonstrated that lung cell isolates may be derived from the vasculature, tissue, or airways (Anderson *et al.*, 2012). By tissue, we are referring to all regions of the lung not directly adjacent to circulating blood or airflow, further distinguishing locations within the tissue is not possible with this technique. To determine the relative change in Tregs in each of these compartments, mice were injected *i.v.* and *i.t.* with different fluorophore conjugated anti-CD45 antibodies, labelling vascular vs airway cells (Fig. 3.4.A). Upon *A.f.* exposure, Tregs in the lung tissue or airways increased from an average of 34% (PBS) to 50% at d12 and 66% at d19, with a corresponding decrease in the percentage of Tregs in the blood at these time points (Fig. 3.4.A). Proportionally, there were significant increases in airway Tregs at d12 and d19, when compared to PBS or d12, and a significant decrease in blood Tregs at d19 when compared to PBS or d12 (Fig. 3.4.A). By cell number, there was a significant increase in Tregs in the lung tissue and airways at d12 and d19, compared to PBS (Fig. 3.4.A). Specifically, a 5.4-fold increase in Treg number in the lung tissue and airways was observed at d19 of *A.f.* treatment, compared to PBS. Without excluding blood Tregs the fold increase was only 1.9-fold (Fig. 3.3&4), highlighting the importance of this discrimination. The number of Tregs in the blood did not significantly change in response to *A.f.* driven AAI (Fig. 3.4.A). Overall, this shows that the expansion of Tregs in the lung is specifically due to increases in the tissue and airways, the site of AAI.

Recent work has suggested that tissue Tregs show specific functional characteristics, and can be identified by expression of the marker KLRG1, and the IL-33R (ST2) (Delacher *et al.*, 2017). Notably previous studies have not utilised intravascular staining to verify tissue location of $KLRG1^+ST2^+$ “tissue Tregs” (Delacher *et al.*, 2017). We observed an increase in “tissue Tregs”, both as cell number and as a proportion of total Tregs, at d12 and d19 of *A.f.* induced AAI (Fig.3.4.B). In line with total Tregs, there was a significant increase in “tissue Tregs” by cell number in the lung tissue and airways, but not blood, at d12 and d19, compared to PBS (Fig. 3.4.B). In PBS treated mice 48% of $KLRG1^+ST2^+$ “tissue Tregs” were in the lung tissue or airways however upon *A.f.* driven AAI this increased to 72% at d12 and 87% at d19, with a corresponding decrease in the percentage of Tregs in the blood at these time points

(Fig. 3.4.B). KLRG1^{+}ST2^{+} “tissue Tregs” were therefore enriched in the tissue, although not uniquely found there. Overall, these data suggest there is a specific expansion of tissue Tregs during *A.f.* induced AAI.

3.2.3. Lung Tregs express activation markers and the regulatory cytokine IL-10 during *A.f.* induced AAI.

Although Treg production of IL-10 has been shown to be vital for their suppressive function in an OVA/alum model of AAI (Rubtsov *et al.*, 2008), it is not known whether this is also the case for fungal AAI. To address this, we utilised VertX IL-10 transcriptional reporter mice (Madan *et al.*, 2009), and found that Treg IL-10 expression increased significantly at d19 of *A.f.* exposure (Fig. 3.5.A). The fact that we noted lung Treg expansion earlier than d19 (Fig. 3.3) might suggest an enhancement of Treg suppressive function between d12 and d19. IL-10 was also expressed by CD4^{+}Foxp3^{-} T cells, with a significant increase in number at d12 and proportion at d19 (Fig. 3.5.B). Comparing these populations, a higher proportion of Tregs expressed IL-10 than CD4^{+} Foxp3^{-} cells at d19 (mean 9.2% vs 2.5%). However, due to the greater number of CD4^{+}Foxp3^{-} T cells, overall there were fewer IL-10 producing Tregs than CD4^{+}Foxp3^{-} T cells at d19 (mean 0.003 vs 0.008x10^{4}/mg lung) (Fig. 3.5.B). Irrespective, increasing Treg IL-10 production over time suggests this could be a mechanism that they use to suppress *A.f.* driven AAI. Compared to IL-10^{-} cells, both IL-10^{+} Tregs, and IL-10^{+} CD4^{+}Foxp3^{-} T cells were significantly enriched in the airways and the tissue, and reduced in the blood, suggesting IL-10 expression is concentrated at the site of AAI (Fig. 3.5.B&C).

Following our identification of enhanced Treg IL-10 expression, we wanted to look at other activation markers, to assess if these too suggested an increase in Treg functionality by d19 of *A.f.* induced AAI. To do this we looked at co-expression of the activation markers CD69 and PD1 (Agata *et al.*, 1996; McGee *et al.*, 2010; Asano *et al.*, 2017; Yu *et al.*, 2018). In line with IL-10 expression, there was a significant increase in the number and proportion of activated CD69^{+}PD1^{+} Tregs at d19, but not d12, of *A.f.* treatment (Fig. 3.5.C). Activated CD69^{+}PD1^{+} Tregs were significantly enriched in the airways and the tissue and reduced in the blood (Fig. 3.5.C). We next wanted to directly assess whether IL-10 was co-expressed with CD69^{+}PD1^{+}. Compared to IL-10^{-} cells, a significantly higher proportion of IL-10^{+} cells were CD69^{+}PD1^{+} (Fig. 3.5.D). Taken together, these data suggest that pulmonary Tregs increased from d12, and had enhanced regulatory function by d19, of *A.f.* driven AAI.

3.2.4 Tregs are required to regulate Th2 responses

Having shown that Tregs expanded in lung tissue, and expressed the regulatory cytokine IL-10, we next wanted to know if they were required to subdue *A.f.* induced type-2/type-17 AAI. We utilised the DEREG mouse strain, which has a bacterial artificial chromosome (BAC) transgene in which *DTr-eGFP* is transcribed under control of the Foxp3 promoter, leading to inducible depletion of Tregs upon DTx administration (Lahl *et al.*, 2009). As long-term depletion is not feasible in this model, due to expansion of BAC transgene-negative (Foxp3^{GFP-}) Tregs (Lahl and Sparwasser, 2011), we chose to deplete for 7 days, between d12 and d19 of *A.f.* treatment. This timing was chosen as it corresponds with when Tregs appear

to gain enhanced suppressive function, increasing IL-10 expression (Fig. 3.5). Lungs were taken at d19, at which point ~65% of lung Foxp3^{+} Tregs, and 96% of transgene-positive Foxp3^{GFP+} cells were depleted in DEREG mice (Fig. 3.6.A&B). Although we observed outgrowth of transgene-negative (Foxp3^{GFP-}) Tregs, this was only partial, with almost complete ablation of transgene-positive Foxp3^{GFP+} Tregs. This is in line with published data, which has shown outgrowth of transgene-negative (Foxp3^{GFP-}) Tregs, that have been shown to be functionally impaired (Lahl and Sparwasser, 2011)

Next, we wanted to assess how Treg depletion affected the inflammation and immunopathology during *A.f.* driven AAI. In Treg depleted DEREG mice a clear increase in expression of lung Th2 (IL-4, IL-5, IL-13) and Th1 (IFNγ) cytokines by CD4^{+} Foxp3^{-} cells was observed, when compared to WT (DTx treated) controls. This was seen by both cell number and proportion in the lung and lung dLN (Fig. 3.6.C). Notably, there was no increase in the proportion of IL-17^{+} CD4^{+} Foxp3^{-} cells in the lung or lung dLN, or in the absolute number of IL-17^{+} CD4^{+} Foxp3^{-} cells in the lung dLN. There was a slight increase in the absolute number of IL-17^{+} CD4^{+} Foxp3^{-} cells in the lung (Fig. 3.6.C). Overall, this suggests that Tregs are more involved in regulating Th2/Th1 cells, than Th17 cells during AAI. Neutrophils and eosinophils have been classically associated with type-17 and type-2 responses, respectively. We therefore wanted to see if the contrasting impact of Treg depletion on Th2 and Th17 responses was also evident in terms of eosinophilia and neutrophilia. Surprisingly, there was an increase in both eosinophilia and neutrophilia in the BAL of DEREG mice, suggesting an uncoupling of Th17 cells and neutrophils in the pulmonary anti-fungal type-17 response (Fig. 3.6.D). Finally, we assessed the overall impact of Treg depletion on lung pathology, identifying an increase in overall lung inflammation in DEREG mice, as determined by histological analysis of lung tissue. This was seen throughout the lung parenchyma, and surrounding airways (Fig. 3.6.E). Overall, these data reveal that Tregs are critical to restrict type-2 inflammation during *A.f.* driven AAI, whilst being less important for controlling the Th17 inflammatory response.

3.2.5 The majority of Tregs are stable during *A.f.* driven AAI

Although we had found Tregs were required to reduce the severity of *A.f.* driven AAI, there was still the possibility that a subset of Tregs could display instability, namely a gain in inflammatory function (Overacre and Vignali, 2016). To assess this, we used a Treg fate reporter mouse (*Il4^{GFP}Foxp3$^{YFP/Cre}$R26R^{FP635}*) (Pelly *et al.*, 2017). In this model, cells that have ever expressed Foxp3 will express the fate reporter FP635 ("Foxp3^{fate+}"), any cells that are currently expressing Foxp3 will be Foxp3^{YFP+}, and any cells that are currently expressing IL-4 will be IL-4^{GFP+} (Fig. 3.7.A). As we anticipated that instability may be more apparent later in the inflammatory response to *A.f.*, we extended this experiment up to d26. Compared to PBS treated mice, during *A.f* driven AAI there was a significant reduction in the proportion of IL-4 expressing Th2 cells that had previously expressed Foxp3 (Th2 exTregs; Foxp3^{fate+} IL-4^{GFP+} Foxp3^{YFP-}) in the lung or lung dLN (Fig. 3.7.B). Although caution should be taken as to whether this decrease is biologically relevant, given the small number of events and the higher baseline proportional expression of Foxp3^{fate} in the PBS group. Notably, comparing

A.f. treated groups, there was no significant change in the proportion of Th2 exTregs in the lung or dLN as inflammation increased from d12 to d26. Looking at absolute numbers, there was a slight but significant increase in Th2 exTregs in the lung at d12, and the lung dLN at d26, when compared to PBS (Fig. 3.7.B). This increase could be due to Tregs downregulating Foxp3 expression, and gaining Th2 effector function, or due to the proliferation of existing exTregs. Overall, this strongly suggests that majority of exTregs do not gain Th2 effector function during *A.f.* driven AAI.

To assess whether active Tregs ($Foxp3^{YFP+}Foxp3^{fate+}$) may become unstable over time, gaining effector function whilst maintaining Foxp3 expression, we utilised the $IL\text{-}4^{GFP}$ reporter in this mouse model to determine if Tregs contributed to the Th2 response. There was a small but significant increase in $IL\text{-}4^{+}$ Tregs in the lung and lung dLN from d19 of *A.f.* exposure, seen as a proportion of Tregs and as absolute cell number (Fig. 3.7.C). By d26 a mean of 7.5% of lung, and 9.5% of lung dLN Tregs expressed IL-4 mRNA (Fig.3.7.C). To summarise, although these data provide no evidence that Tregs downregulated Foxp3 during *A.f.* driven AAI, a small proportion of Tregs upregulated IL-4 during *A.f.* driven AAI.

At this point, we had elucidated that during *A.f* .driven AAI, a proportion of Tregs had increased expression of markers associated with enhanced suppressive function (IL-10), as well as instability (IL-4). It was not clear how these markers related to the intrinsic suppressive capacity of Tregs. To better understand this, we sorted the following lung and lung dLN Treg populations: $IL\text{-}4^{+}$ Tregs from d19 *A.f.* exposed mice; $IL\text{-}4^{-}$ Tregs from d19 *A.f.* exposed mice; and $IL\text{-}4^{-}$ Tregs from PBS mice. These different Treg populations were cultured *ex vivo* with $CD4^{+}Foxp3^{-}$ T responder ("Tresp") cells, from d19 *A.f.* treated mice, at varying ratios. Tresp cells were stained with a proliferation dye (CTV) and suppression was measured as the ability to inhibit Tresp proliferation (Fig 3.8.A). To control for well-to-well variation, numbers of proliferated cells were normalised to the well with the maximal Tresp proliferation. All sorted Tregs were able to supress T cell proliferation at the 1:1 Tresp: Treg ratio (Fig.3.8.B&C). Despite the phenotypic changes in lung Tregs observed *in vivo* (Fig. 3.4&5) there was no significant change in intrinsic suppressive capacity of lung Tregs at any Treg: Tresp ratios (Fig. 3.8.C). However, in the lung dLN $IL\text{-}4^{+}$ *A.f.* Tregs at the 4:1 ratio had significantly reduced suppressive capacity than $IL\text{-}4^{-}$ *A.f.* Tregs and PBS Tregs (Fig 3.8.B). No significant difference in suppressive capacity was observed between $IL\text{-}4^{-}$ *A.f.* Tregs and PBS Tregs in either tissue at any ratio (Fig. 3.8.B&C). Together, these data suggest that Treg IL-4 expression in the context of *A.f.* AAI can correlate with a decline in suppressive function and may therefore be a sign of functional instability.

3.2.6 Summary

- *A.f.* driven AAI induces a mixed Th2/Th17 response, with pronounced airway eosinophilia, neutrophilia and extensive airway remodelling (Figs. 3.1&2)
- During *A.f.* driven AAI Tregs expand in the lung, lung dLN and BAL (Fig. 3.3)
- During *A.f.* driven AAI there is an increase in lung tissue Tregs (Fig. 3.4)
- During *A.f.* driven AAI Tregs increase expression of the regulatory cytokine IL-10, as well as markers associated with activation (Fig. 3.5)
- Treg depletion during *A.f.* driven AAI leads to increased lung inflammation, airway eosinophilia, neutrophilia and Th2 cytokine production (Fig. 3.6)
- During *A.f.* driven AAI there is no evidence of downregulation of Treg Foxp3 expression, but a small proportion of Tregs express the Th2 cytokine IL-4 (Fig. 3.7)
- IL-4 expression during *A.f.* driven AAI can be associated with significantly reduced intrinsic suppressive capacity (Fig. 3.8)

3.3. Discussion

In this chapter, we have elucidated the role of Tregs in a relevant model of fungal AAI. Building from basic cell number measurements, we have utilised a number of strategies to elucidate Treg function. We have revealed a requirement for Tregs in controlling Th2/Th17 aspects of fungal AAI, although notably not Th17. Our identification of increased IL-10 expression by Tregs during fungal AAI provides a potential suppressive mechanism. Through use of *in vitro* suppression assays, we have shown that the majority of Tregs retain suppressive capacity during AAI. Further, although we found no evidence for downregulation of Foxp3 in Tregs during fungal AAI, functional instability of a subset of $Foxp3^+$ Tregs was observed – with an increase in IL-4 mRNA expression associated with their reduced suppressive capacity. Taken together these novel data reveal a critical role for Tregs in suppressing pulmonary inflammation during fungal AAI, despite signs of some functional instability.

3.3.1 Treg requirement and dynamics during fungal AAI

Throughout this chapter we have utilised a murine model of fungal AAI to dissect the role of T regulatory cells. Evaluating this model is critical to assess how translatable it is to human disease, and therefore the relevance of our discoveries using this system. The allergen we chose in this study was *A.f.*, a ubiquitous and clinically relevant fungal airborne allergen (Maurya *et al.*, 2005; Agarwal *et al.*, 2011; Alshareef and Robson, 2014). Repeat intranasal exposure of *A.f.* has been previously shown to promote mixed Th2/Th17 responses in mice (Porter *et al.*, 2011).

We have confirmed that repeat intranasal exposure of *A.f.* drives a mixed Th2/Th17 response, with early airway neutrophilia followed by dominant eosinophilia (Fig.3.1). This is reminiscent of what is observed in humans - Th2 cytokines have classically been associated with promoting the allergic immune response, while Th17 cells have more recently been implicated in asthma pathogenesis (Grunewald *et al.*, 1998; Cosmi *et al.*, 2010; Cosmi, Liotta and Annunziato, 2016). Moreover, asthmatic individuals may show eosinophilic, neutrophilic or mixed sputum phenotypes (Svenningsen and Nair, 2017).

Prior to the work described in this Chapter, it was unclear how tissue remodelling develops in *A.f.* driven AAI, even though this would be critical to assess translational relevance. We found that repeat *A.f.* exposure promoted early (d12) epithelial thickening, followed by goblet cell hyperplasia (d19) and lung fibrosis (d26) (Fig.3.2). Increased epithelial thickness, goblet cell hyperplasia and fibrosis are all features of human asthma (Cutz, Levison and Cooper, 1978; Cohen *et al.*, 2007). Moreover, these features are linked to disease severity, for instance increased airway mucus as a result of goblet cell hyperplasia can occlude airways (Kuyper *et al.*, 2003). These features have been observed other AAI models (Temelkovski *et al.*, 1998; Johnson *et al.*, 2004; Chen *et al.*, 2009; Piyadasa *et al.*, 2016; Lemessurier *et al.*, 2018; Woo *et al.*, 2018). To further assess the translational relevance of this model it will be necessary to study lung function and airway hyperresponsiveness, key features of human asthma (Lougheed *et al.*, 2012), this could be done via plethysmography (Lundblad, 2012; Walker, Kraft and Fisher, 2013)

Having established that repeat exposure to *A.f.* generates a relevant model of fungal AAI, we wanted to understand how it was regulated. Due to their central importance in regulating diverse inflammatory conditions, we focused on Tregs (Baru *et al.*, 2010; Smith *et al.*, 2016). A key initial step to understand the role of Tregs in fungal AAI was to track their change in number as inflammation developed. We observed an expansion of pulmonary Tregs during AAI, as has been observed previously in Cockroach Ag/alum asthma model (Jang *et al.*, 2017), and in fungal (cryptococcal) lung infection (Schulze *et al.*, 2014). This was matched by an expansion of $Foxp3^{-}$ $CD4^{+}$ T cells in the dLN and BAL (Fig. 3.3.). In the lungs however, an observed increase in Tregs as a percentage of $CD4^{+}$ T cells implied a shift to a more regulatory $CD4^{+}$ T cell pool (Fig. 3.3.A.). Selective expansion of tissue Tregs suggested a key role for this cell type in the lung, a prospect that we went on to directly address using a Treg depletion strategy.

Experimental Treg depletion has been used to demonstrate a requirement for Tregs to dampen inflammation in differing airway inflammation models, including OVA/alum driven AAI, and fungal (cryptococcal) infection (Baru *et al.*, 2010; Schulze *et al.*, 2014; Jang *et al.*, 2017). However, these past studies relied on anti-CD25 depletion, which targets not only Tregs, but CD25 expressing effector T cells (Couper *et al.*, 2009). More refined transgenic Treg depletion models having not yet been used in fungal or other repeat airway exposure models of AAI, which better reflect the recurrent exposure of asthmatics to aeroallergens (Martorano and Erwin, 2018). Utilising an inducible depletion model (DEREG) (Lahl *et al.*, 2007), we observed increased type-2 inflammation, elevated overall lung pathology and increased numbers of inflammatory granulocytes (eosinophils and neutrophils) in the airways of Treg depleted mice during fungal AAI (Fig. 3.6.). Using this approach we have demonstrated, for the first time, that Tregs are required to reduce the severity of fungal AAI. Despite a partial rebound of transgene-negative (GFP^{-}) Tregs (Fig. 3.6.A), we observed a striking increase in inflammation post depletion, supporting published work showing transgene-negative (GFP^{-}) Tregs to be functionally impaired (Lahl and Sparwasser, 2011). It could be argued that total Treg depletion is a blunt tool to understand Treg function. A more refined approach could be to use intranasal DTx, allowing depletion of Tregs just at the effector site, thereby reducing any systemic effects of Treg depletion (Roberts *et al.*, 2015). Alternatively, deeper understanding of specific Treg requirements could be investigated by depleting particular Treg “subsets”, for example peripheral vs thymic Tregs (Josefowicz *et al.*, 2012). Contrastingly, adoptive transfer or therapeutic expansion of Tregs could provide a complementary approach to depletion to investigate the importance of Tregs during fungal AAI. This strategy has been used in a model of fungal airway infection where Tregs, therapeutically expanded by IL-2 complex injection, reduced Th2 cell cytokine expression and mucus production (Schulze *et al.*, 2016).

Overall, we have revealed that Tregs expand and are required to limit pulmonary inflammation using a relevant model of fungal AAI, results that now raise a number of questions. Notably, a proportional increase in Tregs was only observed in the lung (Fig. 3.3.A). What is the impact of lung tissue location on Treg function? Moreover, beyond broad depletion strategies, how

can we best define Treg subsets and functionality during fungal AAI? Although we have shown that Tregs are generally stable and required to limit inflammation during developing AAI, would this be the same in more chronic stages of AAI?

3.3.2 Tissue Tregs – the importance of location

As Tregs will likely be selectively recruited to, or expanded at, sites where they are required during an ongoing immune response, defining Treg location may help better understand their function. Furthermore, the lung tissue environment has been shown to dramatically affect function of immune cells, including macrophages (Svedberg *et al.*, 2019; Szabo *et al.*, 2019). Several recent papers have also shown Tregs adapt to their non-lymphoid tissue environment (including the gut, skin, fat and lung), expressing "tissue Treg" characteristic markers such as ST2, KLRG1, Gata3 and IL-10 (Feuerer *et al.*, 2009; Delacher *et al.*, 2017; Miragaia *et al.*, 2019). However, identifying tissue-resident cells in highly vascularised tissues can be a challenge, as tissue responses may be obscured by recirculating cells.

Intravascular and intratracheal staining is an effective way to differentiate cellular location of lung cell populations (Patel *et al.*, 2015; Svedberg *et al.*, 2019) and more accurate than perfusion, which damages the delicate vasculature of the lung (Anderson *et al.*, 2014). Notably, this technique does not allow for more precise anatomical location of the cells within the lung parenchyma, including all cells that are not in direct contact with circulating blood or airflow. Utilising intravascular staining was critical to identify the location of Tregs in the lung during AAI (tissue and airways) (Fig. 3.4.A). Furthermore, we found an increase in lung tissue Tregs expressing characteristic "tissue Treg" markers ST2 and KLRG1, notably although these cells were enriched in the tissue, they were also present in the vasculature (Fig. 3.4.B). Intravascular staining has not been used previously to validate they use of ST2 and KLRG1 to demarcate lung tissue Tregs, and our work suggests that $ST2^{+}KLRG1^{+}$ lung "tissue Tregs" may in fact include cells that are either in, or in contact with the vasculature. Further research into the precise anatomical location of these cells within the tissue is needed.

$ST2^{+}KLRG1^{+}$ Tregs have a characteristic transcriptional profile, although their roles in inflammation are unclear. Recent studies have shown that at steady state $ST2^{+}KLRG1^{+}$ lung and fat tissue Tregs express a mixed markers characteristic of Th2 cells, including Gata-3, and ST2 (Zheng and Flavell, 1997; Tibbitt *et al.*, 2019), as well as regulatory/repair markers IL-10 and AREG (Arpaia *et al.*, 2015; Delacher *et al.*, 2017) Conversion of lymphoid Tregs to a tissue Treg phenotype is observed *in vitro* upon IL-4 treatment, suggestive that these cells are associated with type-2 inflammation (Delacher *et al.*, 2017). The IL-33 receptor ST2 has been shown to be upregulated in response to the alarmin IL-33 in GvHD, lung injury and AAI (Matta *et al.*, 2014, 2016; Schiering *et al.*, 2014; Chen *et al.*, 2017; Liu *et al.*, 2019) $ST2^{+}$ Tregs can drive type-2 responses, expressing IL-13 post lung injury, and during IL-33 driven AAI, notably post lung injury Treg IL-13 was required to inhibited inflammatory cytokine (IL-6/G-CSF) production (Chen *et al.*, 2017; Liu *et al.*, 2019).

3.3.3 Assessing Treg function

Although the work described in this chapter has revealed that Tregs play a key role in restricting pulmonary inflammation during fungal AAI, the question remains of how, specifically, they may be doing this.

While we saw a dramatic increase in Th1 and Th2 cytokines, eosinophilia and neutrophilia following Treg depletion during fungal AAI, we found that $CD4^+$ T cell IL-17 was less dependent on Treg control (Fig.3.6.C.). This has been observed before, utilising DEREG mice in a model of cryptococcal lung infection, where no increase in IL-17 production was evident following Treg depletion (Schulze *et al.*, 2014). A more recent study, again using DEREG mice, depleted Tregs in a neutrophilic/Th17 dominated AAI model induced by intranasal challenge with Cockroach Ag (CA) after sensitisation with CA/Complete Freund's Adjuvant. Again, Treg depletion in this model did not affect $CD4^+$ T cell IL-17 production (Jang *et al.*, 2017). In contrast, Tregs have been shown to be able to inhibit Th17 cells in other situations. For example, Treg depletion in an eosinophilic/Th2 dominant AAI model induced by CA/alum sensitisation increased the number of IL-17 producing T cells (Jang *et al.*, 2017). Colitis studies have also supported the role of Tregs in inhibiting IL-17 production by T cells (Chaudhry *et al.*, 2009; Huber *et al.*, 2011; Joller *et al.*, 2014). It is unclear why Tregs seem to be important for regulating Th17 responses in some models, but not others. Utilising *in vitro* suppression assays, to compare Treg ability to suppress IL-17 production would provide evidence for a Treg intrinsic inability to regulate IL-17 responses.

Both Tregs and $Foxp3^-$ $CD4^+$ T cells have previously been shown to express IL-10, which is critical for regulation of multiple aspects of the immune response (Saraiva and O'Garra, 2010). Treg-specific IL-10 has been shown to be required to limit inflammation in an OVA/alum model of AAI (Rubtsov *et al.*, 2008). Here, we have shown Tregs and $Foxp3^-$ $CD4^+$ T cells make IL-10 during *A.f.* driven AAI. The observed increase in Treg IL-10 was coincident with an increase in expression of the activation markers CD69 and PD1 (Fig. 3.4). Although IL-10 expression is characteristic of the "tissue Treg" phenotype, we observed lung-specific Treg expansion earlier than Treg IL-10, which did not increase until d19, suggesting there is another mechanism regulating IL-10 expression. CD69 and PD1 were both originally described as markers of T cell activation (Lanier *et al.*, 1988; Agata *et al.*, 1996). More recently PD1/PDL-1 interactions have been shown to regulate the survival and function of Tregs (Francisco *et al.*, 2009) and CD69 expression has been associated with increased suppressive potential (Cortés *et al.*, 2014; Yu *et al.*, 2018). The timing of IL-10 and CD69 expression we have observed fits with published work, which has shown that Treg CD69 overexpression increased the expression of IL-10 (Yu *et al.*, 2018). Taken together, this suggests Treg activation is required for IL-10 expression. Moreover, when assessing IL-10 expression is not possible, our work suggests CD69 and PD1 co-expression may be used to identify activated tissue Tregs with enhanced IL-10 production potential. As for the function of IL-10, it has been shown to act directly on Th2 cells, limiting T cell survival during AAI (Wilson *et al.*, 2011). Notably, this must be tightly regulated in the case of fungal AAI, as individuals with genetic IL-10 overexpression have reduced antifungal immunity, predisposing

them to invasive aspergillosis (Cunha *et al.*, 2017). One limitation of our data on IL-10 is that it is wholly observational. The importance of IL-10 in *A.f.* induced AAI could be further investigated by using mice with Treg specific IL-10 deficiency (*Foxp3*^{cre} *Il10*$^{fl/fl}$) (Rubtsov *et al.*, 2008).

How the intrinsic suppressive capacity of Tregs changes during AAI is not known. To directly address this, we sorted lung or lung dLN Tregs *ex vivo* from PBS or *A.f.* exposed mice and performed an *in vitro* Treg suppression assay. We found no difference in Treg suppressive ability when comparing IL-4^{-} Tregs from PBS or *A.f.* treated mice (Fig.3.8). This is intriguing, as there were clear phenotypic signs of increased regulatory potential during *A.f.* driven AAI (Fig. 3.4). One explanation could be Treg-extrinsic factors, in that it is not possible to recapitulate all aspects of the lung environment in an *in vitro* suppression assay. For instance, tissue factors such as TGFβ, which is found in abundance in the lung, could be required to confer full suppressive ability on Tregs (Worthington *et al.*, 2015; Flanders *et al.*, 2016). Future work analysing regulatory cytokines in the supernatants of Treg suppression assays could provide evidence to support this hypothesis. It may also be possible to test Treg suppressive potential *in vivo*, by adoptively transferring Tregs from mice with *A.f.* driven AAI, or PBS treated controls, into mice with *A.f.* driven AAI. Isolating sufficient lung Tregs to transfer would be a key technical difficulty, we are able to sort 1x10^4 lung tissue Tregs per PBS treated mouse. Previous studies have administered 5x10^4 Tregs *i.n.* or 5x10^6 Tregs *i.v.* or *i.p.* to ameliorate AAI, which would require sorting Tregs from 5-500 mice per recipient (Leech *et al.*, 2007; Xu *et al.*, 2012; Faustino *et al.*, 2013; Gollwitzer *et al.*, 2014)

Taken together, we have utilised a variety of techniques to assess Treg function, finding a specific role for Tregs in inhibiting Th2/Th1 but not Th17 responses, and with Tregs from *A.f.* treated mice displaying elevated expression of IL-10 and markers of regulatory function. Surprisingly, however, these hallmarks of increased Treg suppressive function did not correlate with enhanced *in vitro* intrinsic suppressive capability.

3.3.4 Treg stability

On balance, we have shown that Tregs are important for dampening *A.f.* induced AAI. However, this does not preclude a small proportion of Tregs becoming unstable, either by down-regulating Foxp3 expression, or gaining inflammatory effector function whilst maintaining Foxp3 expression. This potential instability is obviously an important issue to consider, given widespread interest in Treg induction as a possible therapeutic approach in immune-mediated disease settings, including AAI (Thorburn and Hansbro, 2010; Baatjes *et al.*, 2015; Ye, Brand and Zheng, 2018).

We found an increase in IL-4 mRNA expression by active Tregs in the lung and lung dLN during *A.f.* driven AAI (Fig. 3.7.C). Although low-level IL-4 expression has been observed previously in WT Tregs during HDM driven AAI (Massoud *et al.*, 2016; Tibbitt *et al.*, 2019). The function of this IL-4 is unclear. Utilising a murine model of food allergy with a genetic IL-4R variant, Rivas *et al.* showed that Tregs could make IL-4 protein, and that specific deletion of IL-4 in Tregs diminished the allergic response (Rivas *et al.*, 2015). Beyond this transgenic

model, the role of IL-4 in Tregs has not yet been studied. Although Tregs expressed IL-4 transcript in our fungal AAI model, it remains possible that they are not in fact producing or secreting IL-4 protein. Transcript production could be confirmed on sorted Tregs by qPCR, while cytokine production could be assayed by the use of IL-4 protein-secretion reporter mice (Mohrs *et al.*, 2005). Alternatively, IL-4 protein could be assessed via intracellular cytokine staining and flow cytometry, or by ELISA on supernatants from *ex vivo* cultured cells. The importance of IL-4 expression could be assessed in our model via *Foxp3*^{cre} *IL4/13*$^{fl/fl}$ mice (Voehringer *et al.*, 2009; Rubtsov *et al.*, 2010; Massoud *et al.*, 2016). It is also possible that the IL-4$^+$ Foxp3$^+$ population could be derived from IL-4$^+$ Th2 cells upregulating Foxp3 expression. An ideal approach to study this would be to transgenic fate marking the IL-4$^+$ Foxp3$^-$ Th2 cells, this approach would require identification of a gene specifically expressed in IL-4$^+$ Foxp3$^-$ cells, and not in IL-4$^+$ Tregs, which would be a challenge due to the overlap in markers (Tibbitt *et al.*, 2019). Th2 to Treg conversion is possible *in vitro*, but has not been shown to occur naturally *in vivo* (Kim *et al.*, 2010).

The approach that we took to investigate the function of IL-4 in Tregs was to isolate IL-4$^+$ Tregs *ex vivo* and assay their suppressive activity *in vitro*. IL-4$^+$ Tregs from the lung dLN, but not the lung, showed significantly reduced suppressive capacity when compared to IL-4$^-$ Tregs (Fig. 3.8.B). To our knowledge, this is the first demonstration of the impact of IL-4 expression on Treg function. Our results support a model in which a small proportion of Tregs become functionally unstable during *A.f.* driven AAI. IL-4$^+$ Tregs may have reduced suppressive capacity because of IL-4 expression, or because their expression of IL-4 is a sign of dysfunction, and therefore decline in other suppressive mechanisms. IL-4 is a survival factor for T cells, increasing their proliferation in culture (Ben-Sasson *et al.*, 2000), therefore Treg IL-4 could be directly responsible for enhanced T cell proliferation and/or survival. Notably, IL-4$^+$ Tregs isolated from the lung dLN had more obviously reduced suppressive capacity than those from the lung tissue (Fig. 3.8). One potential explanation for this is that Tregs sorted from the lung dLN appeared to express more IL-4 per cell (i.e. displayed a higher fluorescence intensity for IL-4eGFP), than the lung Tregs (Fig. 3.7.C). To further understand the significance of Treg IL-4, it would be first interesting to test the supernatants of the Treg suppression assays for IL-4 protein, as it could be expected that there would be increased IL-4 in the IL-4$^+$ Treg wells. Alternatively, there could be reduced expression of soluble suppressive factors – such as TGFβ or IL-10 – by IL-4$^+$ Tregs, which could again be assayed by measuring the level of these factors in the cell supernatant. A more objective approach to investigate potential functional differences in IL-4 expressing Tregs would be mRNA sequencing of FACS isolated IL-4$^+$ vs IL-4$^+$ Tregs.

Despite a subpopulation of Foxp3$^+$ Tregs showing potential signs of reduced regulatory and increased inflammatory effector function, there was no evidence that Tregs down-regulated Foxp3 expression during *A.f.* driven AAI. As our phenotyping of Tregs during AAI has shown increased expression of Th2 associated markers, for instance ST2 and, notably, IL-4 (Fig.7.C), we therefore wondered if this was a sign of Treg to Th2 instability. To measure this we used a fate reporter mouse (*Il4*^{GFP}*Foxp3*$^{YFP/Cre}$*R26R*^{FP635}), which allowed us to track both

current and historic Foxp3 expression, as well as expression of the Th2 cytokine IL-4 (Pelly *et al.*, 2017). In our model there was no increase in Th2-exTregs proportionally in the lung or lung dLN, and by absolute cell number in the lung (Fig. 3.7.B). This is in contrast to previous work using the same transgenic mice, in which Tregs were found to be unstable during repeat helminth infection (Pelly *et al.*, 2017). However, we could see no evidence for downregulation of Foxp3 expression during *A.f.* driven AAI (Fig. 3.7.B). This would support other published work which has argued that Tregs stably express Foxp3 both at steady state and during inflammation (Rubtsov *et al.*, 2010). Other strategies, such as adoptive transfer of fate-marked Tregs, could be utilised to further address this question of potential instability. Taken together, we have shown that during fungal AAI a subset of Tregs displayed functional instability, with a gain in inflammatory effector function - IL-4 expression. Notably this occurred without any evidence for a decreased Foxp3 expression.

In this chapter, we set out to define the role of Tregs in fungal AAI. Our novel data reveal a striking increase of tissue Tregs to be a key feature of fungal AAI, with those Tregs playing a critical role in limiting Th2 and Th1, but not Th17, pulmonary inflammation. We have identified IL-10 as a potential suppressive mechanism. In terms of their stability, we have no evidence to suggest that the mixed Th2/Th17 inflammation apparent during fungal AAI is associated with Foxp3 transcriptional instability in Tregs. Moreover, we have shown that the intrinsic suppressive capability of Tregs does not change during AAI, unless those Tregs express IL-4, expression of which is associated with reduced Treg suppressive potential. Together these data increase our fundamental understanding of the generation, maintenance, function and stability of Tregs during fungal AAI.

In the next chapter, we go on to ask how the expansion of Tregs occurs. Specifically, we look at the cross-talk between Tregs and DCs, and the potential role of DCs in inducing Tregs.

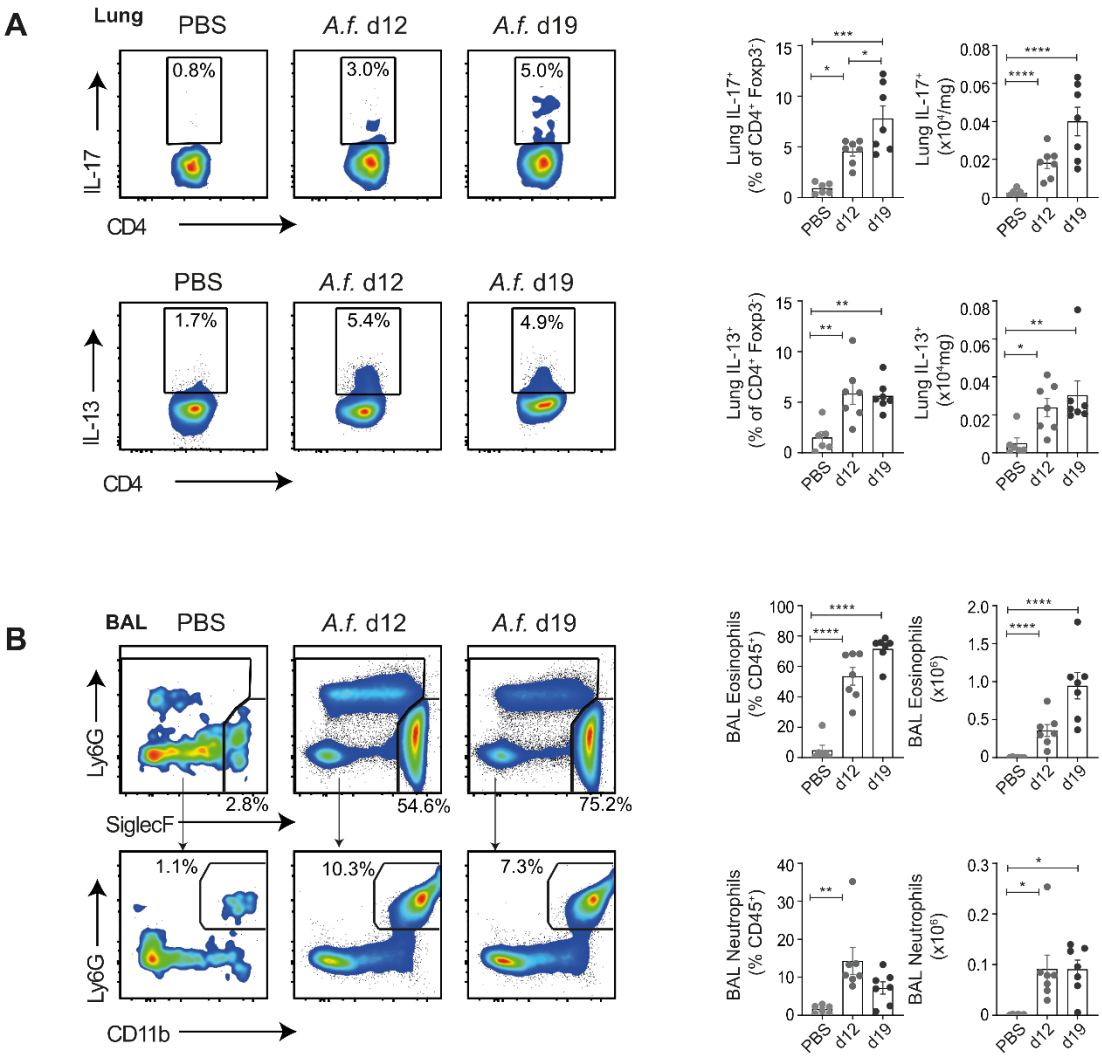

Figure 3.1. Repeat *A.f.* exposure generates concurrent type-2 and type-17 pulmonary inflammation.

C57BL/6 mice were exposed to 0.4x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At days 12 and 19 lungs were taken. A) Lung cell isolates were restimulated with PMA/ionomycin and assessed by flow cytometry. Representative flow cytometry plots depict CD4$^+$ cell cytokine expression, with gate frequencies showing % of CD4$^+$ Foxp3$^-$ cells. B) BAL cell isolates were assessed by flow cytometry. Representative flow cyometry plots gating for SiglecF$^+$ Eosinophils, and Ly6g$^+$ CD11b$^+$ neutrophils. Gate frequencies show % of CD45$^+$ cells. Combined from 2 experiments (n=6-7 per group). Each data point is an individual mouse. Linear mixed effect modelling applied, with experimental repeat as a random effect variable. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

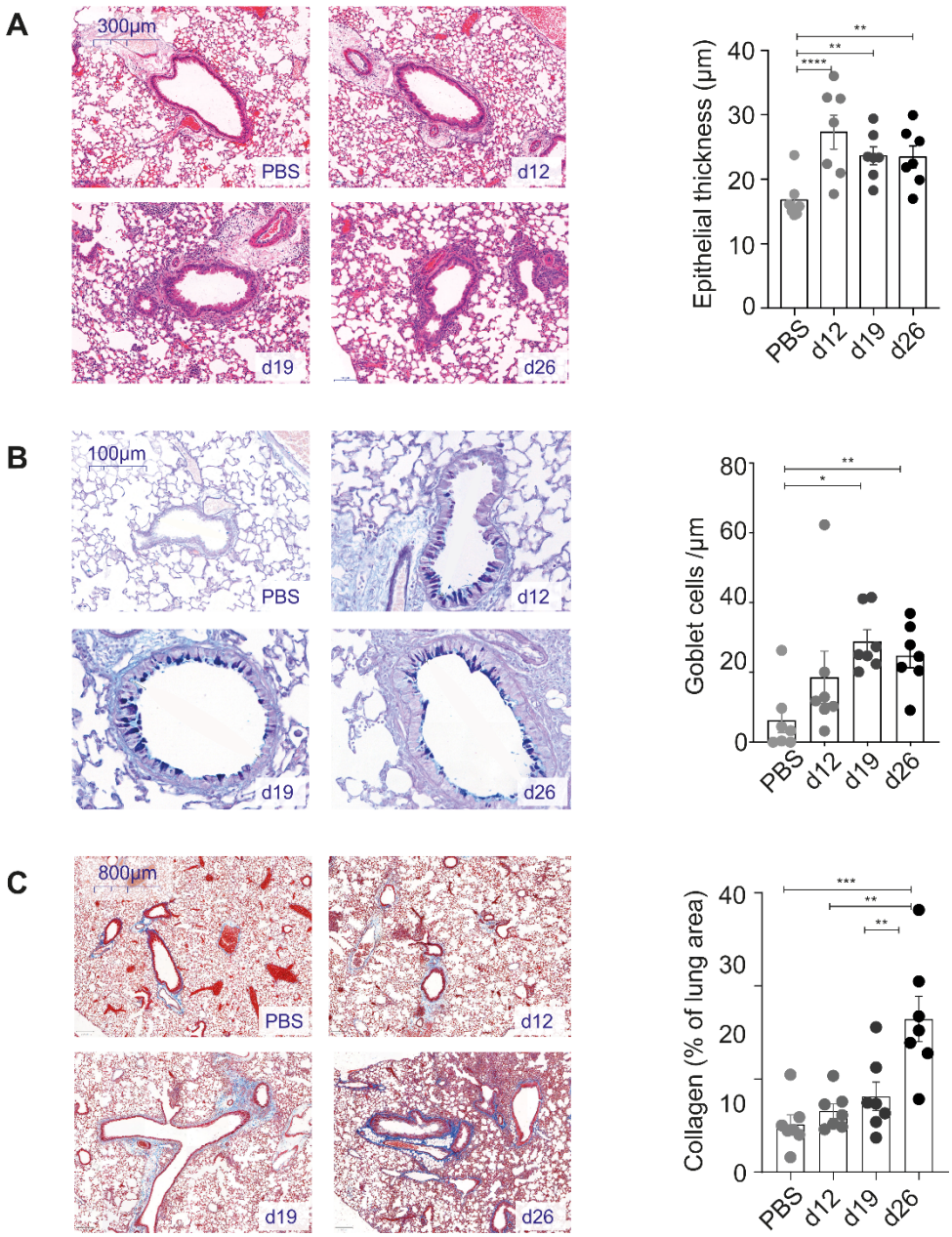

Figure 3.2. Repeat *A.f.* exposure replicates key pathological features of AAI
C57BL/6 mice were exposed to 0.4 x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At days 12, 19 & 26 lungs were taken. A) Lungs were stained with H&E and epithelial thickness measured from the basement membrane to the airway lumen. Representative images are shown. Combined from 2 experiments (n= 6-7 per group). B) Lungs were stained with AB-PAS and goblet cells counted, and normalised to airway circumference. Representative images are shown. Combined from 2 experiments (n=6-7 per group). C) Lungs were stained with Masson's Trichrome and the proportion of lung tissue stained blue (collagen) staining quantified. Representative images are shown. Combined from 2 experiments (n=6-7 per group). Each data point is an individual mouse. Linear mixed effect modelling applied, with experimental repeat as a random effect variable. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

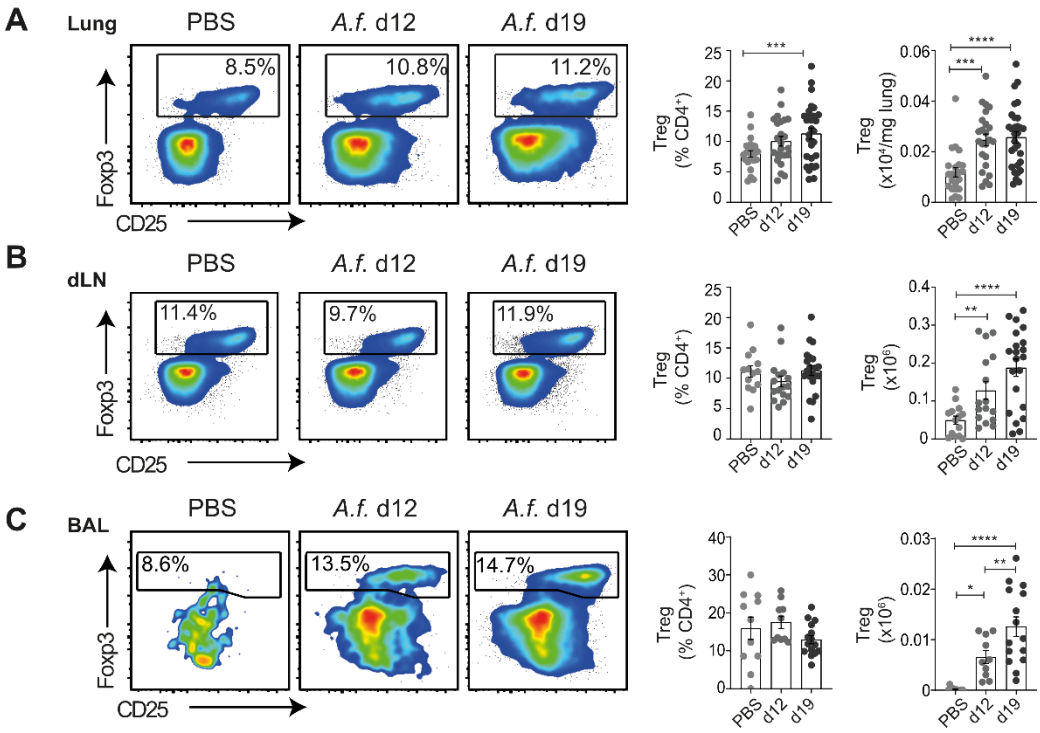

Figure 3.3. Pulmonary Treg expansion in response to *A.f.* driven AAI.
C57BL/6 mice were exposed to 0.4 x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At days 12 & 19 lungs were taken. (A) Representative flow cytometry plots depict lung Tregs within the $CD4^+$ population. Lung data are combined from seven experiments total (n = 23-29 per group). Each data point is an individual mouse. (B) Representative flow cytometry plots depict lung dLN Tregs within the $CD4^+$ population. Lung dLN data are combined from five experiments total (n = 13-21 per group). (C) Representative flow cytometry plots depict BAL Tregs within the $CD4^+$ population. BAL data are combined from four experiments total (n = 11-15 per group). Each data point is an individual mouse. Linear mixed effect modelling applied, with experimental repeat as a random effect variable. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

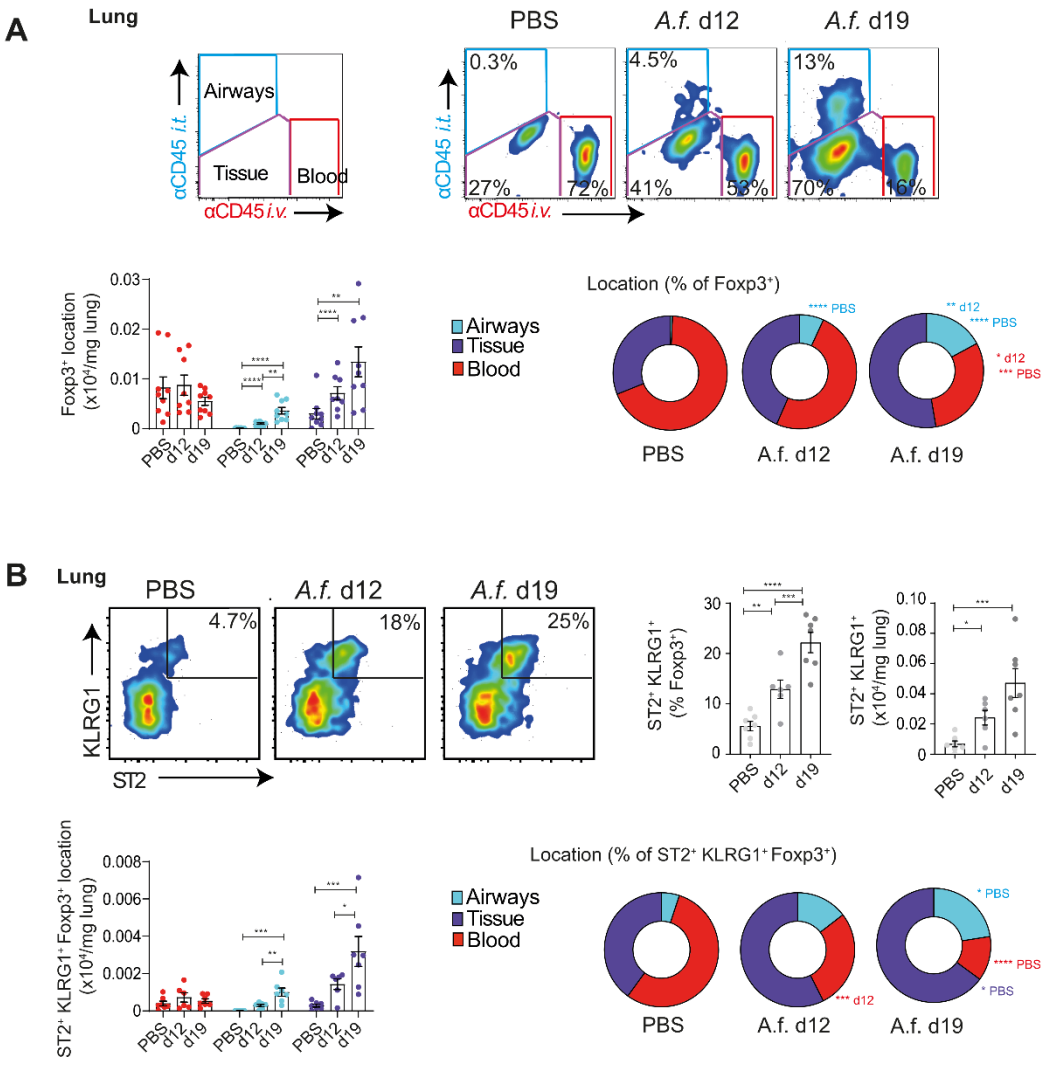

Figure 3.4. Expansion of lung tissue Tregs in response to *A.f.* driven AAI.
VertX *Il10*^{GFP} mice were exposed to 0.4 x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At days 12 & 19 lungs were taken. (A) Mice were injected *i.v.* with anti-CD45-APC, euthanased with $C0_2$ and then injected *i.t.* with anti-CD45 PE. Representative flow cyometry plots depict location of lung Foxp3$^+$ Tregs within the CD4$^+$ population. Treg location as absolute number, and proportion of the Treg pool is shown. Combined from three experiments (n = 8-9 per group). (B) Representative flow cyometry plots depict location of lung ST2$^+$ KLRG1$^+$ "tissue Tregs" within the Foxp3$^+$ CD4$^+$ population. "Tissue Treg" location as absolute number, and proportion of the "Tissue Treg" pool is shown. Combined from two experiments (n = 6-7 per group). Each data point is an individual mouse. Linear mixed effect modelling applied, with experimental repeat as a random effect variable. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

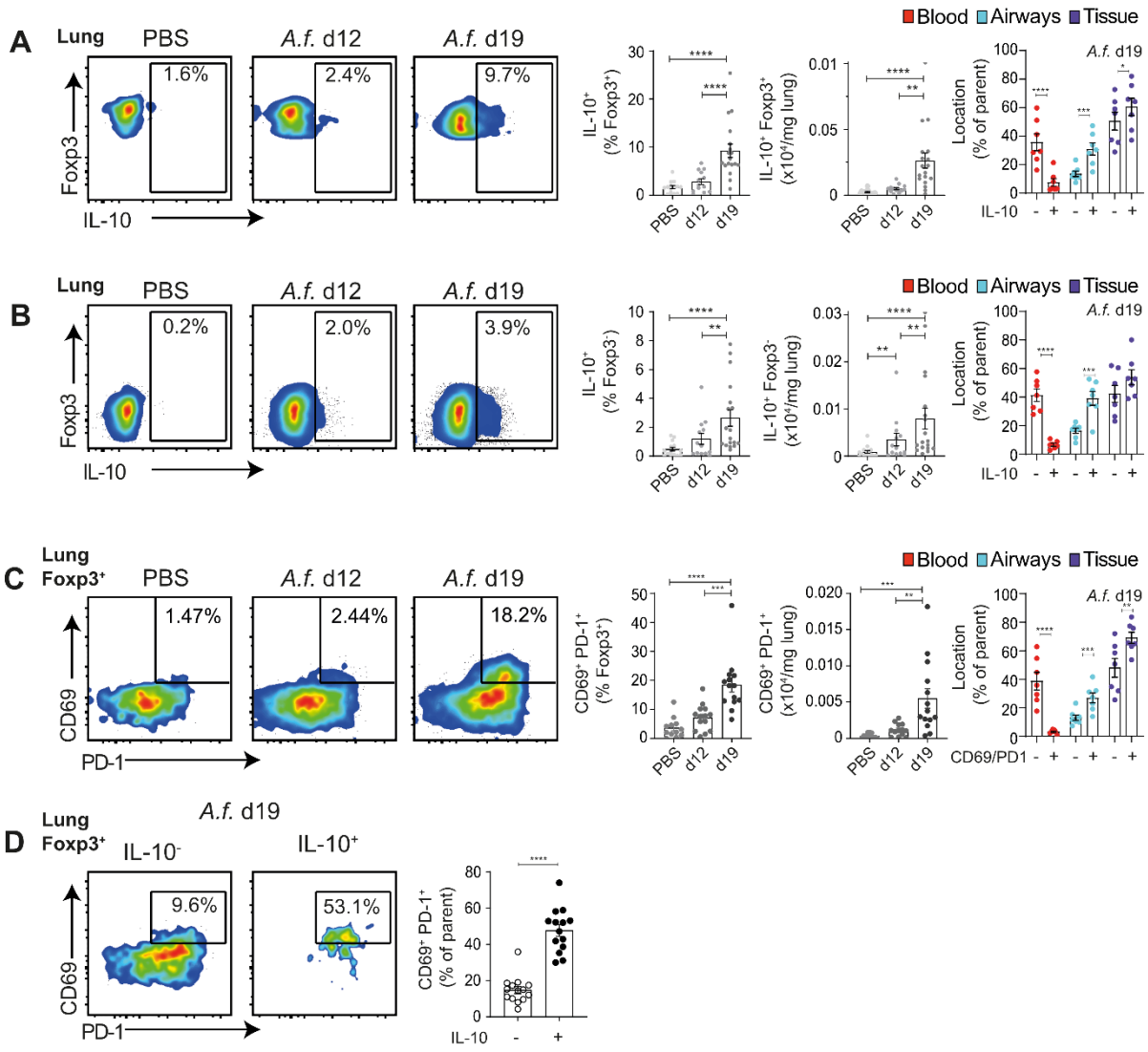

Figure 3.5. Lung tissue and airway IL-10 expression by CD4⁺ Foxp3⁻ T cells and Tregs in *A.f.* driven AAI.

VertX *Il10*^{GFP} mice were exposed to 0.4 x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At days 12 & 19 lungs were taken. In some experiments mice were injected *i.v.* with anti-CD45-APC, euthanased with CO_2 and then injected *i.t.* with anti-CD45 PE to distinguish tissue location. (A) Representative flow cytometry plots depict IL-10eGFP expression within the Foxp3⁺ CD4⁺ population. Tissue location of IL-10⁺ or IL-10⁻ Tregs at d19 by *i.t./i.v.* staining. (B) Representative flow cytometry plots depict IL-10eGFP expression within the Foxp3⁺ CD4⁻ population. Tissue location of IL-10⁺ or IL-10⁻ Foxp3⁺ CD4⁻ cells at d19 by *i.t./i.v.* staining. Graphs in (A) and (B) were combined from five experiments total (n = 14-19 per group), location graphs combined from two experiments (n=7 per group). (C) Representative flow cytometry plots depict activated CD69⁺PD1⁺ Lung Tregs. Tissue location of CD69⁺PD1⁺ or CD69⁻/PD1⁻ Tregs at d19 by *i.t./i.v.* staining. (D) Representative flow cytometry plots depict activated CD69⁺PD1⁺ cells within the IL-10⁺ or IL-10⁻ Lung Treg population. Graphs in (C) and (D) were combined from four experiments total (n = 12-14 per group), location graphs combined from two experiments (n=7 per group). Each data point is an individual mouse. Linear mixed effect modelling applied, with experimental day as a random effect variable. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

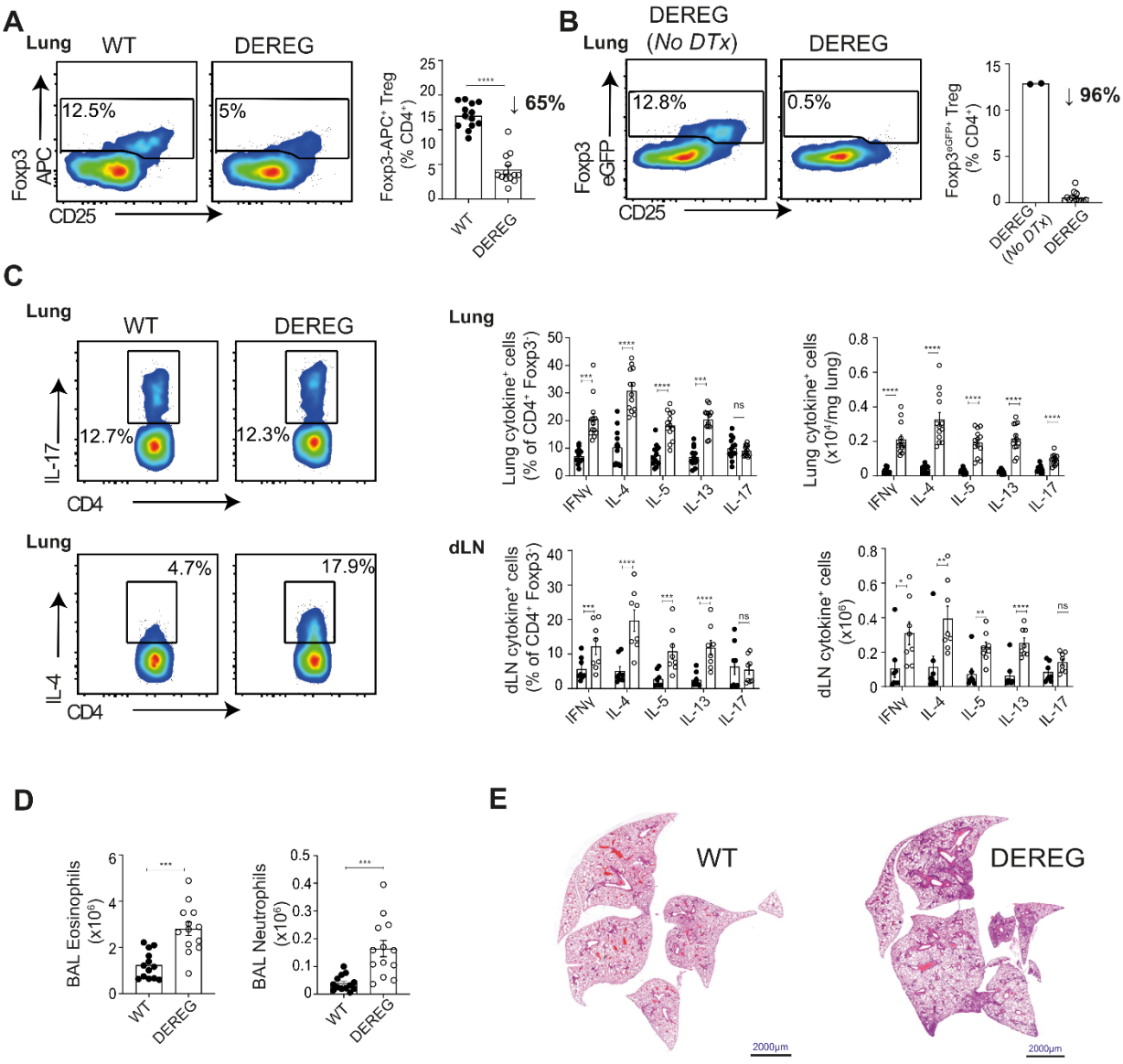

Figure 3.6 Treg depletion results in increased type-2 lung and airway inflammation. DEREG mice were exposed to 0.4 x10^6 *A.f.* spores or PBS *i.n.* 3x per week. Unless otherwise stated, DTx was administered *i.p.* to all mice on days 12, 15 and 17. At day 19 lungs were taken. (A) Representative flow cytometry plots depict lung cell isolates used to assess efficiency of Treg depletion by antibody staining. Percent depletion shown. (B) Representative flow cytometry plots depict lung cell isolates used to assess efficiency of Treg depletion by detection of the $Foxp3^{GFP}$ transgene, compared to control non-DTx treated DEREG mice. Percent depletion shown. Combined from 3 experiments (total n = 2-13 per group). (C) Lung or lung dLN cell isolates were stimulated with PMA/ionomycin, and intracellular cytokines assessed. Representative flow cytometry plots are shown. Lung dLN data are combined from 2 experiments (total n = 8 per group). (D) BAL cell isolates were assessed by flow cytometry to determine eosinophil or neutrophil numbers. (E) Lungs were inflated with formalin, and stained with Hematoxylin & Eosin. Representative images are shown. Unless otherwise stated, data are combined from 3 experiments (total n = 13 per group). Each data point is an individual mouse. Graphs show mean ± SEM (error bars). Linear mixed effect modelling applied, with experimental day as a random effect variable. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001

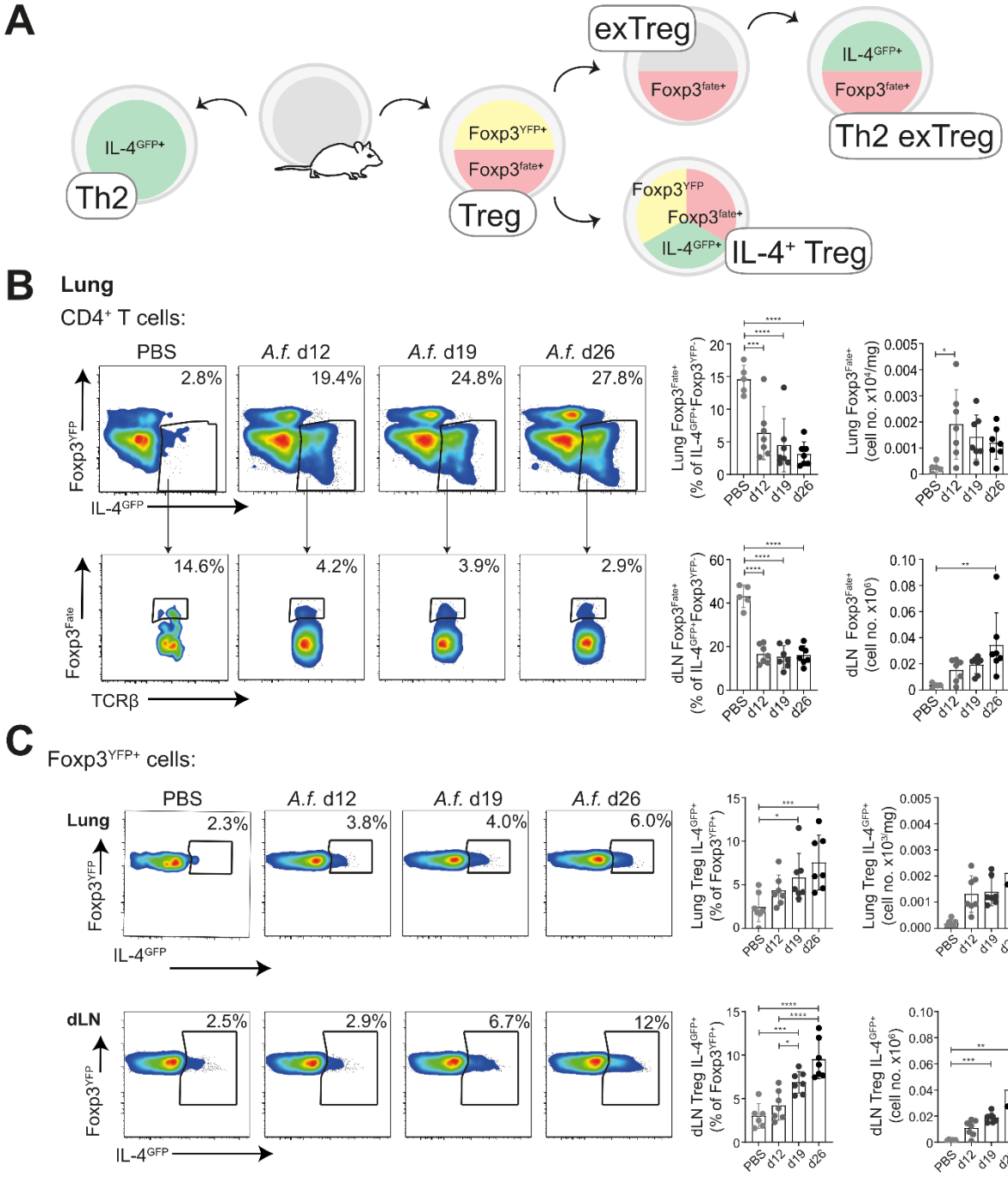

Figure 3.7. Tregs are stable, but begin to express IL-4 in *A.f.* driven AAI.
Foxp3$^{YFP/Cre}$ *R26R*^{FP635} *IL4*^{GFP} mice were exposed to 0.4 x10^6 *A.f.* spores or PBS *i.n.* 3x per week, lungs and lung dLN were harvested on days 12, 19, 26 and cell isolates assessed via flow cytometry. (A) Representative flow cytometry plots depict gating of lung Th2 (IL-4^{GFP+}) ex Tregs R26R^{FP635+} (referred to as Foxp^{Fate+}) mice, following gating out of Foxp3^{YFP+} Tregs. (B) Representative flow cytometry plots depict IL-4^{GFP} expression from lung and lung dLN Foxp3^{YFP+} Tregs. Combined from 2 experiments (total n = 5-7 per timepoint). For all graphs each data point is an individual mouse. Graphs show mean ± SEM (error bars). Linear mixed effect modelling applied, with experimental day as a random effect variable. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001

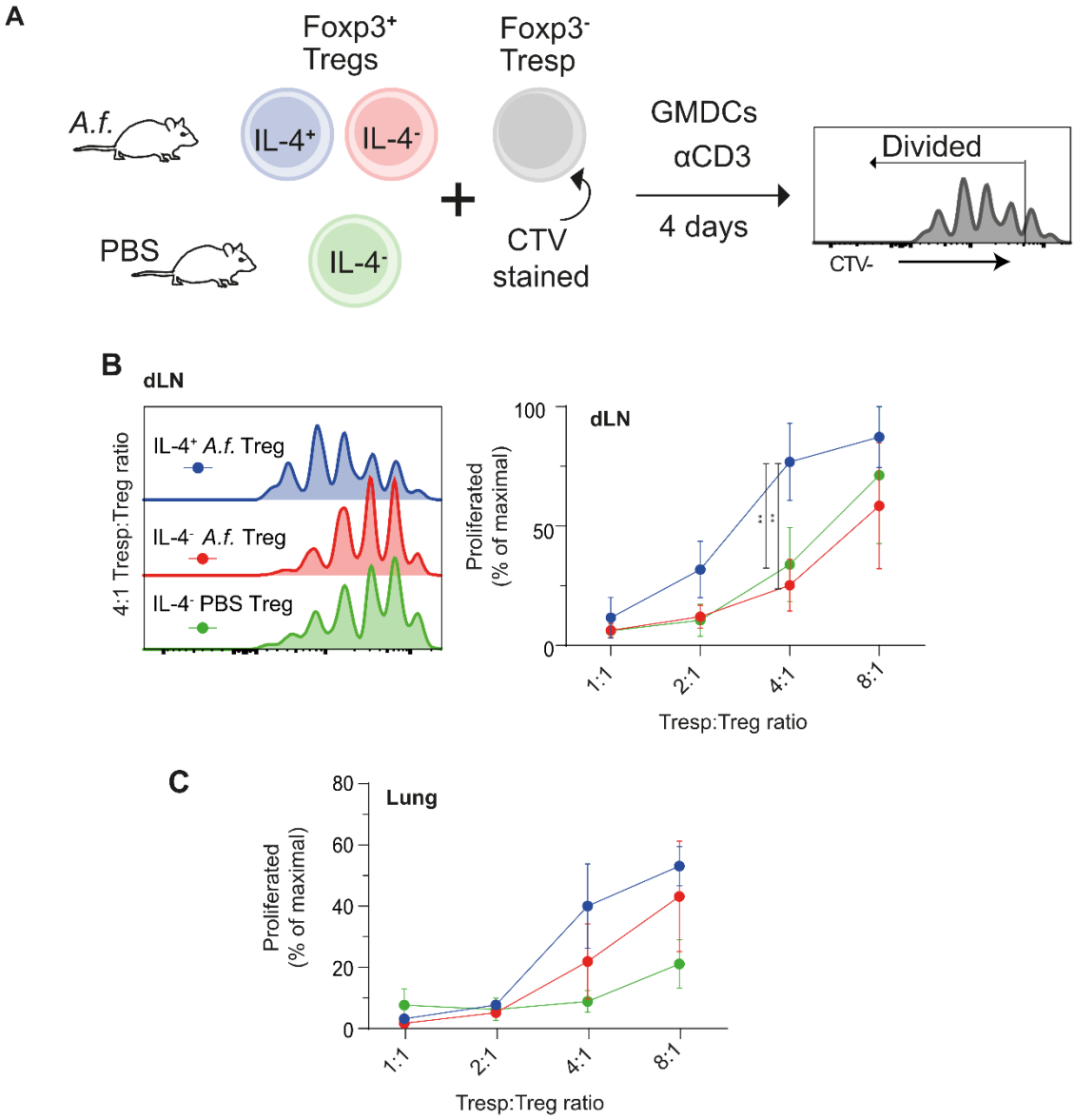

Figure 3.8. IL-4⁺ Tregs have reduced suppressive function *in vitro*.
Foxp3$^{YFP/Cre}$ *R26R*^{FP635} *Il4*^{GFP} mice were exposed to 0.4 x10^{6} *A.f.* spores or PBS *i.n.* 3x per week for 19 days. (A) Tregs (IL-4^{+} or IL-4^{-}) were sorted from lung draining lymph nodes or lungs of *A.f.* or PBS treated mice, and mixed at the ratios stated with Foxp3- Tresponder (Tresp) cells. Proliferation of Tresp cells after 4 days in the presence of differing Tregs was measured via cell trace violet (CTV) staining. (B) Representative CTV proliferation plots are shown for lung draining LN. (B) and (C) Proliferation of Tresp at various Tresp:Treg ratios is shown, normalised to maximal Tresp proliferation. Combined from 3 experiments (total n = 2-3 per group). Graphs show mean ± SEM (error bars). Linear mixed effect modelling applied, with experimental day as a random effect variable. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001

Chapter 4

Innate cell requirements for Treg expansion during *A.f.* driven AAI

Chapter 4 - Innate cell requirements for Treg expansion during *A.f.* driven AAI

4.1 Introduction

DCs are a specialised cell type, responsible for integrating tissue signals, sampling, processing and presenting Ag, and directing the polarisation of T cells and therefore, the immune response (Banchereau and Steinman, 1998; Merad *et al.*, 2013). DCs can be divided into different subsets, based on ontogeny and function, including cDC1s, cDC2s, pDCs and moDCs (Durai and Murphy, 2016). The ontogeny of moDCs has recently been questioned, with a suggestion that this subset may in fact represent cDCs that have upregulated monocyte features, in comparison to monocytes that have differentiated to moDCs (Bosteels *et al.*, 2020). In response to certain parasites and extracellular bacteria cDC2s, and particularly $MGL2^+$ cDC2s, are required to induce type-2 and type-17 responses (Lewis *et al.*, 2011; Kumamoto *et al.*, 2013; Satpathy *et al.*, 2013; Tussiwand *et al.*, 2015). Notably, $CD11b^+$ cDC2s have been shown to be sufficient to drive type-2 responses in a HDM model of AAI (Plantinga *et al.*, 2013). In addition to promoting inflammation, DCs are critical to maintain immune tolerance at steady state (Ohnmacht *et al.*, 2009). However, the role of particular DC subsets in directing lung Treg expansion during ongoing AAI is still unclear.

Classically, DC-mediated tolerance is evident following exposure to Ags in the absence of danger signals, leading to deletion or anergy of Ag-specific cells (Bonasio *et al.*, 2006; Ohnmacht *et al.*, 2009; Takenaka and Quintana, 2016). DCs can be treated with regulatory factors, such as IL-10, dexamethasone or IL-27, to become tolerogenic (Zhang-Hoover, Finn and Stein-Streilein, 2005; Koya *et al.*, 2007; Nayyar *et al.*, 2012; Mascanfroni *et al.*, 2013), and administration of these tolerogenic DCs can prevent or reverse AAI (Zhang-Hoover, Finn and Stein-Streilein, 2005; Koya *et al.*, 2007; Nayyar *et al.*, 2012). DC subset specific roles have also been detailed, with cDC1s and pDCs implicated in promoting Treg-mediated tolerance to airway allergens (de Heer *et al.*, 2004; Lombardi *et al.*, 2012; Khare *et al.*, 2013; Gollwitzer *et al.*, 2014). Airway exposure to the Ag OVA, without adjuvant, leads to the development of tolerance, meaning an inflammatory reaction will not occur following OVA/adjuvant challenge (de Heer *et al.*, 2004; Khare *et al.*, 2013). Utilising this model, depletion of pDCs or cDC1s has been shown, in separate studies, to ablate Ag tolerance (de Heer *et al.*, 2004; Khare *et al.*, 2013). Airway transfer of OVA-loaded pDCs has also been shown to induce tolerance to subsequent OVA/alum sensitisation and challenge (de Heer *et al.*, 2004; Lombardi *et al.*, 2012). Notably, during ongoing inflammation it is unclear how DCs promote tolerogenic responses, and whether a specific DC subset is required.

Regulatory responses can occur concurrently with inflammatory responses, as shown in Chapter 3, in which we observed an expansion of Tregs alongside Th2 cells in *A.f.* driven AAI. DC-driven Treg expansion has recently been elucidated in a lung cancer model, attributed to a regulatory expression profile seen in both cDC1s and cDC2s (Maier *et al.*,

2020). Another recent study, during lung influenza infection, identified IRF4-dependent cDC2s as specifically required for Treg expansion (Ainsua-Enrich *et al.*, 2019). Overall, it is still not clear whether Treg expansion during inflammation in general, and AAI specifically, is dependent upon a particular DC subset, attributable to a regulatory function common to the whole DC compartment, or even DC independent.

DCs can induce and expand Tregs by a variety of mechanisms, including TGFβ activation (Worthington *et al.*, 2011). Tregs can develop from naïve T cells *in vitro* with TGFβ stimulation (Chen *et al.*, 2003). In the intestine it has been shown that expression of the TGFβ activating integrin αVβ8 is critical for steady-state Treg induction by cDC1s (Coombes *et al.*, 2007; Worthington *et al.*, 2011). Treg expansion has also been shown to be promoted by IL-2 (Fontenot *et al.*, 2005). Interaction of Tregs with DCs enhances Treg expansion *in vitro* and *in vivo*, further enhanced by IL-2 provided via $Foxp3^{-}CD4^{+}$ T cells (Zou *et al.*, 2010). The mechanisms by which DCs can induce Tregs during ongoing AAI are not known.

In this chapter, utilising the *A.f.* model of AAI described in Chapter 3, we aimed to further elucidate the role of DCs in Treg expansion during AAI. First, we assessed numeric changes in the DC compartment. Next, to assess if one subset was preferentially responsible for Treg expansion, we systematically depleted pDCs, moDCs, cDC1s and $MGL2^{+}$ cDC2s during ongoing *A.f.* driven AAI. Depletion of pDCs, moDCs, cDC1s had only minor effects on lung inflammation, and no effect on Treg number. In contrast, we found that $MGL2^{+}$ $CD11c^{+}$ cells were required to induce both Th2 and Treg responses during ongoing *A.f.* driven AAI. Surprisingly, *in vitro*, sorted cDC2s from *A.f.* treated mice did not show enhanced Treg induction ability. Finally, we showed that expression of the integrin αVβ8 on $CD11c^{+}$ cells was required to promote lung Treg expansion during *A.f.* driven AAI, suggesting that TGFβ activation may be a key mechanism for DC mediated Treg expansion in this setting.

4.1.1 Key questions:

Our overall hypothesis was that a subset of DCs would be critical for Treg expansion during *A.f.* driven AAI, and that DC αVβ8 mediated-TGFβ activation would be centrally involved in this process. Specific questions were:

1. How does the DC compartment change during fungal AAI?
2. Which DC subset – pDCs, moDCs, cDC1s or cDC2s – is required for Treg expansion during fungal AAI?
3. What is the mechanism of DC Treg induction during fungal AAI? Is there a role for activation of TGFβ via αVβ8?

4.2. Results

4.2.1 Expansion of cDC2s in *A.f.* driven AAI

To understand the role of DCs during *A.f.* driven AAI, we began by assessing proportional and absolute changes in the DC compartment. To ensure accuracy in DC identification lymphocytes, macrophages and granulocytes were excluded prior to gating on DC subsets, as shown in Fig. 2.2. As in Chapter 3, mice were dosed *i.n.* with *A.f.* spores, and tissues harvested at d12 or d19 post initial dose (Porter *et al.*, 2011). There was no significant increase in pDCs (PDCA1$^+$) by cell number or as a proportion of total leukocytes (CD45$^+$) during *A.f.* driven AAI (Fig. 4.1.A). Repeat *A.f.* exposure led to a significant increase in total cDCs (MHCII$^+$ CD11c$^+$) by d12, both as absolute number and as percent of total leukocytes (CD45$^+$) (Fig. 4.1.B). Stratifying the DC compartment revealed this expansion was due to an increase in cDC2s (CD11b$^+$), which significantly increased by cell number and as a proportion of the total cDC compartment by d12 (Fig. 4.1.C). In contrast, cDC1s (XCR1$^+$) did not change by cell number, and significantly decreased as a proportion of the total DC compartment by d12 of *A.f.* driven AAI (Fig. 4.1.C). As cDC2s have been shown to be heterogenous (Han *et al.*, 2018; Brown *et al.*, 2019; Bosteels *et al.*, 2020), we further divided this compartment based on expression of MGL2, which is expressed on DCs required for driving type-2 responses in the skin (Kumamoto *et al.*, 2013). MGL2$^+$ cDC2s significantly increased by cell number and as a proportion of the total DC compartment by d12 of *A.f.* driven AAI (Fig. 4.1.D). There was a trend for an increase in MGL2$^-$ cDC2 cell number by d12, but as a proportion of the total cDC compartment these cells remained constant during *A.f.* driven AAI (Fig. 4.1.D). Together, this showed MGL2$^+$ cDC2s expand by d12 of *A.f.* driven AAI, remaining constant to d19, and were predominantly responsible for the numeric increase in the overall cDC compartment at these timepoints.

4.2.2 MGL2$^+$ CD11c$^+$ cells, and not pDCs, cDC1s or CCR2 dependent cells are required for Treg expansion during *A.f.* driven AAI

At this point, we had shown a specific increase in MGL2$^+$ cDC2s during *A.f.* driven AAI, however the function of MGL2$^+$ DCs, or the other DC subsets was still unknown. To investigate this, we utilised transgenic mouse models to systematically ablate myeloid and DC subsets during *A.f.* driven AAI. We were primarily interested in the requirement of particular DC subsets for Treg expansion and activation at d19 of *A.f.* driven AAI, as the role of DCs in Treg induction during ongoing allergic inflammation is not clear. Activation of Tregs was assessed via co-expression of CD69 and PD1 (Agata *et al.*, 1996; McGee *et al.*, 2010; Asano *et al.*, 2017; Yu *et al.*, 2018), bearing in mind that CD69$^+$PD1$^+$ Tregs were lung tissue and airway located (Fig. 3.5.C), with increased expression of the regulatory cytokine IL-10 (Fig. 3.5.C). To understand the inflammatory context of observed Treg changes CD4$^+$ T cell polarisation and activation was evaluated by intracellular cytokine staining, and cellular AAI by measuring BAL granulocytes and macrophages.

First, we assessed the role of pDCs. pDCs were depleted with *Bdca2*^{Dtr} mice, which the DTr is specifically expressed under control of the human pDC gene promoter *BDCA2* (Swiecki *et al.*, 2010). We administered DTx to deplete pDCs from d13-d19, this range being chosen to include the period in which Tregs expanded and were activated during *A.f.* driven AAI (Fig. 3.3.A & 3.3D). pDCs (PDCA1^{+}) were depleted by cell number (75%), as well as proportion of total leukocytes (72%) in *Bdca2*^{Dtr} mice (Fig. 4.2.A). There were no changes in DC numbers either as absolute cell number, or as proportion of the total cDC compartment (Fig. 4.2.A).

After establishing efficient pDC depletion, we assessed resultant inflammatory or Treg changes. There was a slight but significant decrease in the proportion, but not absolute number, of IL-17 expressing cells, but no changes in other CD4^{+} T cell cytokines assessed in pDC depleted mice (Fig. 4.2.B). Convincing CD4^{+} T cell IL-10 expression was not observed post PMA/ionomycin stimulation during *A.f.* driven AAI, with less than 1% of CD4^{+} T cells IL-10^{+}, and so this cytokine is not displayed in this figure or subsequent figures. In terms of airway cellular inflammation, there was a slight but significant increase in neutrophils by cell number, but not proportion of total BAL leukocytes, in pDC depleted mice (Fig. 4.2.C). There was a slight significant increase in lung Tregs as a proportion of CD4^{+} T cells, but no difference as absolute cell number in pDC depleted mice at d19 (Fig. 4.2.D). Similarly, there was no changes in Treg activation in pDC depleted mice (Fig. 4.2.D). Taken together, we found pDC depletion during ongoing AAI to have very little effect on overall inflammation, and no effect on Treg activation or cell number.

Next, we utilised mice deficient in the chemokine receptor CCR2 (*Ccr2*$^{-/-}$) (Boring *et al.*, 1997), to understand the role of CCR2 dependent DCs. In *Ccr2*$^{-/-}$ mice monocytes are unable to egress from the bone marrow, leading to a reduction in monocytes and cells of monocyte descent in the tissues (Serbina and Pamer, 2006). Fittingly, measured at d19 of *A.f.* driven AAI, there was a dramatic decrease in lung monocytes (Ly6C^{+} CD11b^{+} CD64^{+}) by cell number (76%) and as a proportion of total leukocytes (77%) in *Ccr2*$^{-/-}$ mice, when compared to WT mice (Fig. 4.3.A). Monocyte derived CD11c^{+}MHCII^{+} moDCs (Menezes *et al.*, 2016), as well as certain pre-DC derived CD11b^{+} cDCs (Scott *et al.*, 2015; Nakano *et al.*, 2017; Bosteels *et al.*, 2020) referred to as inf-cDC2s, have also been shown to be CCR2 dependent (Bosteels *et al.*, 2020), and should therefore be reduced in the lung in *Ccr2*$^{-/-}$ mice. Our DC panel lacked the antibody Mar-1, and therefore we were not able to specifically identify inf-cDC2s (Bosteels *et al.*, 2020). However, there was a slight but significant decrease in cDC2s, in particular MGL2^{+} cDC2s, as a proportion of the total cDC compartment in *Ccr2*$^{-/-}$ mice (Fig. 4.3.A), potentially explained by ablation of CCR2 dependent cDC2s or moDCs. Potential changes in the pDC compartment were not assessed, due to their demonstrated negligible importance in this system (Fig. 4.2).

After establishing efficient depletion of CCR2 dependent cells, we assessed resultant inflammatory changes, and the effect on lung Treg number and activation. There was a slight but significant proportional decrease in Th2 cytokine expression (IL-4, IL-5, and IL-13) by CD4^{+} T cells in *Ccr2*$^{-/-}$ mice (Fig. 4.3.B). There was no change in Th1 (IFNγ) or Th17 (IL-17)

cytokine expression, or any significant change in cytokine expressing $CD4^+$ T cell numbers. This reduction in type-2 responses was also observed in the BAL, where there was a slight but significant decrease in eosinophils as a proportion of total leukocytes ($CD45^+$), but no change by cell number (Fig. 4.3.C). Importantly, there was no change in lung Tregs, either as a proportion of $CD4^+$ T cells or by cell number (Fig. 4.3.D). There was also no change in activated ($CD69^+PD1^+$) Tregs either as a proportion of Tregs, or by cell number (Fig. 4.3.D). Overall, CCR2 dependent cells were not required for Treg expansion or activation, but partially contributed to the type-2 response during *A.f.* driven AAI.

We next wanted to understand the role of cDC1s utilising *Batf3*$^{-/-}$ mice, which are deficient in cDC1s, throughout *A.f.* driven AAI (Hildner *et al.*, 2008; Edelson *et al.*, 2010). There were significantly fewer cDC1s ($CD103^+$) in the lungs of *Batf3*$^{-/-}$ mice, both by cell number (93%) and as a proportion of the total cDC compartment (96%) (Fig. 4.4.A). Surprisingly, there was also a slight but significant decrease in $MGL2^-$ cDC2 numbers in *Batf3*$^{-/-}$ mice (Fig. 4.4.A), while there was a reciprocal increase in $MGL2^+$ cDC2s in *Batf3*$^{-/-}$ mice as a proportion of the total cDC2 compartment, presumably due to the reduced cDC1 proportion (Fig. 4.4.A).

Inflammatory and Treg responses were assessed in cDC1 deficient mice. There was a significant increase in Th2 cytokine expression (IL-4, IL-5, and IL-13) as a proportion of $CD4^+$ T cells, but not by cell number, in *Batf3*$^{-/-}$ mice at d19 (Fig. 4.4.B). There were no changes in the expression of Th1 (IFNγ) or Th17 (IL-17) cytokines in BATF3$^{-/-}$ mice, or in airway cellular inflammation (Fig. 4.4.B&C). There was no change in Tregs as a proportion of $CD4^+$ T cells or as absolute cell number in *Batf3*$^{-/-}$ mice (Fig. 4.4.D). There was a significant decrease in activated $CD69^+PD1^+$ Tregs as a proportion of the Treg pool, but not as overall cell number, in *Batf3*$^{-/-}$ mice (Fig. 4.4.D). Taken together, cDC1s reduce Th2 cytokine responses during *A.f.* driven AAI, do not affect Treg expansion, but may be required for Treg activation.

Having found no role for CCR2-dependent DCs, pDCs or cDC1s in Treg expansion during *A.f.* driven AAI, we next depleted cDC2s. No depletion models efficiently target the entire cDC2 population (Durai and Murphy, 2016; Anderson *et al.*, 2021), therefore we chose to deplete $MGL2^+$ cDC2s, which showed the most dramatic numeric changes during *A.f.* driven AAI (Fig.4.1.D). To do this we utilised *Mgl2*^{DTr} mice (Kumamoto *et al.*, 2013). DTx was administered from d13-d19, during ongoing AAI. $MGL2^+$ cDC2s were significantly decreased by cell number (86%) and as a proportion of the total cDC compartment (77%) (Fig. 4.5.A). In line with this, there was a corresponding decrease in total cDC2 numbers and proportion, as well as a reciprocal increase in the proportion of cDC1s and $MGL2^-$ cDC2s at d19 of *A.f.* driven AAI (Fig. 4.5.A). MGL2 expression was also observed on alveolar and interstitial macrophages, and the number and proportion of both $MGL2^+$ macrophage subsets were significantly decreased in *Mgl2*^{DTr} mice (Fig. 4.5.A). Notably however, there was no decrease in absolute cell number of total alveolar or interstitial macrophages (Fig. 4.5.A).

Finally, having established efficient depletion of $MGL2^+$ cDC2s, we next assessed resultant inflammatory and Treg changes during *A.f.* driven AAI. As absolute cell number, and as proportion of $CD4^+$ T cells, there was a notable and significant decrease in Th2 cytokines (IL-

4, IL-5 and IL-13) in *Mgl2*^{DTr} mice (Fig. 4.5.B). There was also a significant decrease in IL-17 by cell number, but not proportion, and a significant increase in IFNγ as a proportion of CD4$^+$ T cells, but not by cell number in *Mgl2*^{DTr} mice. (Fig. 4.5.B). In terms of airway cellular inflammation, there was a significant decrease in eosinophils by cell number and as a proportion of BAL leukocytes (CD45$^+$) BAL cells (Fig. 4.5.C), as well as a significant decrease in neutrophils by cell number, and a significant increase in macrophages as a proportion of BAL leukocytes (Fig. 4.5.C). Notably, there was a significant decrease in Tregs by cell number and proportion in *Mgl2*^{DTr} mice at d19, which was also observed in the lung dLN (Fig. 4.5.D). This change in cell number was associated with a significant decrease in the number, but not proportion, of PD1$^+$CD69$^+$ activated Tregs (Fig. 4.5.D). Taken together, these data reveal that MGL2$^+$ cDC2s are required for the expansion of lung and lung dLN Tregs during *A.f.* driven AAI, in addition to being required to sustain type-2 inflammatory responses.

4.2.3 *In vitro* assays do not reveal intrinsic differences in Treg induction capacity of DC subsets in *A.f.* driven AAI

At this point, we had established that MGL2$^+$ cDC2s were required *in vivo* to promote lung Treg expansion during *A.f.* induced AAI. However, it was unclear if this was due to cDC2s having an intrinsically greater ability to induce Tregs. To address this, we isolated lung and lung dLN cDC subsets from mice at d19 of *A.f.* driven AAI, and cultured them with splenic Foxp3$^-$ CD4$^+$T cells to assess their Treg induction ability (Fig. 4.6.A), following previously published protocols (Worthington *et al.*, 2011). To obtain sufficient numbers of sorted DCs, lungs and lung dLNs were pooled from 12 mice, purity of sorted subsets was over 94%, as displayed in Fig. 2.7. To account for possible co-operation between MGL2$^+$ and MGL2$^-$ cDC2s a bulk cDC2 population was re-combined post sort. Low levels of Treg induction were observed, with no significant difference in intrinsic Treg induction capability between sorted DC subsets (Fig. 4.6.B). There was however an overall trend for increased Treg number when compared to the no DC control (Fig. 4.6). We hypothesised that these low levels of Treg induction could be due to sub-optimal assay conditions, and therefore to some wells added exogenous active TGFβ (Fig. 4.6.C). There was no difference in Treg induction capacity between sorted DC subsets with exogenous TGFβ, when measured as a proportion of CD4$^+$ T cells or by cell number (Fig. 4.6.B). As this experiment was only performed twice, with 2-3 technical replicates, statistical analysis was not possible.

4.2.4 αVβ8 expressing CD11c$^+$ cells are required to drive Treg expansion during *A.f.* driven AAI.

These seemingly contradictory findings, of superior Treg expansion capacity of MGL2$^+$ cDC2s *in vivo* (Fig. 4.5.D), but not *in vitro* (Fig. 4.6.), led us to question if interaction with a component of the tissue microenvironment was required for Treg expansion. Specifically, we wondered if adding in active TGFβ bypassed a requirement for αVβ8, a DC expressed integrin that activates latent TGFβ, abundant in the lung, to induce Tregs (Travis *et al.*, 2007; Worthington *et al.*, 2013; Flanders *et al.*, 2016). CD11cΔαVβ8 mice were used to assay the role of αVβ8 expression on CD11c$^+$ cells (including DCs and macrophages) during *A.f.* driven AAI (Travis

et al., 2007). Having established key roles for DC subsets in Treg expansion, we first wanted to check the effect of αVβ8 depletion on DC number. There was a slight but significant increase in MGL2^{-} cDC2s and decrease in MGL2^{+} cDC2s in CD11cΔαVβ8 mice, but no change in cell number of DC subsets (Fig. 4.7.A). While Th2 cytokine production (IL-4, IL-5 and IL-13) was not altered, CD11cΔαVβ8 mice displayed dramatically reduced CD4^{+} T cell IL-17, and increased IFNγ, by cell number and proportion (Fig. 4.7.B). There were no significant changes in airway cellular inflammation in Cd11cΔαVβ8 mice at d19 of *A.f.* driven AAI (Fig. 4.7.C).

Next, we assessed changes in Tregs in CD11cΔαVβ8 mice during *A.f.* driven AAI, where we observed a significant decrease in lung Tregs by cell number and proportion (Fig. 4.7.D). The proportion of activated (CD69^{+}PD1^{+}) lung Tregs also decreased in CD11cΔαVβ8 mice (Fig. 4.7.D). In contrast, in the lung dLN there was a significant proportional increase in Tregs in Cd11cΔαVβ8 mice, although there was no change in number (Fig. 4.7.D). Overall, αVβ8 expression on CD11c^{+} cells was required for Treg expansion and activation in the lung, but not lung dLN, whilst also being vital for promotion of Th17 and inhibition of Th1 lung responses.

4.2.5 Summary

- During *A.f.* induced AAI, MGL2$^+$ cDC2s specifically expand in the lung (Fig. 4.1).
- CCR2-dependent cells, pDCs and cDC1s are not required to promote lung Treg expansion during *A.f.* driven AAI (Fig. 4.2-4).
- MGL2$^+$ CD11c$^+$ cells are required to promote lung and lung dLN Treg expansion during *A.f.* induced AAI (Fig. 4.5).
- Lung cDC2s isolated *ex vivo* from *A.f.* treated mice are not intrinsically superior at Treg induction than cDC1s (Fig. 4.6).
- Expression of the TGFβ activating integrin αVβ8 on CD11c$^+$ cells is required to promote Treg expansion and activation in the lung, but not lung dLN during *A.f.* driven AAI (Fig. 4.7).

4.3 Discussion

In Chapter 3 we showed that lung Tregs expand during *A.f.* induced AAI, increasing both as a proportion of $CD4^+$ T cells, and by absolute number (Fig. 3.3.). In this chapter we hypothesised that a subset of DCs may be required to mediate the observed Treg expansion. It should be noted that Tregs are present in the lung at steady state, and an integral part of immune homeostasis (Brunkow *et al.*, 2001). DC depletion during *A.f.* induced AAI was therefore not expected to completely ablate lung Tregs, but instead to reduce them to levels closer to those seen at steady state. The word expansion refers to increases in Treg proportion and number that could result from increased *de novo* Foxp3 induction, or enhancement of the proliferation, survival, or lung migration of existing Tregs (Travis *et al.*, 2007; Darrasse-Jèze *et al.*, 2009; Mayer, Berod and Sparwasser, 2012; Döhler *et al.*, 2017).

We began with phenotyping changes in the DC compartment during *A.f.* induced AAI, showing an increase in $MGL2^+$ cDC2s (Fig. 4.1). Next, using a range of transgenic models, we have shown that CCR2 dependent DCs, as well as pDCs and cDC1s were not required for Treg expansion (Fig. 4.2-4). We found that $MGL2^+$ $CD11c^+$ cells (cDC2s or macrophages) were required during ongoing inflammation to maintain type-2 inflammation, as well as to promote Treg expansion (Fig. 4.5). *In vitro* studies provided no evidence for intrinsically superior Treg induction capability of $MGL2^+$ cDC2s in *A.f.* driven AAI (Fig. 4.6), when tested outside of the allergic lung, leading us to investigate tissue-dependent mechanisms. Focusing on TGFβ activation, which has previously been shown to be required for Treg induction (Worthington *et al.*, 2011), we found that αVβ8 expression on $CD11c^+$ cells was required for Treg expansion (Fig. 4.7). Taken together, this reveals Treg expansion during *A.f.* induced AAI requires $MGL2^+$ $CD11c^+$ cells, and expression of the TGFβ activating integrin αVβ8 by $CD11c^+$ cells.

4.3.1 CCR2-dependent cells, pDCs and cDC1s are not required for Treg expansion during *A.f.* driven AAI

The DC subset(s) responsible for generation of Treg responses during ongoing AAI are not known. Both pDCs and cDC1s have been associated with Treg induction in models of airway tolerance, but their role has not been investigated after AAI has begun (Lombardi *et al.*, 2012; Khare *et al.*, 2013). Surprisingly, however, neither of these subsets, or CCR2-dependent DCs, were required for Treg expansion during fungal AAI (Fig. 4.2-4). Together, this suggests a model in which Treg induction and expansion is delegated to certain DC subsets, and not an overall feature of the DC compartment. Below, I will discuss these findings in more detail for each DC subset, as well as addressing potential caveats with each depletion model.

CCR2-dependent cells were not required for lung Treg expansion or expression of activation markers during *A.f.* induced AAI (Fig. 4.3). CCR2-dependent cells include monocytes, as well as some cDC2s (Boring *et al.*, 1997; Serbina and Pamer, 2006; Scott *et al.*, 2015; Nakano *et al.*, 2017; Bosteels *et al.*, 2020). Notably, CCR2 has been recently described as required for the migration of 'inf-cDC2s', a population with superior capacity to prime naïve T cells during viral infection, and post exposure to HDM. One limitation of our study was that we were unable

to specifically verify depletion of inf-cDC2s in our *Ccr2*$^{-/-}$ experiments, as we did not use the Mar-1 antibody, which recognises FcεRI, CD64 (FcγRI) and FcγRIV (Tang, Jung and Allen, 2019). Crucially, however, CCR2 deficiency did not affect Treg cells, suggesting these populations are not critical for Treg expansion during fungal AAI (Fig. 4.3.D). This fits with CCR2-dependent DCs being reported to be pro-inflammatory rather than regulatory, promoting type-2 inflammation in HDM driven AAI (Plantinga *et al.*, 2013). Treg CCR2 expression has recently been proposed to be critical for lung Treg CD25 expression and suppressive function at steady state, independent of monocyte/cDC CCR2 expression (Zhan *et al.*, 2020). We did not observe reduced Treg CD25 expression (Fig. 4.3.D) in our study. Moreover, we observed a significant reduction in Th2 cytokine expression by CD4^{+} T cells in *Ccr2*$^{-/-}$ mice, suggesting Treg inhibition of Th2 cells was intact (4.3.B). Previous studies at steady state have found no change in lung Treg numbers or proportion in *Ccr2*$^{-/-}$ mice, a finding we have now extended to inflammation (Zhan *et al.*, 2020). We did not observe any immune changes, either in cytokine production, BAL cellularity, or T cell cytokines, in PBS treated *Ccr2*$^{-/-}$ mice, however this will need to be confirmed with an increased sample size.

Perhaps surprisingly, due to their published importance in driving tolerogenic responses in the airways (de Heer *et al.*, 2004; Lombardi *et al.*, 2012), Tregs were not altered in pDC depleted mice during AAI (Fig. 4.2). In the primary work describing pDCs in mouse lungs, de Heer *et al*., found pDCs were required for tolerance to inhaled Ag (OVA), although whether this was attributable to Tregs is not known (de Heer *et al.*, 2004). Notably, in this study anti-Gr1 (Ly6G/Ly6c) antibody was used to target pDCs, the specificity of this strategy is questionable, with Ly6G and Ly6G expressed on numerous granulocyte and monocyte origin cells (Bronte *et al.*, 2016), potentially confounding interpretation. Transfer of Ag (OVA) pulsed pDCs prior to sensitisation with OVA and adjuvant (alum) challenge prevents the development of AAI, increasing OVA-specific Tregs (de Heer *et al.*, 2004; Lombardi *et al.*, 2012). In contrast, we did not observe Treg number or activation to be dependent on pDCs (Fig. 4.2.D), although our study was during ongoing AAI, and looked at bulk, not Ag-specific Tregs, in comparison to Lombardi *et al* (Lombardi *et al.*, 2012). Depleting pDCs with the *Bdca2*^{DTr} mouse model showed that pDC depletion during the onset of viral bronchiolitis led to a reduction in lung Treg numbers in neonatal mice (Lynch *et al.*, 2018). Neonatal pDC depletion led to long-term changes in the Treg compartment, making them unable to suppress AAI when intranasally transferred, unlike wild type Tregs (Lynch *et al.*, 2020). Notably, no difference is observed in lung Tregs when pDCs are depleted in adult mice during viral bronchiolitis, in agreement with our finding that pDCs were not critical for Treg expansion in adult lung inflammation (Lynch *et al.*, 2018). Further research is required to evaluate the effects of pDC depletion in the absence of *A.f.* driven AAI.

Next, we studied cDCs, finding that cDC1s were not required for lung Treg expansion during AAI (Fig. 4.4). This is in line with a previous study, in which cDC1 deficient *Batf3*$^{-/-}$ mice do not display altered Treg induction in a HDM model of AAI (Conejero *et al.*, 2017). But perhaps in contrast with an alternative study, showing *Batf3*$^{-/-}$ mice are not able to induce Ag(OVA)-specific Tregs in a model of inhalational tolerance (Khare *et al.*, 2013). Together, these

reports suggest that the DC subset that promotes Treg expansion is likely to differ between tolerance/steady state models and during ongoing inflammation. Our identification of enhanced lung Th2 cytokines in *Batf3*$^{-/-}$ mice has been shown before, in HDM allergy as well as schistosome infection, and is attributed to steady state IL-12 production by cDC1s antagonising Th2 cytokine expression (Everts *et al.*, 2016; Conejero *et al.*, 2017). Although to a lesser extent, this was replicated in PBS treated mice, with a significant increase in IL-5 and IL-17 expression in *Batf3*$^{-/-}$ mice (data not shown). BATF3 has also been proposed to have a cell-intrinsic role in Tregs, antagonising Foxp3 expression, leading to increased lung Treg proportions in *Batf3*$^{-/-}$ mice at steady state (W. Lee *et al.*, 2017). We did not find an increase in Treg proportion during lung inflammation in *Batf3*$^{-/-}$ mice (Fig.4.4.D), however this was seen in PBS treated mice (data not shown). During lung inflammation there was a significant decrease in the proportion, but not number, of activated (CD69^{+}PD1^{+}) Tregs in *Batf3*$^{-/-}$ mice (Fig.4.4.D). This reduction in activated Tregs could in part explain the increased Th2 response observed in *Batf3*$^{-/-}$ mice.

Taken together, this data implies that CCR2-dependent APCs, pDCs or cDC1s are not vital for Treg expansion during *A.f.* induced AAI. Notably, redundancy is a key mechanism in immunological systems (Dyer *et al.*, 2019), and depleting single DC subsets in this way could lead to another cell type taking on a Treg promoting role. Depletion of multiple subsets sequentially via transgenic crosses, for example *Bdca2*^{DTr} x *Batf3*$^{-/-}$ may address this problem. Notably, depletion of CCR2-dependent cells, pDCs or cDC1s resulted in only partial changes cytokine expression or inflammatory cell responses in *A.f.* driven AAI (Fig. 4.2-4.B&C), as well as not affecting Treg expansion (Fig. 4.2-4.D). It is possible that these subsets are kept anatomically separate from the site of the inflammatory response in the lung, as such segregation is seen at steady state, with cDC1s and cDC2s occupying distinct LN and lung tissue regions (Sung *et al.*, 2006; Thornton *et al.*, 2012; Gerner *et al.*, 2017).This hypothesis could be tested via imaging studies, in which intra-tissue locations of DCs could be established. Since accurate identification of DC subsets requires simultaneous evaluation of multiple markers (Misharin *et al.*, 2013), a multiparameter technique, such as imaging mass cytometry (Giesen *et al.*, 2014), would be preferable.

4.3.2 MGL2^{+} CD11c^{+} cells are required for lung Treg expansion during *A.f.* induced AAI

As stated, depletion of MGL2^{+} CD11c^{+} cells led to a reduction in the number, and proportion of Tregs in the lung, including a decrease in activated CD69^{+}PD1^{+} Tregs (Fig. 4.5.D). To our knowledge, this is first time MGL2^{+} CD11c^{+} cells have been shown to promote Treg responses during ongoing AAI. Using CD11cΔIRF4 mice (Persson *et al.*, 2013; Schlitzer *et al.*, 2013) in which there is a reduction in lung cDC2s, it has been shown that IRF4 dependent DCs are required for lung and lung dLN Treg expansion following influenza infection (Ainsua-Enrich *et al.*, 2019). Again using CD11cΔIRF4mice (Persson *et al.*, 2013; Schlitzer *et al.*, 2013) a recent paper has implicated cDC2s in lung Treg induction at steady state, and MGL2^{+} TNFR2^{+} cDC2s in lung Treg induction in response to intranasal OVA (Mansouri *et al.*, 2020). However, there were a few technical issues with this paper, in particular their description of a

lack of lung Tregs in steady state CD11cΔIRF4 mice, which contradicts previous studies (Ainsua-Enrich *et al.*, 2019), and work later in this thesis (Fig. 5.5). Additionally, to establish lung MGL2$^+$ TNFR2$^+$ cDC2s role in Treg induction, the authors supposedly intranasally transfer 500,000 lung MGL2$^+$ TNFR2$^+$ cDC2s per recipient. With their published sorting strategy and previous upper estimates of total sorted DC yield per mouse lung (100,000) (Bosteels, Lambrecht and Hammad, 2018), these experiments would require the lungs of at least 300 mice, which seems implausible.

In addition to promoting Treg expansion, we found that MGL2$^+$CD11c$^+$ cells were required to maintain type-2 inflammatory responses during *A.f.* driven AAI. The requirement for MGL2$^+$CD11c$^+$ cells to initiate type-2 responses during *A.f.* driven AAI has been shown before following depletion between d5 and d12 of *A.f.* exposure (Cook *et al.,* unpublished). We have extended these results by depleting between d13-d19 of *A.f.* driven AAI, showing that MGL2$^+$CD11c$^+$ cells are required maintain (as well as initiate) type-2 responses (Fig. 4.5B&C). Further research is required to evaluate the effects of MGL2$^+$CD11c$^+$ depletion in the absence of *A.f.* driven AAI. MGL2$^+$CD11c$^+$ cells have previously been shown to be required for the initiation of type-2 immunity in the skin and bladder following exposure to OVA/alum or bacterial infection, respectively (Kumamoto *et al.*, 2013; Jianxuan Wu *et al.*, 2020). The requirement of DCs to maintain, as well as initiate, immune responses may be due to their continued tissue location (Hemann and Legge, 2014). We have shown that DCs expand in the lung tissues during AAI (Fig. 4.1), and colocalization of DCs/T cells in the tissue has been shown to be critical for T cell proliferation, for instance in viral liver infection (Huang *et al.*, 2013).

The ability of a specific DC subset to induce inflammatory and regulatory responses simultaneously has been seen before: in *Batf3*$^{-/-}$ cDC1-deficient mice, a reduction in both inflammatory responses and Neuropilin-1$^-$ Tregs was observed in *Helicobacter pylori* infection (Arnold *et al.*, 2019). Moreover, IRF4 dependent lung cDC2s were required to promote CD8$^+$ T cell memory responses, as well as Tregs, in influenza infection (Ainsua-Enrich *et al.*, 2019). DCs have been shown to be required to promote Ag-specific Th1 IFNγ production, as well as Treg IL-10 production in response to intradermal ear injections of Ag/adjuvant (McLachlan *et al.*, 2009). Notably, the concurrent reduction of Treg and Th2 responses we have observed when MGL2$^+$CD11c$^+$ cells are depleted makes it is unclear whether Treg expansion is via a direct action of MGL2$^+$CD11c$^+$ cells on Tregs, inducing proliferation or *de novo* Treg induction, or indirect via another aspect of inflammation that then promotes or sustains Tregs. The dual role of MGL2$^+$CD11c$^+$ cells in promoting both Treg and Th2 expansion could be due to enhancement of T cell proliferation. A key factor required for Treg expansion *in vivo* and *in vitro* is IL-2 (Fontenot *et al.*, 2005; Singh *et al.*, 2011), the required cellular source of which is unknown during AAI. DCs can provide IL-2 for both T cell and Treg proliferation *in vivo* (Granucci *et al.*, 2001). Alternatively, by expanding IL-2 producing Th2 cells (Ditoro *et al.*, 2018), DCs could indirectly increase IL-2 levels. At steady state, IL-2 production by T cells, and not DCs, is required for splenic Treg homeostasis (Owen *et al.*, 2018). To understand the role of DCs in supporting Treg proliferation, *in vitro* Treg proliferation assays could be

performed, testing the ability of different DC subtypes to support proliferation of existing Tregs (Zou *et al.*, 2010). A potential requirement for Th2 cell help to support Treg proliferation could also be tested in such assays, with IL-4$^+$ Th2 cells and Tregs identified via the use of reporter mice (Pelly *et al.*, 2017), as used in Chapter 3. The role of IL-2 could be studied using conditional IL-2 depletion models (*Il2*$^{fl/fl}$ mice) (Popmihajlov *et al.*, 2012; Owen *et al.*, 2018), crossed to a DC specific (*Cd11c*^{cre} or *Zbtb46*^{cre}) (Caton, Smith-Raska and Reizis, 2007; Loschko, Rieke, *et al.*, 2016), or a T cell specific (*Cd4*^{cre}) (Lee *et al.*, 2001) line. Utilising an IL-13^{cre}IL-2$^{fl/fl}$ (Price *et al.*, 2010; Popmihajlov *et al.*, 2012) cross could reveal a specific requirement for Th2 cell IL-2, although care would have to be taken to exclude the role of other IL-13 expressing cells such as ILCs (Neill *et al.*, 2010).

DCs can promote Tregs by multiple different mechanisms, which we have not assessed here. For instance via expression of the regulatory cytokines TGFβ or IL-10 (Henry *et al.*, 2008; Kushwah *et al.*, 2010), or via expression of enzymes such as RALDH2, or IDO which can catalyse production of the regulatory metabolites RA and kynurenine, which can promote Treg induction (Benson *et al.*, 2007; Mucida *et al.*, 2007; Elias *et al.*, 2008; Manicassamy *et al.*, 2009; Mezrich *et al.*, 2010; Yeste *et al.*, 2016). Finally, DCs may promote Treg induction by cell-cell interactions with co-inhibitory receptors and ligands, such as PDL1 and BTLA (Wang *et al.*, 2008; Francisco *et al.*, 2009; Yogev *et al.*, 2012; Jones *et al.*, 2016). Investigation into the expression of these regulatory factors by DC subsets could greatly increase our understanding of how MGL2$^+$ DCs could contribute to Treg expansion. This could be done by sorting DC subsets, followed by qPCR assessment of gene expression.

Further work is required to be able to definitively state that the reduction in Treg number and proportion observed by depletion of MGL2$^+$ cells is dependent upon MGL2$^+$ cDC2s and does not involve MGL2$^+$ macrophages. Our focus on these cell types only as MGL2 expressors is supported by ssRNAseq studies of murine lung immune cells, which reveal MGL2 expression specific to DCs and macrophages in the lung (Han *et al.*, 2018). In Fig. 4.5.A. we show that MGL2 is expressed by both interstitial and alveolar macrophages, and that these populations were depleted by DTx treatment. There are several observations which argue against the role of lung macrophages in Treg expansion. First, the absolute number of lung macrophages did not change upon depletion of MGL2$^+$ cells, suggesting a potential compensatory expansion of MGL2$^-$ macrophages, which makes it difficult to assert that either interstitial or alveolar macrophages are required for Treg expansion (Fig. 4.5.A). Second, Treg numbers were reduced in the lung dLN as well as the lung (Fig. 4.5.D) suggesting that the cell type responsible for promoting Treg expansion is present in both locations, a criterion which is filled by MGL2$^+$ cDC2s, but not macrophages. Notably, however MGL2 expressing macrophages have been shown to carry out a number of unique functions, including regulation of body weight, and effective wound healing (Kumamoto *et al.*, 2016; Shook *et al.*, 2016). More specific depletion of lung macrophage subsets could be carried out by crossing a macrophage-specific Cre line, to a Cre-inducible DTr line (Buch *et al.*, 2005). However, in many “macrophage-specific” lines (including *LysM*^{cre}, *CD68*^{cre} and *CX3CR1*^{cre}), Cre is also expressed by DCs (McCubbrey *et al.*, 2017). A better strategy could therefore be to utilising

a DC specific inducible depletion line to show DC requirement for Treg expansion. The *Zbtb46* lox stop lox DTr (zDC^{ISIDTR}) line (Loschko, Rieke, *et al.*, 2016), which utilises the DC specific transcription factor *Zbtb46* (Meredith *et al.*, 2012; Satpathy *et al.*, 2012) and can be crossed to a CSF1r^{cre} line for inducible depletion of all DC subsets (Loschko, Rieke, *et al.*, 2016) could be used for this. Ideally, a MGL2^{cre} line would be generated to deplete specifically MGL2^{+} cDC2s by crossing to the zDC^{ISIDTR} line. Failing that, cDC2s could theoretically be depleted by crossing the zDC^{ISIDTR} line to a *Cd11b^{cre}* line (Ferron and Vacher, 2005). The relative abilities of MGL2^{+} macrophages and DCs to promote Treg expansion could be tested in *in vitro* Treg induction assays, as in Fig.4.6, or in Treg proliferation assays as discussed above. Taken together, dissecting the relative contributions of MGL2^{+} macrophages vs MGL2^{+} cDC2s in Treg expansion is possible, though not simple, with DC specific depletion, being potentially the most technically feasible.

Although recent work, especially single-cell RNA sequencing studies, has revealed the cDC2 compartment to be heterogenous, it is still unclear whether this is dictated by development, maturation, or location (Han *et al.*, 2018; Brown *et al.*, 2019; Bosteels *et al.*, 2020; Maier *et al.*, 2020). Notably, we were not able to deplete, and therefore investigate, the role of MGL2^{-} cDC2s in our studies. A recent sequencing study suggested that expression of the transcription factor Tbet could distinguish 'more regulatory' Tbet^{+} cDC2s from a 'more inflammatory' Tbet^{-} cDC2 subpopulation, with MGL2^{-} cDC2s being predominantly found within the 'regulatory' Tbet^{+} population (Brown *et al.*, 2019). Notably, despite being considered regulatory by gene expression profile, Tbet^{+} cDC2s do not display enhanced Treg induction capability (Brown *et al.*, 2019). Notably, other proposed cDC2 deficient models do not deplete all lung cDC2 populations: CD11cΔIRF4 mice are only deficient in CD24^{+} or MGL2^{+} CD11b^{+} cDC2s (Gao *et al.*, 2013; Persson *et al.*, 2013; Ainsua-Enrich *et al.*, 2019), while *Cd11c^{cre}Klf4$^{fl/fl}$* mice also are only deficient in the double positive CD24^{+}CD11b^{+} lung DC population, which were also identified to be MGL2^{+} (Tussiwand *et al.*, 2015). Novel DC depletion models are therefore required to target the MGL2^{-} cDC2 DC subset. Crossing *Tbet^{cre}* with the zDC^{ISIDTR} line could potentially achieve this aim, as only *Tbet^{+}* cDC2s, which are MGL2^{-}, would be expected to co-express Zbtb46 and Tbet (Loschko, Rieke, *et al.*, 2016; Brown *et al.*, 2019).

4.3.3 cDC2s isolated *ex vivo* from *A.f.* induced AAI do not display enhanced Treg induction ability *in vitro*

Despite the requirement for MGL2^{+}CD11c^{+} cells, for Treg induction during *A.f.* driven AAI, we observed no difference between the Treg induction capabilities of *ex vivo* isolated MGL2^{+} cDC2s (Fig.4.6).

This result may indicate that MGL2^{+} cDC2s do not have an intrinsic ability to induce higher levels of Tregs, and the requirement for MGL2^{+}CD11c^{+} cells for Treg expansion *in vivo* may be due to an alternative mechanism. For instance, MGL2^{+} cDC2s could have a superior role in provoking survival or proliferation of existing Tregs, which could be tested by incubating sorted DC subsets with Tregs. Alternatively, Treg proliferation *in vivo* could be measured by

assaying markers of the cell cycle, such as Ki67, or by injection of BrDU, a synthetic nucleotide that incorporates into cells undergoing the synthesis phase of the cell cycle (Jang *et al.*, 2017). The mechanism by which DCs promote Treg expansion may also change over time, and it is possible that DCs sorted from d19 of *A.f.* driven AAI are no longer expressing factors that promote Treg expansion, and that repeating the assay at an earlier timepoint, day 12 for instance, may be required.

Another reason for the discrepancy between the ability of MGL2$^+$ cDC2s to drive Treg expansion *in vivo* and *in vitro* could be the presence of tissue specific factors. Another lung myeloid cell type, alveolar macrophages, have enhanced responsiveness to type-2 stimuli when taken out of their lung microenvironment, suggesting a niche-specific factor is regulating their responsiveness (Svedberg *et al.*, 2019). It is possible that lung DCs also show altered function when taken out of their microenvironment, which could be accounted for by adding back in lung specific factors. We have shown that increasing levels of active TGFβ lead to enhanced Treg induction (Fig. 4.6). However, by adding in the active form of this cytokine, we could be bypassing a potential DC subset specific role in activating TGFβ, which is abundant in the lung in its inactive form (Flanders *et al.*, 2016). As we have shown in Fig. 4.7. CD11c$^+$ cell expression of the TGFβ activating integrin αVβ8 (Travis *et al.*, 2007) is required to promote lung Treg expansion. It is not yet known if there is DC subset-specific expression of αVβ8 during AAI, as discussed further in section 4.3.4. However, if there is subset-specific expression, addition of exogenous inactive TGFβ to the *in vitro* DC Treg induction assays could be required to reveal true differences in Treg induction potential of DC subsets. DCs have been shown to induce Tregs via expression of enzymes (RALDH2 and IDO) that catalyse the production of the regulatory factors RA and kynurenine (Chen *et al.*, 2008; Khare *et al.*, 2013). Addition of the precursors (retinal and tryptophan) of these factors into the *in vitro* system may be required to reveal subset specific differences in Treg induction ability.

Finally, instead of relying upon *in vitro* induction strategies, adoptive transfer assays could be used to assess sufficiency of MGL2$^+$ cDC2s for Treg expansion *in vivo*. MGL2$^+$ cDC2s isolated from mice during *A.f.* driven AAI, could be intranasally transferred, and lung Treg expansion assayed (Plantinga *et al.*, 2013), although sorting sufficient numbers of MGL2$^+$ cDC2s could be a technical challenge, as discussed previously (Bosteels, Lambrecht and Hammad, 2018; Mansouri *et al.*, 2020).

4.3.4 αVβ8 expression on CD11c$^+$ cells is required for Treg lung expansion

In Fig. 4.7. we showed CD11c$^+$ cell αVβ8 expression was important for Treg lung expansion during *A.f.* driven AAI. This is the first study to show Treg dependency on CD11c$^+$ cell αVβ8 expression during lung AAI. In a model of Treg-dependent resolution of LPS driven lung inflammation, mice with myeloid cell deficiency of αV integrins have reduced Treg proportions, and are unable to resolve lung inflammation (Zhang *et al.*, 2020). Cd11cΔαVβ8 mice have been used previously in a study of OVA/alum driven AAI, in which the authors also find a requirement for αVβ8 for Th17 cell expansion (Kudo *et al.*, 2012). However, this study does not quantify Treg number, and there is a non-significant trend for a decrease in Treg

proportion in Cd11cΔαVβ8 mice (Kudo *et al.*, 2012). The difference in Treg results between that study and our work could be due to differences in models, as different allergens and dosing regimens were employed in this published study, and the work presented in this thesis. Alternatively this could be due to microbiota differences between mice housed in different institutes: Treg frequencies in the lamina propria have been shown to differ between C57BL/6 mice, depending upon where the mice have been housed, and therefore their microbiota (Ivanov *et al.*, 2008). The lung microbiota is also dependent upon where mice have been housed, and therefore it is feasible that it may influence Treg expansion (Dickson *et al.*, 2018).

As stated in the previous section, to link the findings of this chapter, a key next step will be to understand which $CD11c^+$ cell types express αVβ8 during *A.f.* induced AAI. Referring to the ImmGen microarray and RNAseq datasets, very low αVβ8 expression has been shown on lung cDC1s and macrophages at steady state (Heng *et al.*, 2008). Recent work utilising a novel αVβ8 reporter mouse, has shown that it is specifically expressed by migratory cDC1s in the mesenteric LNs, and not peyers patches, at steady state and during rotavirus infection (Nakawesi *et al.*, 2020). In Fig. 4.7. the reduction in Treg number and proportion was only observed in the lung, and not the lung dLN, suggesting that αVβ8 expression by $CD11c^+$ cells may also be location dependent during *A.f.* driven AAI. Upregulation of DC αVβ8 expression in response to inflammatory stimuli has been observed in the lung in response to intratracheal LPS and apoptotic cells (Zhang *et al.*, 2020), supporting dynamic expression of αVβ8 in this tissue site. αVβ8 expression levels could be determined by cell sorting and qPCR on DC or macrophage subsets or by exposing the recently developed αVβ8 reporter mouse to *A.f.* driven AAI (Nakawesi *et al.*, 2020).

In summary, in this chapter we set out to define the role of DCs in promoting Treg expansion during fungal AAI. We have shown that $MGL2^+CD11c^+$ cells, likely cDC2s, were required to induce Treg expansion during AAI. This subset was also responsible for maintaining the type-2 response, implying that a single DC subset is responsible for both the pro- and anti-inflammatory balance in AAI. Unexpectedly, lung cDC2s isolated *ex vivo* did not show enhanced ability for *de novo* Treg induction *in vitro*, suggesting either the assay needs to be adjusted to better reflect the lung environment, or that $MGL2^+$ cDC2 dependent *in vivo* Treg expansion is reliant upon Treg an alternative mechanism, for instance proliferation of existing Tregs. Finally, we have shown that $CD11c^+$ cell αVβ8 expression was required for lung Treg expansion, although future work will be required to unequivocally identify the pulmonary $CD11c^+$ cell type that expresses αVβ8.

In the next chapter, we will investigate how immune responses are regulated in another model of type-2 pulmonary inflammation – schistosomiasis.

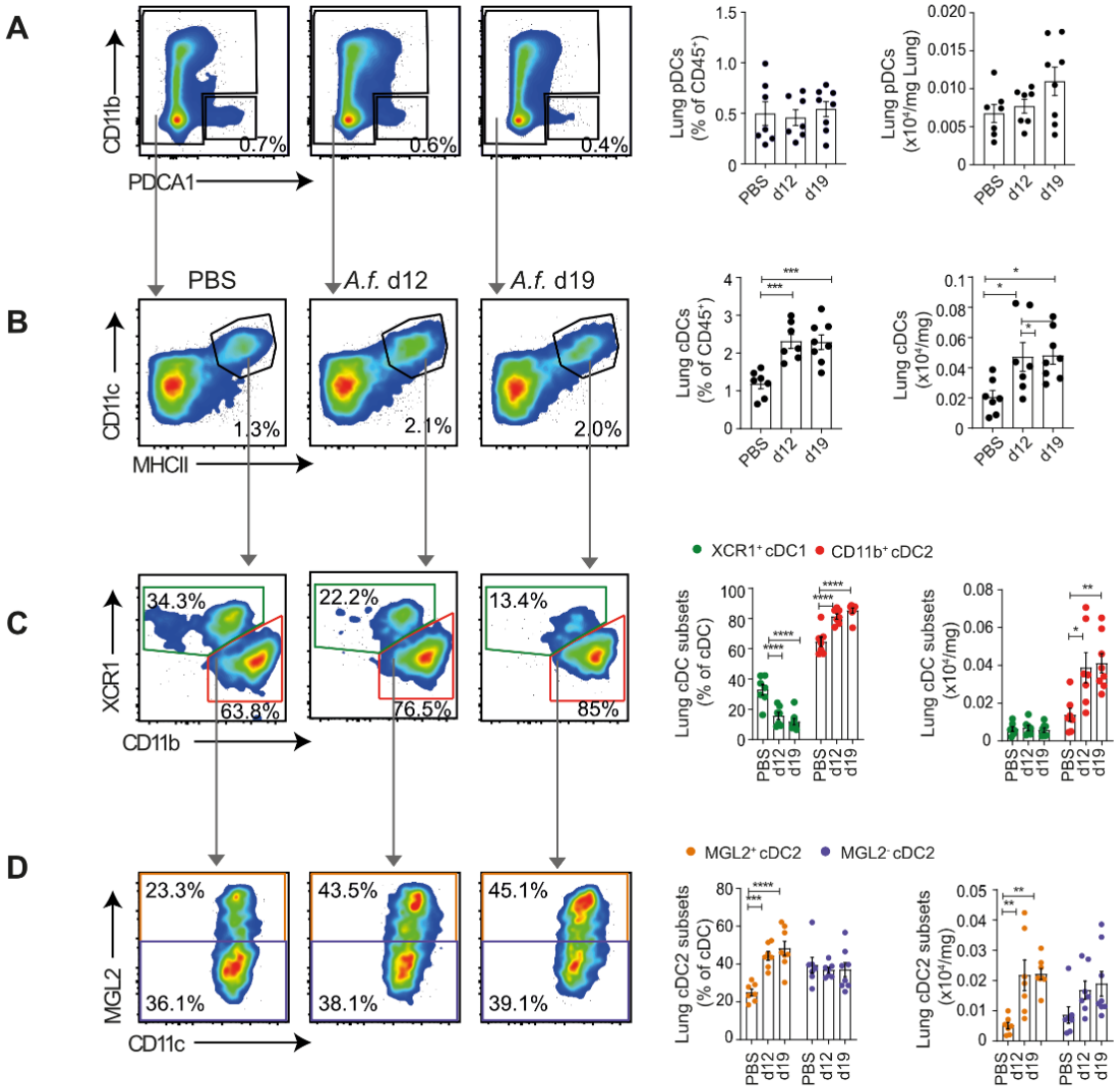

Figure 4.1. Expansion of cDC2s in *A.f.* driven AAI

C57BL/6 (VertX) mice were exposed to 0.4x10^6 *A.f.* spores or PBS i.n. 3x per week. At days 12 and 19 lungs were taken. A) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict pDCs (PDCA1$^+$) post exclusion of doublets, dead cells, Lineage$^+$ (CD3, CD19, Ly6G, Ter119, NK1.1) cells, macrophages, neutrophils, and eosinophils, with gate frequencies showing % of CD45$^+$ cells. B) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict cDCs (CD11c+ MHCII$^+$) with gate frequencies showing % of CD45$^+$ cells. C) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict gating of green cDC1s (XCR1$^+$) and red cDC2s (CD11b$^+$), with gate frequencies showing % of cDCs. D) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict gating of orange MGL2$^+$ cDC2s and pink MGL2$^-$ cDC2s, with gate frequencies showing % of cDCs. Data is from 2 experiments (n=7-8 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Tukey's HSD test was used.*=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

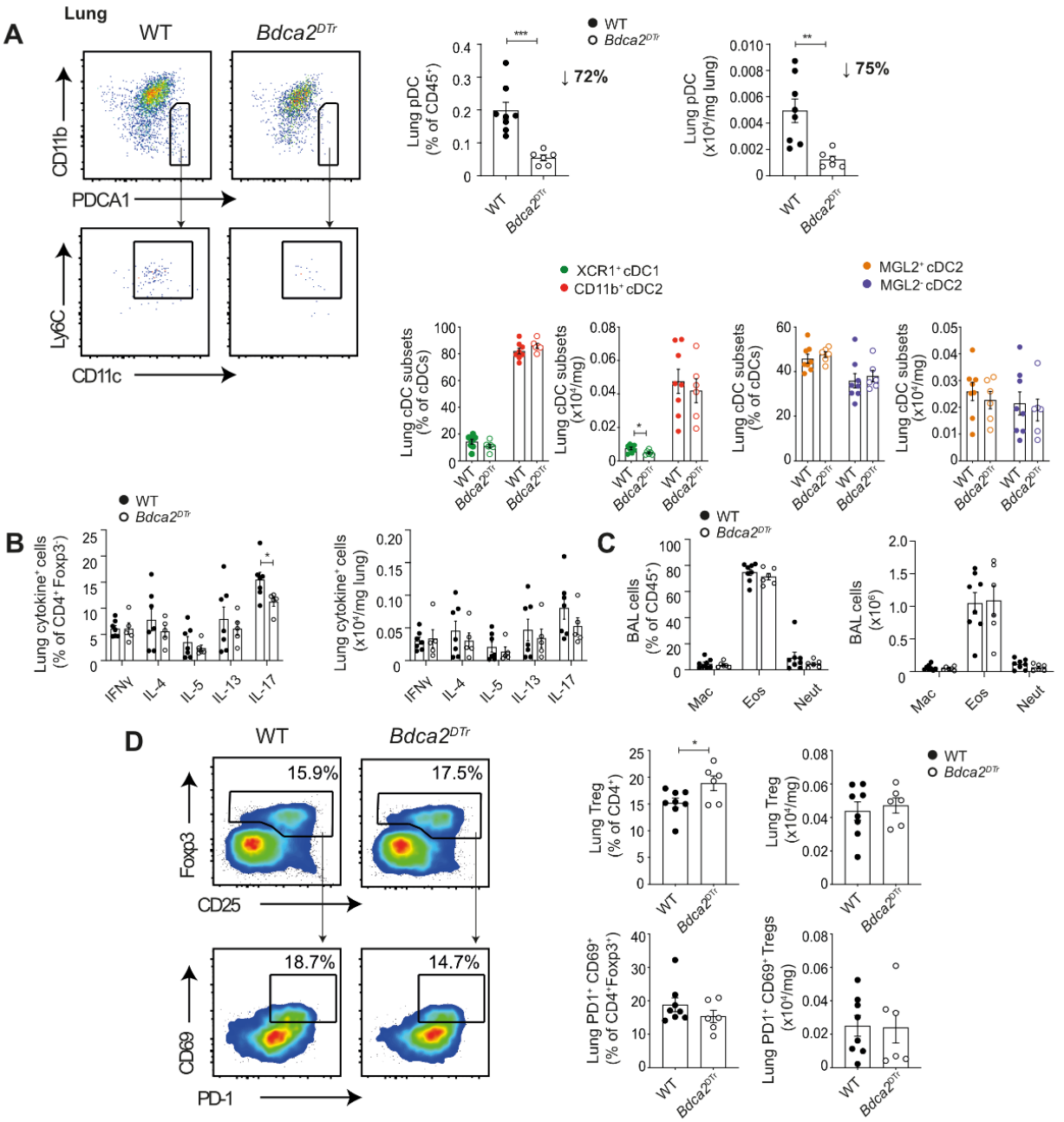

Figure 4.2. pDCs are not required for lung Treg expansion

Bdca2^{DTr} or WT controls were exposed to 0.4x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At day 19 lungs were taken. On day 13, 15 and 17 mice were dosed with DTx A) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots show changes in cDC populations, as well as pDCs (gated as Fig. 4.1, with an additional Ly6C$^+$ CD11c$^+$ gate included to ensure purity of the pDC population). Gate frequencies show, respectively, % of cDCs and % of CD45$^+$ cells. Absolute and proportional depletion is quantified. B) Lung cell isolates were stimulated with PMA/ ionomycin, and intracellular cytokines assessed by flow cytometry. C) BAL cell isolates were assessed by flow cytometry. D) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict gating of Tregs, as well as PD1$^+$CD69$^+$ cells withing the Treg population. Data is from 2 experiments (n= 7-8 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test (pairwise) were used. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

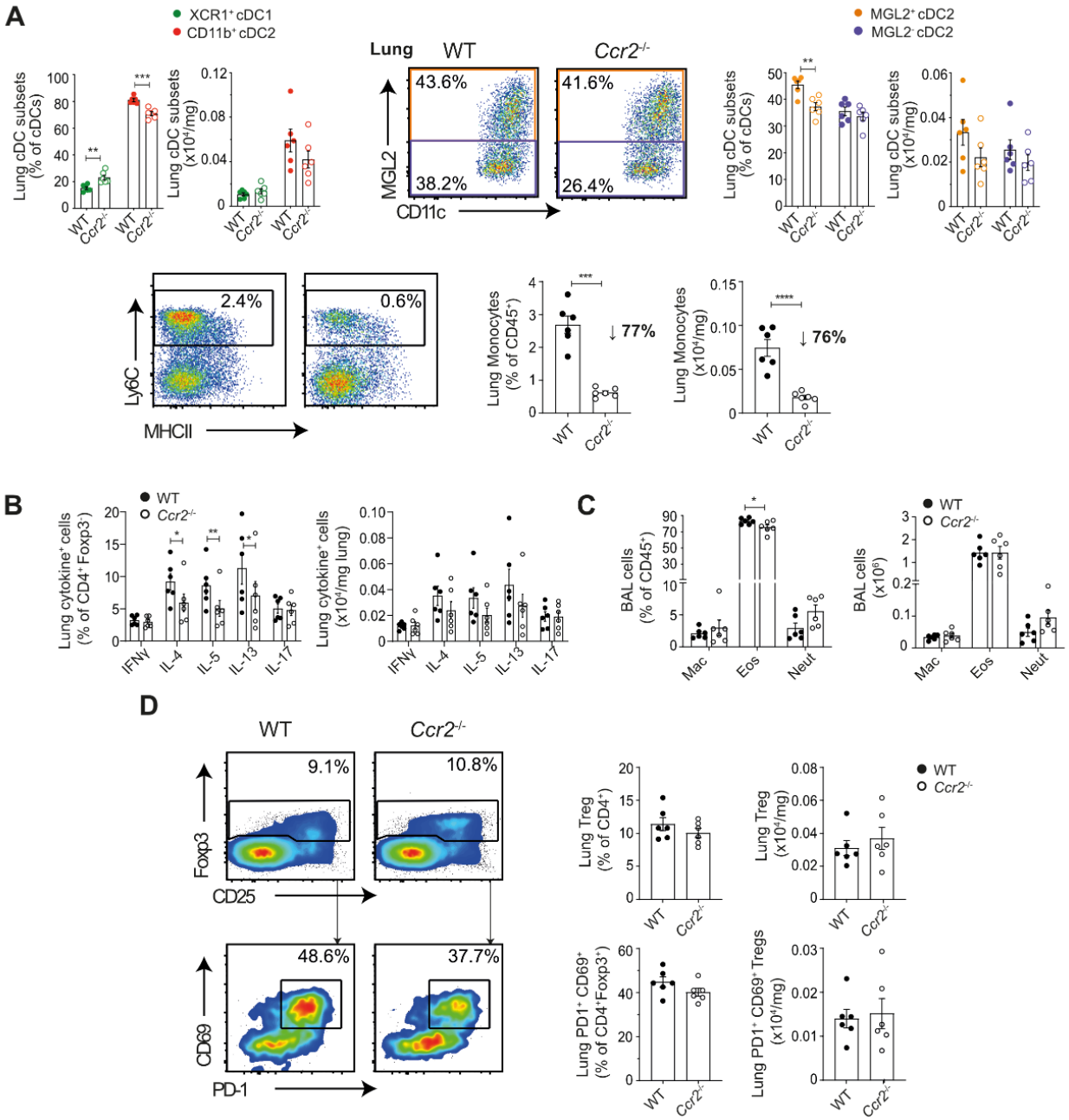

Figure 4.3. CCR2-dependent myeloid cells are not required for lung Treg expansion

Ccr2$^{-/-}$ or WT controls were exposed to 0.4x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At day 19 lungs were taken. A) Lung cell isolates were assessed by flow cytometry. cDCs (CD11c$^+$ MHCII$^+$) and monocytes (CD11b$^+$ CD64$^+$) were gated post exclusion of pDCs, as shown in Fig. 4.1. for cDCs, with monocytes further characterised by Ly6C expression, and cDCs further subsetted by XCR1, CD11b and then MGL2 expression. Representative flow cytometry plots show changes in cDC populations, as well as Ly6C$^+$ monocytes. Gate frequencies show, respectively, % of cDCs and % of CD45$^+$ cells. Absolute and proportional depletion is quantified. B) Lung cell isolates were stimulated with PMA/ionomycin, and intracellular cytokines assessed by flow cytometry. C) BAL cell isolates were assessed by flow cytometry. D) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict Tregs, as well as PD1$^+$CD69$^+$ cells within the Treg population. Data is from 2 experiments (n= 6 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test was used. *=P <0.05, **=P <0.01, ***=P<0.001, ****=P<0.0001.

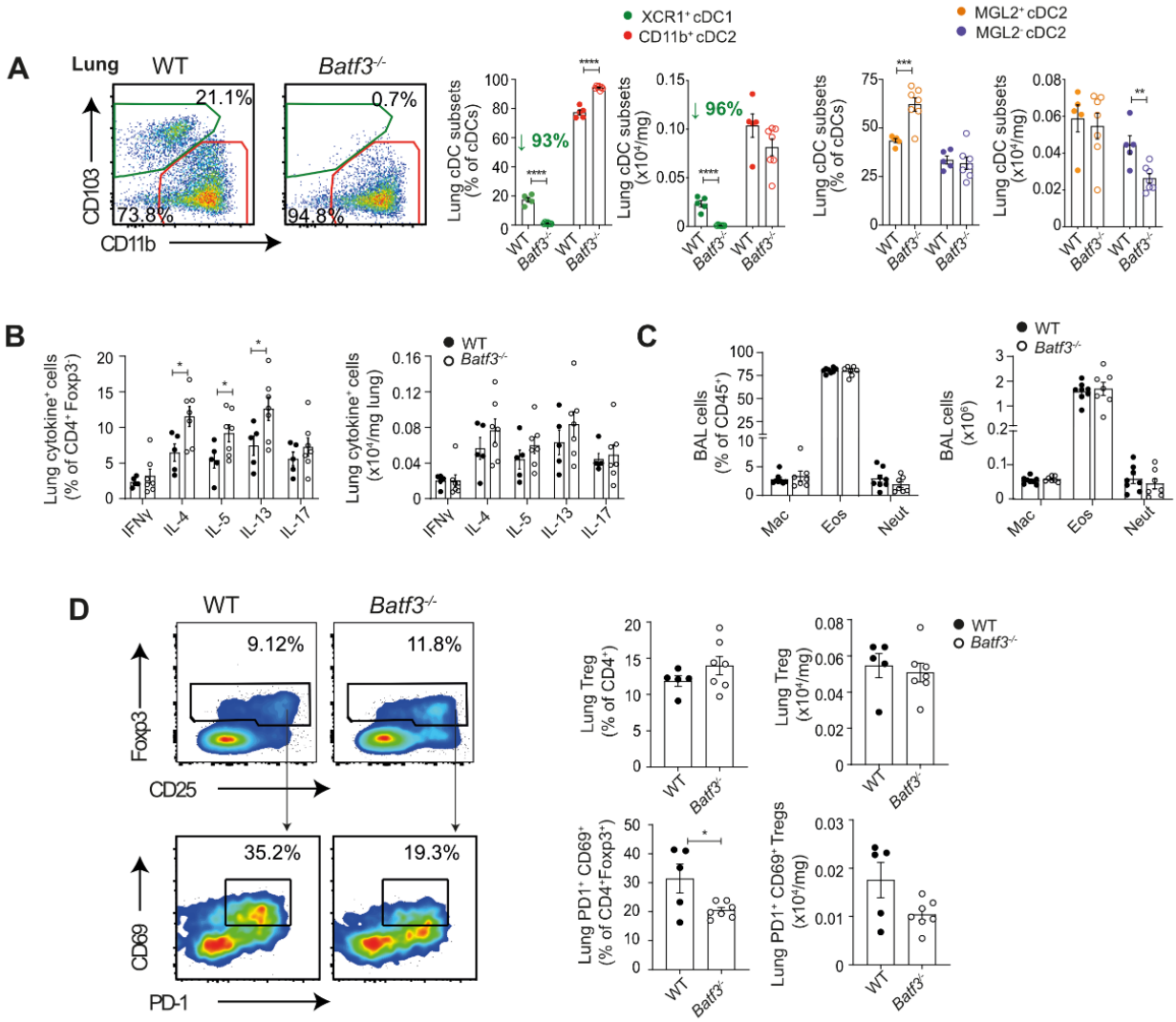

Figure 4.4. cDC1s are not required for lung Treg expansion

Batf3$^{-/-}$ or WT controls were exposed to 0.4x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At day 19 lungs were taken. A) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots show changes in DC populations (gated as described in Fig. 4.1). Gate frequencies show % of cDCs. Absolute and proportional depletion of cDC1s is quantified. B) Lung cell isolates were stimulated with PMA/ionomycin, and intracellular cytokines assessed by flow cytometry. C) BAL cell isolates were assessed by flow cytometry. D) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict gating of Tregs, as well as PD1$^+$CD69$^+$ cells withing the Treg population. Data is from 2 experiments (n= 5-7 per group for all except BAL analysis, in which n=8-7 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test (pairwise) was used.*=P<0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

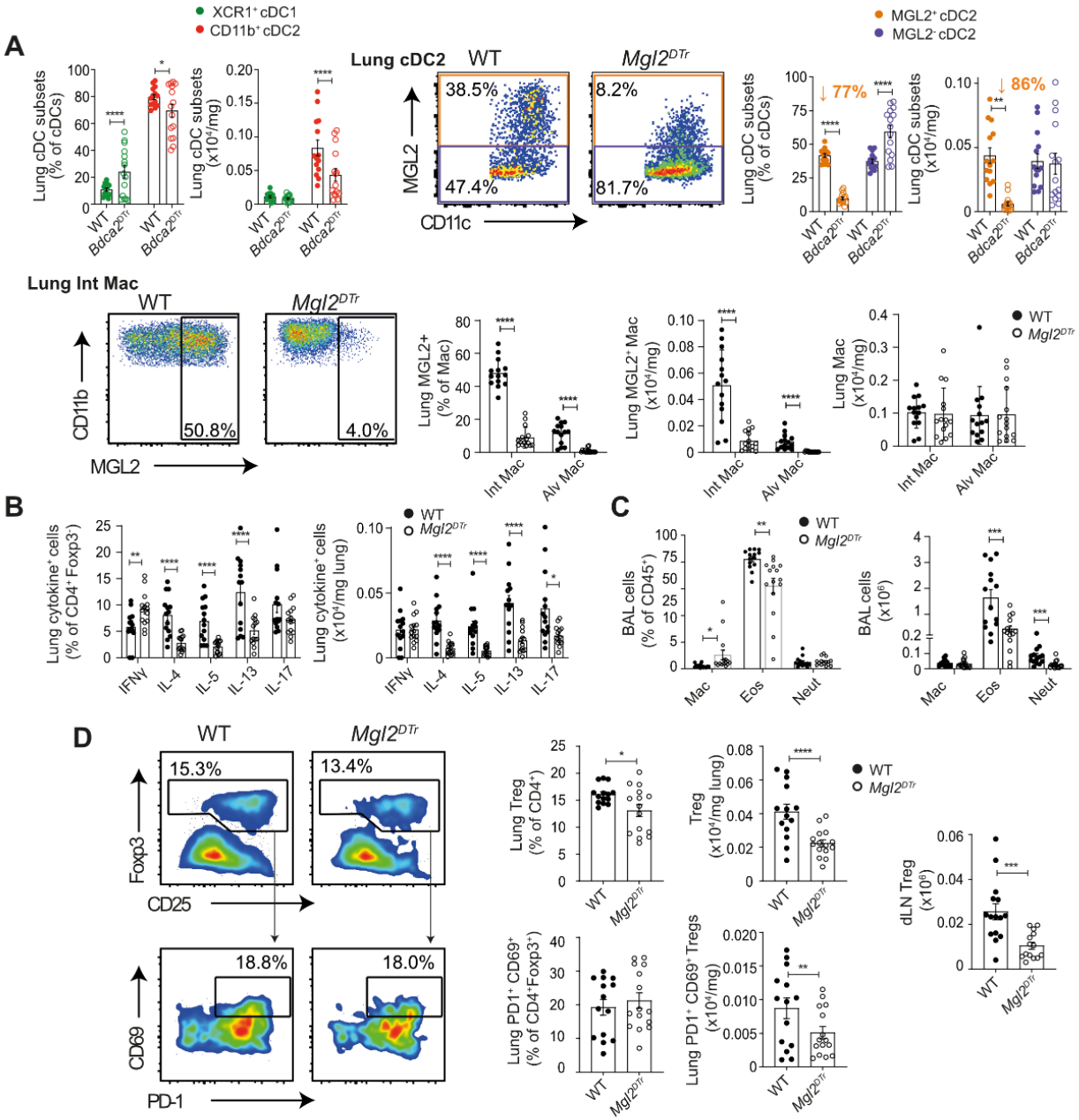

Figure 4.5. MGL2⁺ cD11c⁺ cells are required for lung Treg expansion

MGL2^{DTr} or WT controls were exposed to 0.4x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At day 19 lungs were taken. On day 13 and 16 mice were dosed with DTx A) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots showchanges in cDC populations (gated as described in Fig. 4.1), as well as macrophages (gated out prior to DCs, as CD64⁺ MerTK⁺, then interstitial CD11b⁺, alveolar SiglecF⁺). Gate frequencies show, respectively, % of cDCs and % of interstitial macrophages. Absolute and proportional depletion is quantified for MGL2⁺ cDC2s. B) Lung cell isolates were stimulated with PMA/ionomycin, and intracellular cytokines assessed by flow cytometry. C) BAL cell isolates were assessed by flow cytometry. D) Lung and lung dLN cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict gating of Tregs, as well as PD1⁺CD69⁺ cells withing the lung Treg population. Data is from 3 experiments (n= 14-15 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test (pairwise) was used. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

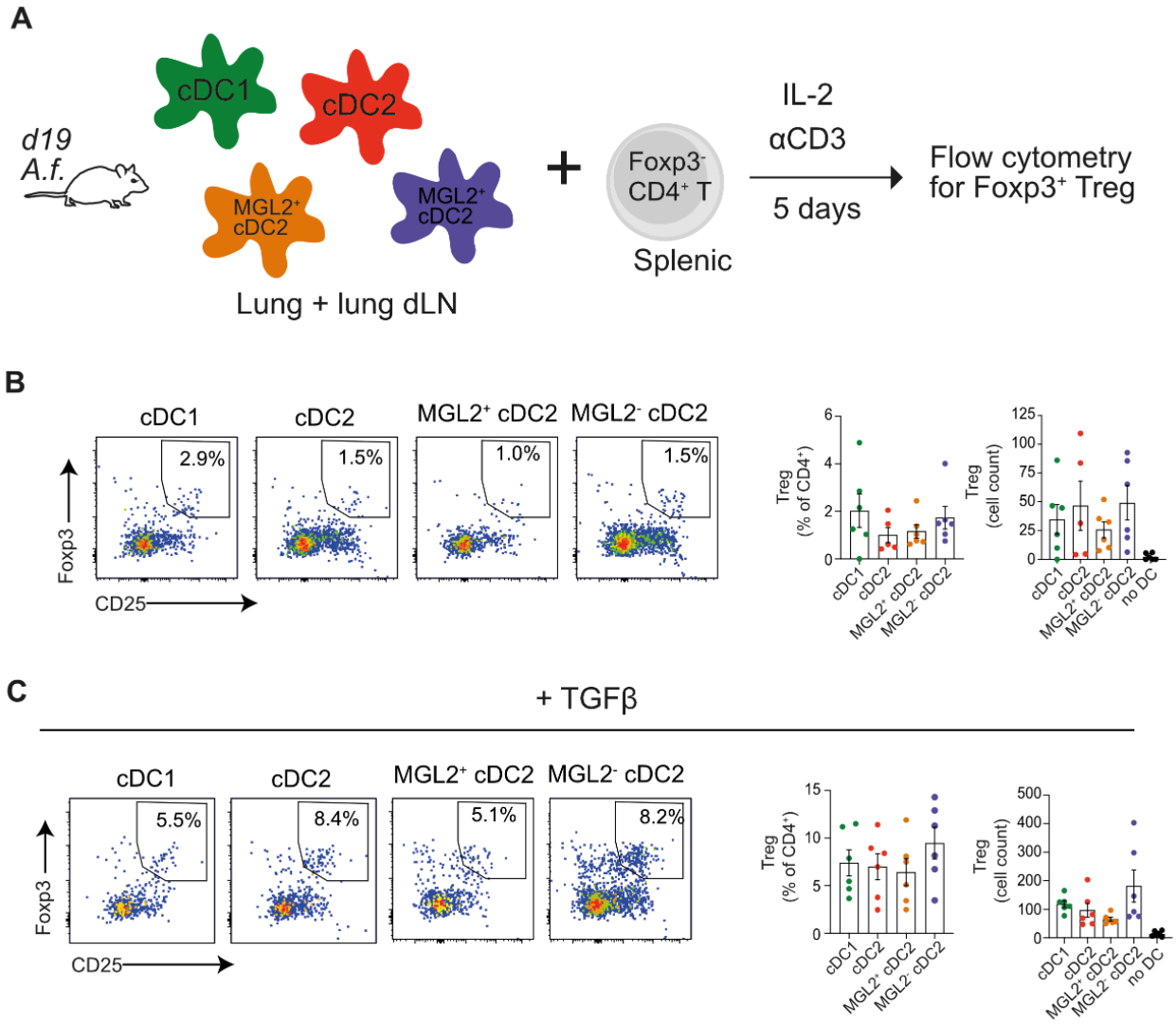

Figure 4.6. DC subsets isolated from lungs and lung dLNs at d19 of *A.f.* driven AAI show comparable *in vitro* Treg induction capability, enhanced uniformly by TGFβ

C57BL/6 mice were exposed to 0.4x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At day 19 lung and lung dLNs were taken. Splenic Foxp3$^-$ CD44$^-$ CD4$^+$ T cells were sorted from naive Foxp3$^{YFP/Cre}$ R26^{RFP635} IL-4^{GFP} mice, based Foxp3^{YFP} negativity. A) Sorted DCs were incubated with sorted Foxp3$^-$ splenic T cells, anti-CD3 and IL-2 for 5 days. To some wells TGFβ was added. (B) Representative flow cytometry plots show Foxp3 induction without exogenous TGFβ as % of CD4$^+$ cells when incubated with the stated DC subsets. Treg induction as a proportion of the CD4$^+$ T cell population, and as cell number is quantified. (C) Representative flow cytometry plots show Foxp3 induction with exogenous TGFβ as % of CD4$^+$ cells when incubated with the stated DC subsets. Treg induction as a proportion of the CD4$^+$ T cell population, and as cell number is quantified. Data is from 2 experiments (n= 5-6 per group).

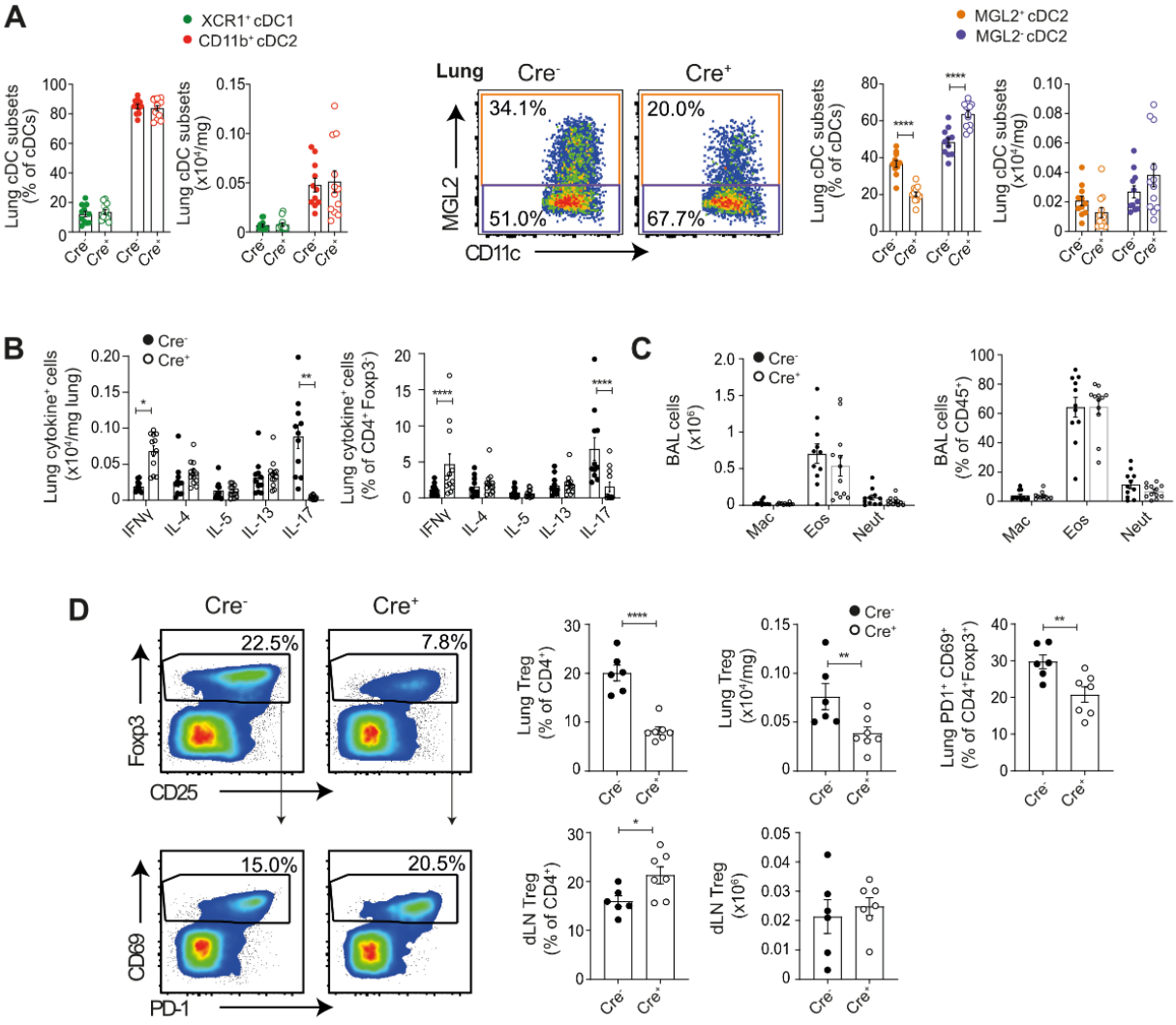

Figure 4.7. αvβ8 expression on CD11c⁺ cells is required for lung Treg expansion during *A.f.* driven AAI

CD11c^{cre} αvβ8^{fl} mice were exposed to 0.4x10^6 *A.f.* spores or PBS *i.n.* 3x per week. At day 19 lungs were taken. A) Lung cell isolates were assessed by flow cytometry. Representative flow cytometry plots show changes in cDC populations (gated as described in Fig. 4.1). Gate frequencies show % of cDCs. B) Lung cell isolates were stimulated with PMA/ionomycin, and intracellular cytokines assessed by flow cytometry. C) BAL cell isolates were assessed by flow cytometry. D) Lung and lung dLN cell isolates were assessed by flow cytometry. Representative flow cytometry plots depict gating of Tregs, as well as PD1$^+$CD69$^+$ cells within the lung Treg population. Data is from 3 experiments (n= 11-12 per group), except for Treg data, which is from 2 experiments (n= 6-7 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test was used. *=P <0.05, **=P <0.01, ***=P <0.001 ***=P <0.001, ****=P<0.0001.

Chapter 5

Pulmonary immune responses in murine and human *S. mansoni* infection

Chapter 5 - Pulmonary immune responses in murine and human *S. mansoni* infection

5.1 Introduction

Type-2 immune responses are thought to have evolved in response to infection with large, tissue damaging parasites (Allen and Sutherland, 2014). In order to gain a more comprehensive understanding of the regulation of pulmonary type-2 immune responses we therefore moved to a different model – studying lung immune responses in *S. mansoni* infection.

Pulmonary manifestations of schistosomiasis must be considered in the context of the life cycle stage of the parasite, within the mammalian host. During larval migration, schistosomula transit through the lungs before maturing to adulthood in the mesenteric vasculature, where pairing and egg production begins (McManus *et al.*, 2018). Although the timescale of schistosome migration in the human lung is unknown, in baboons *S. mansoni* first arrives in the lung at day 2, peaking at day 5, with the majority of the worms having left by day 9 (Wilson *et al.*, 1990). In human acute schistosomiasis, which occurs from week 2-10 post infection, pulmonary symptoms include cough and shortness of breath (Schwartz, Rozenman and Perelman, 2000). These symptoms may occur prior to, and independent, of egg production and have been proposed to be an immune mediated response to migrating or maturing larvae (Schwartz, Rozenman and Perelman, 2000; Lucas *et al.*, 2012; Cnops *et al.*, 2020). Pulmonary manifestations can continue post egg production in the acute phase of schistosomiasis, as well as in the chronic phase (post 10-12 weeks) (Gobbi *et al.*, 2020). In this chronic phase eggs can transit to the lungs, leading to focal inflammation and contributing to potentially fatal pulmonary arterial hypertension (Shaw and Ghareeb, 1938; Gobbi *et al.*, 2017).

Thus far, there have been no comprehensive immunological studies on the human immune response to lung migrating schistosomula, in the first 3 weeks of infection. Our understanding of immune responses from week 4 onwards has been increased by controlled human infection studies, in which individuals were infected with single sex (male) schistosomes, to eliminate the possibility of egg induced pathology. This study found a mixed type-1/type-2 inflammatory response at 4-12 weeks post infection, with enhanced serum levels of IFNγ induced protein 10 (IP-10) as well as an increase in Ag-specific Th2 cytokine (IL-4, IL-5 and IL-13) producing $CD4^+$ T cells in the blood at week 4 post infection, correlated to acute symptoms such as fever at week 4 (Langenberg *et al.*, 2020). An observational study of naturally infected individuals in Brazil revealed enhanced secretion of pro-inflammatory cytokines IL-1, IL-6, and TNFα from peripheral blood mononuclear cells (PBMCs) of patients with acute schistosomiasis, in comparison to those with chronic schistosomiasis (de Jesus *et al.*, 2002). Although informative, the aforementioned studies utilise systemic outputs, rather than specifically measuring lung responses. There are a small number of studies that

measure lung responses directly, for instance one study used transbronchial biopsy in a patient with acute schistosomiasis to reveal eosinophilic inflammation in pulmonary airspaces (Schwartz, Rozenman and Perelman, 2000). During chronic schistosomiasis, which can continue for years post infection, autopsy studies have revealed eosinophils and other leukocytes surrounding ova and worms in the lung (Shaw and Ghareeb, 1938). There have been no studies utilising advanced immunological techniques, such as flow cytometry or multiplex cytokine analysis in lung samples during human acute and chronic pulmonary schistosomiasis. This is in comparison to the detailed and refined understanding of immune responses seen today in other conditions such as asthma, in which multiple human lung flow cytometric and even ssRNAseq studies exist (Vidal *et al.*, 2012; Kuo *et al.*, 2017; Vieira Braga *et al.*, 2019).

Our understanding of pulmonary immune responses in schistosomiasis is informed by a small number of murine studies. During *S. mansoni* infection, schistosomula enter the murine lung from day 4, peaking at days 10-12, with most having left by day 20 (Wheater and Wilson, 1979; Wilson, Coulson and Dixon, 1986). Acute schistosomiasis has conventionally been regarded as a Th1 dominated process (Pearce *et al.*, 1991). However, more recent work has revealed a low level mixed Th1/Th2 response to migrating *S. japonicum* larvae, characterised by eosinophilia (Burke *et al.*, 2011), and an increase in thoracic LN Th2 cell IL-4 expression in the first 3 weeks of *S. mansoni* infection (Redpath *et al.*, 2015). Pulmonary immune responses have also been studied histologically in chronic murine schistosomiasis, in which egg deposition in the lung peaks at week 17 (Crosby *et al.*, 2010). In these studies there was a mixed type-1/type-2 response observed, with increases lung IL-12p70 and TNFα, as well as IL-4, with a positive correlation between pulmonary egg numbers and lung mRNA expression of the Th2 cytokine IL-13 (Crosby *et al.*, 2015). Notably, as in human pulmonary schistosomiasis, there is still a lack of detailed understanding of murine pulmonary immune responses to schistosomiasis. This understanding is required to underpin any future translational research, utilising murine models to gain mechanistic understanding of what might constitute a protective immune response.

Conventional DCs have been shown to be required to induce systemic and hepatic Th2 responses in chronic schistosomiasis (Phythian-Adams *et al.*, 2010; Lundie *et al.*, 2016). During schistosome infection cDCs upregulate costimulatory markers, and when the entire DC population is depleted, utilising $CD11c^{DTr}$ mice, at week four post *S. mansoni* infection, the onset of egg production, reduced systemic and liver Th2 cytokine production is observed (Phythian-Adams *et al.*, 2010; Lundie *et al.*, 2016). Depletion of pDCs did not alter Th2 induction during schistosomiasis, suggesting cDCs are the critical cell type (Lundie *et al.*, 2016). There is evidence for specific roles of cDC subsets in schistosome infection, in particular, IRF4 dependent cDC2s may be crucial for promoting responses to schistosomes, with one study showing that development of Th2 responses to injected eggs in the small intestine was specifically dependent on cDC2s, and another revealing that KLF4 dependent cDC2s were required for survival in *S. mansoni* infection (Tussiwand *et al.*, 2015; Mayer *et al.*, 2017). From these studies, in line with previous work in other type-2 models, it is clear

that cDC2s are heterogenous, with depletion of particular subsets of DCs possible by different models, for instance $MGL2^+$ cDC2s being shown to be reliant on KLF4 and IRF4 expression, and required for the development of type-2 responses (Gao *et al.*, 2013; Tussiwand *et al.*, 2015). In contrast, cDC1s are not required for the development of Th2 responses during *S. mansoni* infection (Everts *et al.*, 2016). There is evidence for functional changes in cDCs during human chronic *S. haematobium* infection, where circulating cDCs were reduced, and found to be functionally impaired (Everts *et al.*, 2010; Nausch *et al.*, 2012). It is now understood that different subsets of human DCs may take on different functional roles, however studies of human DCs during schistosomiasis are yet to take this into account, confounding our interpretation (Collin and Bigley, 2018). It is not known how pulmonary DCs are affected, either in mice or humans, during lung migratory or patent (egg-producing) phases of schistosomiasis. Sampling pulmonary DCs in humans is a challenge, however utilisation of sputum, an accessible proxy for lung immune responses, could reveal their role during pulmonary schistosomiasis. This approach has been used in other pulmonary conditions, such as asthma, in which increases in Th2 cells were observed in PBMC, sputum and bronchial biopsy samples, supporting the idea of sputum as a readout of lung responses (Hinks *et al.*, 2015)

5.1.1 Key questions:

The overall hypothesis of this chapter was that schistosome infections would lead to an inflammatory response, that would differ between lung migratory and patent phases of infection and be observable in both human and murine infection. My specific questions were:

4. How does pulmonary inflammation change during lung migrating and patent schistosomiasis?
5. How does murine and human pulmonary inflammation compare during schistosome infection?
6. What is the role of DCs in schistosome mediated lung inflammation?

5.2. Results

5.2.1 Expansion of lung cDC2s and Th2 immune responses in lung migrating and patent murine *S. mansoni* infection.

To understand the immune response in lung migrating schistosomiasis, mice were infected percutaneously with 180 cercariae, with lung immune responses measured at day 21 (Fig. 5.1.A). Previous studies on lung migrating schistosomiasis often use doses of 450 cercariae, whereas studies on patent infection more typically infect with 40-80 cercariae, to avoid overt immunopathology (Crabtree and Wilson, 1986; Phythian-Adams *et al.*, 2010). The chosen dose, 180 cercariae, was a compromise between these two approaches, provoking a relatively consistent, and therefore mechanistically dissectible immunological phenotype. This timepoint was chosen to follow the peak of lung migration, day 10-12, and therefore allow for development of adaptive lung immune responses to the migrating larvae (Wheater and Wilson, 1979; Wilson, Coulson and Dixon, 1986).

Firstly, we assessed overall inflammatory responses to lung migrating schistosomula. As a basic readout of inflammation, we assessed lung and BAL cellularity at d21 (Fig. 5.1.B). There was no significant difference in lung cellularity post infection, however there was an approximately twofold increase in cell number in the BAL (Fig. 5.1.B). To understand why BAL cellularity was increasing, we measured macrophages, eosinophils and neutrophils, a significant increase in eosinophils was observed, both proportionally and by cell number in response to lung migrating schistosomula (Fig. 5.1.C). A slight but significant increase in BAL macrophage number was also observed, but there was no change in neutrophils (Fig. 5.1.C). Soluble immune mediators associated with type-2 inflammatory responses, Ym-1, Relmα, CCL17 and CCL22 were measured (Fig. 5.1.D). Whilst all showed at least a modest trend to increase at d21, only Relmα, a protein released from macrophages in during type-2 inflammation (Nair, Cochrane and Allen, 2003; Svedberg *et al.*, 2019), was significantly increased at d21 of lung migratory schistosomiasis (Fig. 5.1.D). To understand adaptive immune responses, we assessed $CD4^+$ T cell cytokine secretion in the lung (Fig. 5.1.E). At day 21 post infection there were significant proportional increases in $CD4^+$ T cell expression of the Th1 cytokine IFNγ, as well as the Th2 cytokines IL-4, IL-5 and IL-13 and low levels of the regulatory cytokine IL-10 (Fig. 5.1.E). A corresponding significant increase in the absolute number of cytokine positive cells was observed for IL-5, but not for the other cytokines (Fig. 5.1.E). There was no evidence for parallel development of a regulatory response, with no change in the proportion of $Foxp3^+$ Tregs in the lung, and a slight but significant decrease in Treg number (Fig. 5.1.F).

To understand how immune responses to lung migrating schistosomula were induced we assessed changes in cDCs. At d21 infected mice had significantly increased cDCs by cell number, and as a proportion of $CD45^+$ cells (Fig. 5.2.A). Investigating further, it was clear that an expansion of $CD11b^+$ cDC2s, was responsible for the observed increase in cDCs, with

significant proportional and absolute increases in cDC2s observed, with decreased XCR1$^+$ cDC1s, as a proportion of the total DC compartment and no changes in absolute numbers of cDC1s (Fig. 5.2.B). We investigated the cDC2 population further, assaying expression of MGL2, which has been shown to demarcate an IRF4 dependent cDC2 subset (Gao *et al.*, 2013), and which we have previously shown to be required to maintain type-2 and regulatory responses in the lung (Chapter 4). In addition, PDL2 expression was assayed, a co-inhibitory ligand that has been reported to be co-expressed with MGL2, and demarcate a DC subset critical for promoting type-2 responses (Gao *et al.*, 2013). We did not find PDL2 and MGL2 to be reliably co-expressed by cDC2s (Fig. 5.2.C) during lung migrating schistosomiasis, and therefore went on to assay these markers individually. There were significant increases in MGL2$^+$ cDC2s by cell number, but not as a proportion of cDC2s during lung migrating schistosomiasis (Fig. 5.2.D). Notably, there was a marked and significant increase both proportionally and by cell number in cDC2s that express PDL2 (Fig. 5.2.E).

In order to increase the translatability of this work to human disease, in which adult patent infection can last many years, we wanted to understand pulmonary immune responses to patent *S. mansoni* infection. Therefore, mice were infected percutaneously with 180 cercariae, with lung immune responses measured at day 49, several weeks after the onset of egg production (Fig. 5.3.A). In contrast to lung migrating infection, there was a significant increase in lung cellularity in patent infection, with no observed change in BAL cell number (Fig. 5.3.B). Surprisingly, despite the lack of any overall changes in BAL cellularity, there was a significant increase in eosinophils observed, both proportionally and by cell number in response to patent schistosomiasis. There was a significant decrease in macrophages as a proportion of the total immune compartment, during patent schistosomiasis, perhaps explaining the overall lack of change in BAL cellularity (Fig. 5.3.C). In line with responses to lung migrating infection, there was a significant increase in the cytokine Relmα in the BAL (Fig. 5.3.D). Although there were more convincing trends for increases in Ym-1, CCL17 and CCL22, none of these were significant (Fig. 5.3.D). To understand adaptive immune responses, we assessed CD4$^+$ T cell cytokine secretion in the lung (Fig. 5.3.E). During patent infection there were significant proportional and cell number increases in CD4$^+$ T cell expression of the Th1 cytokine IFNγ, as well as the Th2 cytokines IL-4, IL-5 and IL-13, the regulatory cytokine IL-10 (Fig. 5.3.E). There was a significant decrease in lung IL-17 production, albeit at low levels with 1% to 0.5% of CD4$^+$ T cells producing this cytokine (Fig. 5.3.E). Significant changes in lung Tregs were observed, but these were contradictory, with a significant decrease in Tregs as a proportion of CD4$^+$ T cells, but a significant increase in Treg absolute number (Fig. 5.3.F). Together this data shows the development of an enhanced type-2, but also potential IL-10 related regulatory response in patent schistosomiasis, when compared to lung migratory stages, this enhanced response is despite the adult schistosome being resident in the mesenteric venules, and not the lungs.

To understand how pulmonary immune responses to patent schistosomiasis were induced we assessed changes in cDCs. At d49 infected mice had significantly increased cDCs by cell number, but, in contrast to the lung migratory stage not as a proportion of CD45$^+$ cells (Fig.

5.4.A). An expansion of CD11b$^+$ cDC2s, was responsible for the increase in cDCs, with significant proportional and absolute increases in cDC2s observed, with decreased XCR1$^+$ cDC1s, as a proportion of the total DC compartment and no changes in absolute numbers of cDC1s (Fig. 5.4.B). Like in lung migratory infection, we did not find PDL2 and MGL2 to be reliably co-expressed by cDC2s, with the majority of PDL2$^+$ DCs not MGL2$^+$ (Fig. 5.4.C) therefore went on to assay these markers individually. There were significant increases in MGL2$^+$ cDC2s by cell number, but not as a proportion of cDC2s during patent schistosomiasis (Fig. 5.4.D). There was a marked and significant increase both proportionally and by cell number in cDC2s that express PDL2 (Fig. 5.4.E), potentially suggesting these cells have a critical role in promoting inflammation to schistosomes.

5.2.2. IRF4 dependent cDC2s promote aspects of the pulmonary type-2 immune response to lung migrating schistosomula

After observing an increase in, and phenotypic changes of, cDC2s during schistosome infection, we wanted to know if these cells were required to direct the inflammatory response to lung migrating schistosomula (Fig. 5.5.A). To do this, we used CD11cΔIrf4 mice, where *CD11c* expressing cells (including DCs) in Cre$^+$ mice are deficient in IRF4, specifically impairing cDC2 migration to the LNs, and therefore their ability to drive Th2 responses (Persson *et al.*, 2013; Mayer *et al.*, 2017). Impairment of cDC2s in Cre$^+$ mice did not lead to an alteration in lung or BAL cell number during lung migrating schistosomiasis (Fig. 5.5.B). Looking at inflammatory cells in the BAL, a significant decrease in eosinophils was observed in Cre$^+$ mice, both proportionally and by cell number (Fig. 5.5.C). Despite the alteration in BAL eosinophils, there was no significant changes in BAL cytokines observed (Fig. 5.5.D). However, there was a significant proportional and cell number decrease in IL-4$^+$ CD4$^+$ T cells (Fig. 5.5.E), suggestive of a reduction in the Th2 response. There was no change in the proportion or number of Foxp3$^+$ regulatory T cells in the lung (Fig. 5.5.F). Together this suggests impairment of IRF4 dependent cDC2s leads to a partial reduction in type-2 responses.

Next, we wanted to confirm the DC phenotype in CD11cΔIrf4 mice during lung migrating schistosomiasis. Unsurprisingly, due to the specific nature of DC subset depletion in these mice, there was no change in overall DC number or proportion (Fig.5.6.A) or in cell number of cDC1s (Fig. 5.6.B). At d21 Cre$^+$ mice had significantly increased XCR1$^+$ cDC1s, and decreased CD11b$^+$ cDC2s, as a proportion of the total DC compartment (Fig. 5.6.B). There was no significant decrease in cDC2s by cell number, although a trend is present (Fig. 5.6.B). We then went on to investigating the cDC2 population further, first confirming again that MGL2 and PDL2 were not reliably co-expressed (Fig. 5.6.C). We found that MLG2$^+$ cDC2s were effectively depleted as a proportion of total cDC2s (81%), as well as by cell number (71%) (Fig. 5.6.D), in line with literature utilising this strain (Gao *et al.*, 2013). Surprisingly, there was no significant change in cDC2s that express PDL2 (Fig. 5.6.E). Taken together, this data reveals a role for MGL2$^+$ IRF4 dependent cDC2s in promoting type-2 responses in lung migrating schistosomiasis, whilst also pointing to a potential unexplored role for IRF4 independent PDL2$^+$ cDC2s.

5.2.3 Inflammatory changes in human sputum in lung migratory *S. mansoni* infection.

Having revealed that IRF4-dependent cDC2s mediate type-2 immune responses in murine pulmonary schistosomiasis, we wanted to see if these results would be similar in human infection. Due to practical limitations in obtaining lung samples from infected individuals, induced sputum was used as an accessible proxy for human lung responses. To understand human lung immune responses to migrating schistosomula, samples were obtained from an ongoing controlled human infection study, with individuals infected percutaneously with 20 male *S. mansoni* cercariae (Langenberg *et al.*, 2020). This was the first ever human controlled schistosome infection study, with the controlled nature of the study allowing direct comparison to murine primary infection. Single sex infection was used to eliminate the risk of long-term egg-induced pathology. This initial study was designed primarily to assess safety, with sputum sampling a late addition to the trial protocol, and therefore we were only able to obtain sputum samples from 3 participants pre-infection (in the preceding week), and 11-14 days post infection (Fig. 5.7.A). Participant demographics are shown in Table 5.1.

Flow cytometry was used to identify changes in sputum cellular infiltrates in response to lung migrating schistosomula, at days 11-14 post 20 cercariae schistosome infection (Fig.5.7.A), gating strategy detailed in methods, Figure 2.5. There were no consistent changes in sputum cell number, either as absolute number or per mg of selected sputum plugs (Fig. 5.7.B). There were no clear trends for changes in sputum eosinophils, neutrophils or B cells post infection as proportion of total leukocytes ($CD45^+$), with neutrophils making up a large proportion of sputum cells as is common for this sample type (Fig. 5.7.C&D) (Vidal *et al.*, 2012). Similarly, no consistent changes in γδT cells, $CD4^+$ T cells and $CD8^+$ T cells were observed post infection (Fig. 5.7.F&G). To assess whether the activation status of the T cell pool may have altered, we analysed expression of CD28, which is downregulated in response to TCR-mediated activation, as well as CD127 (IL-7R), which is downregulated in response to IL-7 and by activated cells (Warrington *et al.*, 2003; Mazzucchelli and Durum, 2007; Kiazyk and Fowke, 2008; Lo *et al.*, 2011; Thome *et al.*, 2014). However, no changes were observed in resting $CD127^+$ $CD28^+$ CD4 or $CD8^+$ T cells, either as a proportion of total leukocytes or $CD4^+$ or $CD8^+$ T cells (Fig. 5.7.G). Together this suggests lung migrating schistosome infection does not promote consistent changes in sputum granulocytes or lymphocytes.

In line with our findings of the importance of DCs in murine pulmonary schistosomiasis, there was a significant increase in cDCs ($HLA\text{-}DR^+CD11c^+$ $CD14^-CD16^-$) as a proportion of total leukocytes (Fig. 5.8.A). No significant changes in classical ($HLA\text{-}DR^+CD11c^+CD14^+$) or non-classical ($HLA\text{-}DR^+CD11c^+CD16^+$) monocytes a proportion of total leukocytes was observed (Fig. 5.8.A). Further investigation of the DC compartment revealed a significant increase in $CD1c^+$ cDC2s as a proportion of total leukocytes (Fig. 5.8.B). No convincing evidence of $Clec9a^+$ cDC1s was observed in sputum (Fig. 5.8.B), although these were evident in blood samples stained concurrently, suggesting this could not be attributable to a technical issue. In addition, the proportion of DCs expressing CD1c did not increase, and so we are unable

to conclude that cDC2s were selectively expanded over CD1c$^-$ cDCs, which have been proposed to be a contaminating monocytic population (Guilliams *et al.*, 2016)(Fig. 5.8.B).

To assess whether schistosomula lung migration induced secretion of soluble immune mediators into the airways, a wide range of cytokines (encompassing type-1, type-2, type-17 and regulatory responses, as well as alarmins, growth factors, and cytokines) were measured in the sputum supernatant (Fig. 5.9), with the cytokines TNFα, IL-1β, IL-6, IP-10, IL-1RA, YKL-4, TLP, MIP-1β, MIP-1α, MCP-1, CCL17, CCL22 and IL-8 within detectable range. There were no significant differences in cytokine levels post infection, though this is likely due to the low number of participants in the study (n = 3) (Fig. 5.9). Overall, there was a general trend for increases in type-1 cytokines (TNFα, IL-1β, IL-6, IP-10) and the regulatory cytokine IL-1RA, with a decrease in the type-2 associated cytokines YKL-40, the human homologue of YM-1 (Sutherland, 2018) and TSLP. There were mixed trends in chemokines, with increases in MIP-1β, MIP-1α, MCP-1, IL-8 and CCL17, with a tendency for a decrease in CCL22 (Fig. 5.9). This mixed response is perhaps reflective of our murine lung migratory infection studies, in which only the type-2 responsive cytokine Relmα significantly increased (Fig. 5.1.D), whilst perhaps suggesting a more Type-1 bias in human responses. Overall, during lung migratory infection in humans, we see a mixed inflammatory response, with hints of increased inflammation which could be confirmed in a larger study. In line with our murine lung migratory work there a significant expansion in pulmonary cDC2s was observed.

5.2.4 Inflammatory changes in human sputum in patent S. mansoni infection.

Although informative, the controlled human infection model was performed in the Netherlands, an area not endemic for schistosomiasis, with differing genetic and immune exposures limiting our translation of these results to endemic populations. Therefore, to understand lung immune response to schistosomiasis in an endemic setting, we performed a case-control study, comparing sputum immune responses in schistosome infected and non-infected individuals in Entebbe, Uganda, detailed in Table 5.2. Those in the case group had patent *S. mansoni* infection, determined both by Kato-Katz and CCA test positivity. However, it is not possible to determine the infectious dose(s) of cercariae these individuals received, or the time post infection. Co-infection with other helminths (determined by Kato-Katz) was rare, though 3 out of 15 individuals (20% cases) had a hookworm co-infection. Cases and controls were well matched in age and sex and were drawn from a small (2km radius) area on the shores of Lake Victoria. To increase the chances of observing pulmonary changes due to lung migrating schistosomula, as well as patent schistosomiasis, cases were predominantly drawn from individuals whose work involved regular lake contact, with controls drawn from nearby colleges. Most individuals (93%), in both case and control groups had not been treated with the schistosomicidal drug praziquantel in the past year.

To understand cellular inflammatory changes during patent schistosomiasis, sputum was analysed by flow cytometry (Fig. 5.10), gating strategy detailed in methods, Figure 2.5. There were no consistent changes in sputum cell number, either as absolute number or per mg of selected sputum plugs (Fig. 5.10.B). There were no clear trends for changes in sputum

eosinophils, neutrophils or B cells in cases compared to controls as proportion of total leukocytes ($CD45^+$) (Fig. 5.10.C&D) (Vidal *et al.*, 2012). There were trends for increases in γδT cells and $CD8^+$ T cells post infection in cases, with a significant increase in $CD4^+$ T cells (Fig. 5.10.E&F). Despite the significant increase in $CD4^+$ T cell number , no changes in activation status were observed, with resting $CD127^+CD28^+$ $CD4^+$ or $CD8^+$ T cells not changing in frequency (Fig. 5.10.F). There were no changes in T cell activation status, when resting $CD127^+$ $CD28^+$ CD4 or $CD8^+$ T cells were measured, either as a proportion of total leukocytes or $CD4^+$ or $CD8^+$ T cells (Fig. 5.10.G). Together this suggests endemic patent schistosome infection provokes an increase in sputum lymphocytes, in particular $CD4^+$ T cells.

In line with our findings in murine and human schistosomiasis, both lung migratory and patent phases, cDCs ($HLA\text{-}DR^+CD11c^+$ $CD14^-CD16^-$) were significantly increased in the sputum of individuals with patent infection as a proportion of total leukocytes (Fig. 5.11.A). There was a non-significant trend for an increase in classical ($HLA\text{-}DR^+CD11c^+CD14^+$) and non-classical ($HLA\text{-}DR^+CD11c^+CD16^+$) monocytes as a proportion of total leukocytes (Fig. 5.11.A). As observed during human lung migratory schistosomiasis (Fig. 5.8) sputum from endemic individuals with patent infection had significant increased cDC2s ($CD1c^+$), as a proportion of total leukocytes (Fig. 5.11.B). Again, as the proportion of DCs expressing CD1c was not altered between infected and non-infected individuals, and so we are unable to conclude that cDC2s were selectively expanded (Fig. 5.11.B). As in human lung migratory schistosomiasis, there were no $Clec9a^+$ cDC1s observed in sputum.

To increase our understanding of pulmonary immune changes in endemic patent infection, cytokines and chemokines were assessed in sputum supernatants, as well as in serum. There were no significant changes observed in sputum cytokines or chemokines detected: IL-1β, IL-6, IP-10, IFNγ, IL-1RA or IL-8 (Fig. 5.12). In serum there was significantly increased levels of the chemokine IP-10 in individuals with patent infection (Fig. 5.13). There were no significant changes in the other serum cytokines and chemokines detected: TNFα, MIP-1α, MIP-1β, RANTES, eotaxin and PDGF-BB (Fig. 5.13). Taken together these results display a muted and mixed cytokine and chemokine response in human schistosomiasis, with consistent immune changes observed with the expansion of cDCs.

5.2.5 Summary

- The murine response to schistosomula migration is characterised by an increase in pulmonary cDC2s, increased Th1 and Th2 cytokine expression as well as BAL eosinophils (Fig. 5.1-2).
- Similarly, the murine pulmonary response to patent schistosome infection is characterised by an increase in pulmonary cDC2s, increased Th1, Th2 and regulatory cytokine expression, as well as BAL eosinophils (Fig. 5.3-4).
- IRF4 dependent cDC2s are partially responsible for determining type-2 responses to lung migrating schistosomula, with their depletion leading to reduced Th2 cell IL-4 cytokine expression and BAL eosinophilia (Fig. 5.5-6).
- Pulmonary responses in human lung schistosomula migration, assayed in sputum, are characterised by an increase in DCs, as well as trends for increases in soluble inflammatory mediators (Figs. 5.7-9).
- In endemic patent schistosomiasis, increased numbers of sputum DCs are also observed, as well as an increase in sputum $CD4^+$ T cells. No changes were observed in sputum inflammatory mediators, although an increase in IP-10 is observed in serum (Figs. 5.10-13).

5.3 Discussion

In this chapter we have extended our studies on pulmonary type-2 immune responses, focusing this time the helminth *S. mansoni*, and how it can influence lung inflammation in both its lung migratory, and patent life cycle stages, a very poorly understood process. To tackle this, we used a unique combination of murine and human studies to reveal schistosomiasis induced inflammatory responses in the lung. One of the most striking results that was evident in both murine and human studies was that schistosome infection increases pulmonary cDC2s. Utilising a mouse model in which cDC2s were depleted (CD11cΔIrf4), we were able to shine light on the potential role of this conserved cDC2 increase – to instigate a pulmonary type-2 immune response.

5.3.1 Mouse studies of pulmonary schistosomiasis reveal a type-2 inflammatory response, driven by cDC2s

Prior to this work, there were few studies that assessed pulmonary immune responses in schistosomiasis. Here, at day 21 post infection (towards the end of the lung migratory phase (Wheater and Wilson, 1979; Wilson, Coulson and Dixon, 1986)) we found an increase in a broad range of Th2 cytokines (IL-4, IL-5 and IL-13), as well as the Th1 cytokine IFNγ, but notably not the Th17 cytokine IL-17 (Fig. 5.1.E). Therefore, this study expands upon a previous report, which showed an expansion of IL-4$^+$ CD4$^+$ T cells in the thoracic LN at day 14 and 21 post schistosome infection (Redpath *et al.*, 2015). In addition to these responses, in this work we were able to assay responses in the BAL, as a readout complementary to lung tissue changes. The low cell number and uniform (>90% macrophage) cellular composition of BAL in naïve mice allowed us to observe relatively subtle cellular changes, that may be lost in the more complex environment of the lung tissue. An expansion in BAL eosinophils was observed (Fig. 5.1.C), in line with previous histological studies (Crabtree and Wilson, 1986; Burke *et al.*, 2011). Note, although increased BAL eosinophilia was not comparable to that seen in *A.f.* driven AAI, where over a million eosinophils were observed (Chapter 3). As eosinophils are typically regarded as a type-2 effector cell, dependent upon IL-5 for tissue recruitment (Mould *et al.*, 1997), this suggests that this influx is caused by increased CD4$^+$ T cell type-2 cytokines (Fig. 5.1.E).

In addition to this early timepoint, responses were measured at day 49 post infection, when adult schistosomes are in the mesenteric vessels, and are patent, producing eggs that will be swept mainly to the liver and gut, with very low numbers of eggs found in the lungs – approximately 2 eggs per lung lobe in 75-100 cercariae infections (Crosby *et al.*, 2010). During patent infection a similar pattern of inflammation was observed to lung migratory infection, with expansion of Th1 (IFNγ) and Th2 (IL-4, IL-5, IL-13) cytokines, BAL eosinophilia, and an increase in the type-2 associated cytokine Relmα (Fig. 5.3.D). Promotion of lung Th2 immune responses to an intestinal helminth (*H. polygyrus*) has been observed previously, and was shown to protect mice from subsequent infection from lung migrating *N. brasiliensis* larvae (Filbey *et al.*, 2019). Schistosome eggs are potent inducers of a Th2 response, both systemically, and at sites of deposition (Grzych *et al.*, 1991; Pearce, 2005), and therefore

their presence at day 49 post infection could explain the enhanced lung Th2 responses, with double (~10% vs 5%) the IL-4 production in lung Th2 cells at d49 compared to d21 (Fig. 5.1.E & Fig. 5.3.E). Cellular and cytokine changes are observed in BAL during patent schistosome infection (Fig. 5.3.D), supporting the argument that a local immune response is being generated in the lung to distal schistosome infection, rather than the observed changes being resultant from intravascular circulating immune cells.

To understand the regulation of type-2 immune responses in pulmonary schistosomiasis, to link to the work in previous chapters, assessed Treg numbers, and IL-10 production. A previous report in the lung dLN revealed no increase in Treg numbers during lung migrating schistosomiasis (Redpath *et al.*, 2015), we have extended this work to lung tissue and reveal a similar lack of Treg expansion, in fact a slight decrease was observed (Fig. 5.1). This differs from larval stages of nematode infections, both with and without pulmonary life cycle stages, in which Tregs have been shown to be selectively expanded, with the enhanced regulatory environment aiding parasite survival (Gillan and Devaney, 2005; Finney *et al.*, 2007; McSorley *et al.*, 2008; Blankenhaus *et al.*, 2011). Notably this is not the only helminth to not induce Tregs, with a report showing Treg induction *in vitro* was not altered in nematode (*Trichinella spiralis*) infection (Ilic *et al.*, 2011). Similarly, at day 49 Tregs constituted a decreased proportion of the $CD4^+$ T cell pool, although they had a roughly two-fold numerical increase (Fig. 5.3.F). This pattern contrasts with that seen during type-2 pulmonary inflammation in Chapter 3 (Fig. 3.3.A), where numerical expansion of Tregs was coincident with their proportional increase within the $CD4^+$ T cell pool. Together, this suggests that although Tregs may be increasing at d49 of schistosome infection, they are expanding slower than Th2 cells, potentially suggesting that inflammatory responses may dominate at this stage. Previous reports from chronic infections with schistosomes have shown an dominance of regulatory responses from week 12 onwards, showing for instance an increase in *Foxp3* mRNA expression at week 16 in the spleen and liver (Singh *et al.*, 2005). Therefore, we can hypothesise that Tregs may be more evident in the lung at later stages of infection. The numeric increase lung Tregs during patent infection (Fig. 5.3.F) may be relevant to reports of chronic schistosome infection can mediate Treg-dependent protection against AAI (van der Vlugt *et al.*, 2012; Layland *et al.*, 2013). Notably, we identified elevated IL-10 production by $CD4^+$ T cells in both lung migrating, and patent schistosomiasis (Fig. 5.1.E&5.3. E). Whether enhanced regulation in the lung at d49, alongside type-2 inflammation, would benefit the parasite or the host is not clear. However, promoting a lung regulatory response may be a host defensive response in the face of dramatic systemic inflammation (gut, liver, spleen), guarding against excessive pulmonary inflammation that would have the potential to interfere with vital physiological functions.

Finally, an increase in cDC2s expressing MGL2 and PDL2 was observed in the lungs in both lung migratory and patent phases of mouse schistosome infection (Fig. 5.2.D-E & 5.4.D-E). Expansion of cDC2s in the liver and mLN has been observed previously in schistosome infection, as well as in schistosome egg injection, but has not been previously reported in the lung (Lundie *et al.*, 2016; Mayer *et al.*, 2017). PDL2 and MGL2 have both been proposed as

markers of DCs that are expanded during type-2 inflammation, and may be required to instigate type-2 immune responses (Gao *et al.*, 2013; Kumamoto *et al.*, 2013; Redpath *et al.*, 2018). Notably, the increase in cDC2s was more dramatic at d49, increasing in line with the Th2 response (Fig. 5.4.B). Expansion of cDC2s is a feature of type-2 inflammation, which we also observed in AAI (Fig 4.1) (van Helden and Lambrecht, 2013). The requirement of cDC2s to drive type-2 inflammation has been shown in this thesis (Fig 4.5.B&C), as well as in models of skin allergy and *N. brasiliensis* infection (Gao *et al.*, 2013) including post injection of *S. mansoni* eggs (Mayer *et al.*, 2017) and during schistosome infection (Tussiwand *et al.*, 2015). Therefore, we hypothesised that the role of these cells would also likely be to promote Th2 responses against lung stage schistosome infection.

To understand the function of cDC2s in this setting, we utilised the CD11cΔIrf4 mouse line, in which IRF4 dependent DCs are selectively impaired (Persson *et al.*, 2013). As this line targets CD11c^{hi} cells macrophages may also be affected, something that needs to be further investigated. A similar pattern of DC depletion followed a similar pattern to the *Mgl2*^{DTR} strain used previously (Figure 4.5), with a significant decrease in MGL2^{+} cDC2s (reduced by 78%) in Cre^{+} mice (Fig. 5.6.D). However, the depletion of IRF4 dependent DCs in this strain is constitutive, throughout the entire period of schistosomula migration. Previous reports have similarly identified depletion of MGL2^{+} DCs in skin draining LNs in Cre^{+} CD11cΔIrf4 mice (Gao *et al.*, 2013). Notably PDL2^{+} cDC2s, were not reduced in the lung, suggesting these to be IRF4 independent (Fig. 5.6.E). There was a reduction in type-2 immune responses when IRF4 dependent cDC2s were depleted (Fig. 5.5). This reduction in IL-4^{+} CD4^{+} T cells, as well as BAL eosinophilia, suggests that IRF4 dependent cDC2s are required, at least partially, to instigate type-2 immune responses against lung migrating schistosomiasis. Notably, although there was a trend for a reduction in lung T cell IL-5 and IL-13, there was no significant impact on these cytokines (Fig. 5.5.E). This could imply there are other factors responsible for driving expression of these Th2 cytokines, one of which could be PDL2^{+} DCs, which have been proposed to have a critical role in promoting Th2 immune responses in allergy and *N. brasiliensis* infection (Gao *et al.*, 2013), and were not fully depleted in Cre^{+} mice. It remains unclear whether the Th2 response induced in lung migratory schistosome infection is important for parasite survival. Accurately quantifying lung migrating schistosomula is technically challenging, with past studies relying upon somewhat inaccurate whole lung digestion and larval counting by microscopy, or radiolabelling cercariae utilising a selenium isotope followed by flattening of organs and autoradiography (Wilson, 2009). In the absence of more refined approaches, it may be preferable to assess schistosomula survival by adult worm or egg counts from d42 onwards, in mice depleted of IRF4 dependent DCs. Nonetheless, the finding that cDC2s can drive certain aspects of type-2 immunity in lung migrating schistosomiasis informs how the natural response to lung migrating schistosomula develops, whilst providing evidence that targeting these cells may be critical to therapeutically modulate this condition. This may be particularly relevant in the context human sputum responses in which cDC2s were specifically increased during lung migrating and patent schistosomiasis (Fig. 5.8.A & 5.11.A).

5.3.2 Human studies of pulmonary schistosomiasis reveal expansion of sputum cDC2s, with contradictory changes in soluble immune mediators.

To translate our results from the mouse to the human systems, and therefore to evaluate their potential to guide future vaccine and therapeutic development, we initiated two pilot studies into pulmonary immune responses in human schistosomiasis. To investigate responses to lung migrating schistosomula we acquired samples from an ongoing study at the LUMC in the Netherlands, utilising controlled human infection with *S. mansoni* cercariae (Langenberg *et al.*, 2020) (Table 5.1). To complement this, we instigated a case control study on patent schistosomiasis in an endemic setting, in collaboration with the MRC/UVRI & LSHTM Uganda Research Unit (Table 5.2). Comparisons between lung migratory, and patent schistosome infection in humans and mice have great potential to reveal immune mechanisms in pulmonary schistosomiasis. However, there are key differences between these studies that must be considered carefully.

In terms of timepoints, samples from lung migratory human and murine infection were both taken in the week following the expected peak of lung migration. Lung migration peaks at day 10 in mice, and day 5 in baboons which provide our best estimate of human lung migratory patterns (Wheater and Wilson, 1979; Wilson, Coulson and Dixon, 1986; Wilson *et al.*, 1990). In murine patent infection, samples were taken at day 49 (week 7) post infection. It is not possible to determine the date of infection of individuals with patent schistosomiasis in the Ugandan samples, except to say it must be over 5 weeks, as this is the point at which adult schistosomes begin to produce eggs (McManus *et al.*, 2018). A recent report showed that 24-31% of Ugandans aged 2-19 had schistosomiasis, with the rate 31% in children aged 2-4 (Makerere University and Johns Hopkins. PMA., 2017). Considering the location of the study cohort, on the shores of Lake Victoria (Table 5.2), it is likely that infected individuals have long term infections, a proposal supported by irregular praziquantel treatment meaning that long-term infections would not be cleared (Table 5.2). The majority of cases (93.3%) had regular lake contact, occurring at the time of sampling or within the last month for 73.3% (Table 5.2). It is therefore likely that these individuals had multiple stages of infection, including ongoing larval lung migration, at the time of sampling. In comparison, all murine infections and the human controlled infection study involved exposure to single infectious dose in hosts that had not previously been exposed to schistosomes. Whereas our study in Uganda involved individuals with pre-existing patent infections and regular exposure to infective cercariae through water contact over an extended period, even in utero, may have occurred with potential immunological consequences (Eloi-Santos et al., 1989; Taylor et al., 2009).

Acquiring lung samples in humans, for instance via biopsy, can cause considerable discomfort, and due to the speculative nature of this study we therefore decided to opt for a relatively non-invasive sampling procedure, using induced sputum as a proxy for lung responses. Soluble immune mediators detected in sputum have been shown to correlate with those detected in BAL, a more invasive procedure involving the insertion of a bronchoscope into the trachea (Rutgers et al., 2000; Röpcke et al., 2012). It may be possible to compliment

future studies by use of non-invasive lung imaging, such as computerised tomography scan, to give an idea of lung tissue responses, as radiographically observed lung lesions are present in both acute and chronic schistosomiasis (Gobbi et al., 2020).

To get an overview of pulmonary immune responses, soluble immune mediators in sputum were assayed. Although non-significant, there was a trend for an increase in sputum cytokines and chemokines during human lung migratory infection. Notably, the type-1 cytokine IP-10 tended to increase (Fig. 5.9), a cytokine that increases in serum from week 4-8 of controlled human infection, with levels at week 8 were significantly correlated to acute stage symptoms at week 4 (Langenberg *et al.*, 2020). Our identification of their increase in the sputum at this early stage, days 11-14, is further evidence that the pulmonary response may be critical for inducing these mediators, associated with acute symptoms. Innate pro-inflammatory cytokines and chemokines such as IP-10, IL-6, IL-1β, TNFα, and the chemokines IL-8, CCL17 and MCP-1 had clearer fold increases, potentially suggesting an innate pro-inflammatory response is crucial for lung migrating schistosomiasis. This might fit with a recent study looking at PBMC responses to schistosomula Ags, in which pro-inflammatory cytokines (IL-1β and TNFα) were induced, potentially suggesting schistosomula Ags may be directly inducing these responses in sputum (Egesa *et al.*, 2018).

Surprisingly, patent infection of endemic individuals was not associated with changes in sputum cytokines (Fig. 5.12). This could be reflective of a reduced importance of lung responses in patent disease when adult worms are in mesenteric vessels. This would contrast with murine patent schistosomiasis, in which BAL cytokines were dramatically increased during patent infection (Fig. 5.3.D). Alternatively, a lack of evident pulmonary response could reflect an increasingly regulatory environment, thought to develop in individuals with chronic schistosomiasis, which is likely considering the constant water exposure and infrequent praziquantel treatment of these individuals (Table 5.2). People with chronic disease display reduced lymphoproliferative responses to soluble egg Ag (SEA) preparations (Colley *et al.*, 1986). Although controversial, with conflicting studies (Santiago and Nutman, 2016), some researchers have found infections with *S. mansoni* to be associated with a reduced incidence of asthma (Medeiros *et al.*, 2003). An increasingly regulatory lung environment could explain this association, and future studies assessing whether endemic infected individuals display any altered lung function (including assessment of asthma or wheeze) would add further clarity. This lack of sputum cytokine response also contrasts to the trends observed in human lung migratory infection, however, differences in age, stage of infection, genetics, and immune exposures, discussed earlier, may in part explain these differing profiles (Quach *et al.*, 2016). Studying sputum immune responses in controlled human *S. mansoni* infection studies in endemic locations, such as Uganda (Elliott *et al.*, 2018; Koopman *et al.*, 2019), could allow for more accurate comparisons between stages.

In contrast to the lack of change observed in sputum, in serum of individuals with patent schistosomiasis there was a significant increase in the inflammatory cytokine IP-10, as well as trends for increase in the chemokines eotaxin and MIP-1α (Fig 5.13). The significant

increase in serum levels of IP-10 is also observed in serum from experimentally infected individuals at week 4-12 post infection, as mentioned previously (Langenberg *et al.*, 2020), but in contrast to an endemic study, which observed no differences in IP-10 in *S. mansoni* infected individuals serum (Castro *et al.*, 2018). Comparison of patent to lung migratory serum responses could be enhanced by future work studying serum responses in controlled human infection at lung migratory timepoints. Contrasting systemic and airway (sputum/BAL) cytokine responses have been observed previously in chronic obstructive pulmonary disease (Morello Gearhart *et al.*, 2017), supporting the importance of using lung, and not systemic, samples to assess pulmonary changes.

In addition to soluble inflammatory mediators, immune cells in sputum were also assayed during lung migratory and patent infection (Figs. 5.7,8,10&11). In lung migratory schistosomiasis, there were no consistent changes in immune cells, except DCs (Fig. 5.8). This is in contrast to our murine studies, in which there were an increase in a number of lung tissue and BAL immune cells (Fig. 5.1). However, this may be explained by the small sample size and inherent variability of human immune responses, as well as our inability to sample lung tissue. Similarly, whilst there were trends for increase in a number of sputum immune cell types during patent human infection, the only significant increases we observed were in $CD4^+$ T cells and DCs (Fig. 5.10&11). Increased $CD4^+$ T cells could suggest an ongoing adaptive immune response in the airways. However, as we were not able to measure any changes in activation status of these cells (Fig. 5.10.G), it is not possible to know their function. Future work, characterising the function of these T cells either by more detailed surface phenotyping, or *ex vivo* stimulation with *S. mansoni* Ags and cytokine analysis would help better understand the role of these cells during *S. mansoni* infection.

Notably, we have identified an increase in DCs, particularly cDC2s, in sputum during pulmonary schistosomiasis, in two separate studies looking at experimental and natural schistosome infection, in either the lung migratory, or patent phases of disease (Fig. 5.8. & Fig. 5.11). DCs were identified as HLA-$DR^+CD11c^+CD16^-CD14^-$, with $CD1c^+$ cells identified as cDC2s. This gating scheme was based upon a recent study, which utilised a similar strategy to convincingly gate cDC2s in human lung (Guilliams *et al.*, 2016). Although Clec9a was used in our work to try to identify cDC1s in sputum, as proposed by recent ssRNAseq and high dimensional analyses (Guilliams *et al.*, 2016; Villani *et al.*, 2017), we observed no convincing staining (Fig. 5.4.E & Fig. 5.6.E). While it is possible that this might have been be due to an unidentified technical issue, the frequency of cDC1s in human lung tissue is known to be much lower than cDC2s (Guilliams *et al.*, 2016). Further, this may be even more exaggerated in the airways, with a recent ssRNAseq study of human bronchial brushing samples revealing a single DC population which expressed cD1c (Vieira Braga *et al.*, 2019).

The consistent increase in pulmonary cDC2s in multiple stages of human schistosome infection suggests an important role for these cells in directing the lung immune response. Our accompanying murine studies (Fig. 5.5-6) suggest that cDC2s are required to induce type-2 immune responses against lung stage schistosomes. Although such a depletion

approach is not possible in humans, *ex vivo* models such as isolating DCs from sputum or PBMCs of schistosome infected individuals and assaying their ability to instigate Th2 priming, could be used to directly assess human pulmonary cDC2 function in schistosomiasis. It is possible that sputum DCs may play a regulatory role in the lung in schistosome infection, with several studies having shown that PBMC or skin derived human DCs take on a regulatory phenotype in response to schistosome infection (Everts *et al.*, 2010; Lopes *et al.*, 2018; Winkel *et al.*, 2018). A follow up study, looking at co-stimulatory and co-inhibitory markers on sputum DCs could help determine the function of DCs during schistosomiasis, this was not possible in the work detailed here as we utilised a broad panel targeting multiple cell types as we did not know what we would find.

In summary, in this chapter we set out to define the pulmonary immune response to schistosome infection in both mouse and human systems. We have shown that a pulmonary type-2 immune response, with expression of Th2 cytokines and BAL eosinophilia, is characteristic of both lung migratory and patent schistosome infection. Observing an increase in cDC2s during murine schistosomiasis, we used transgenic cDC2 deficiency to reveal a requirement for cDC2s to promote type-2 responses to lung migrating schistosomula. Taking this further, we have performed novel studies on the human immune response to schistosomiasis, using sputum sampling to reveal contrasting patterns of soluble immune mediators during lung migratory and patent schistosome infection. Finally, we have shown a consistent increase in cDC2s in people with either lung migratory or patent schistosomiasis, suggesting a key role for this cell type.

Taken together, this chapter builds upon the work of the previous chapters, highlighting the critical role for DCs, and in particular cDC2s in type-2 pulmonary inflammation.

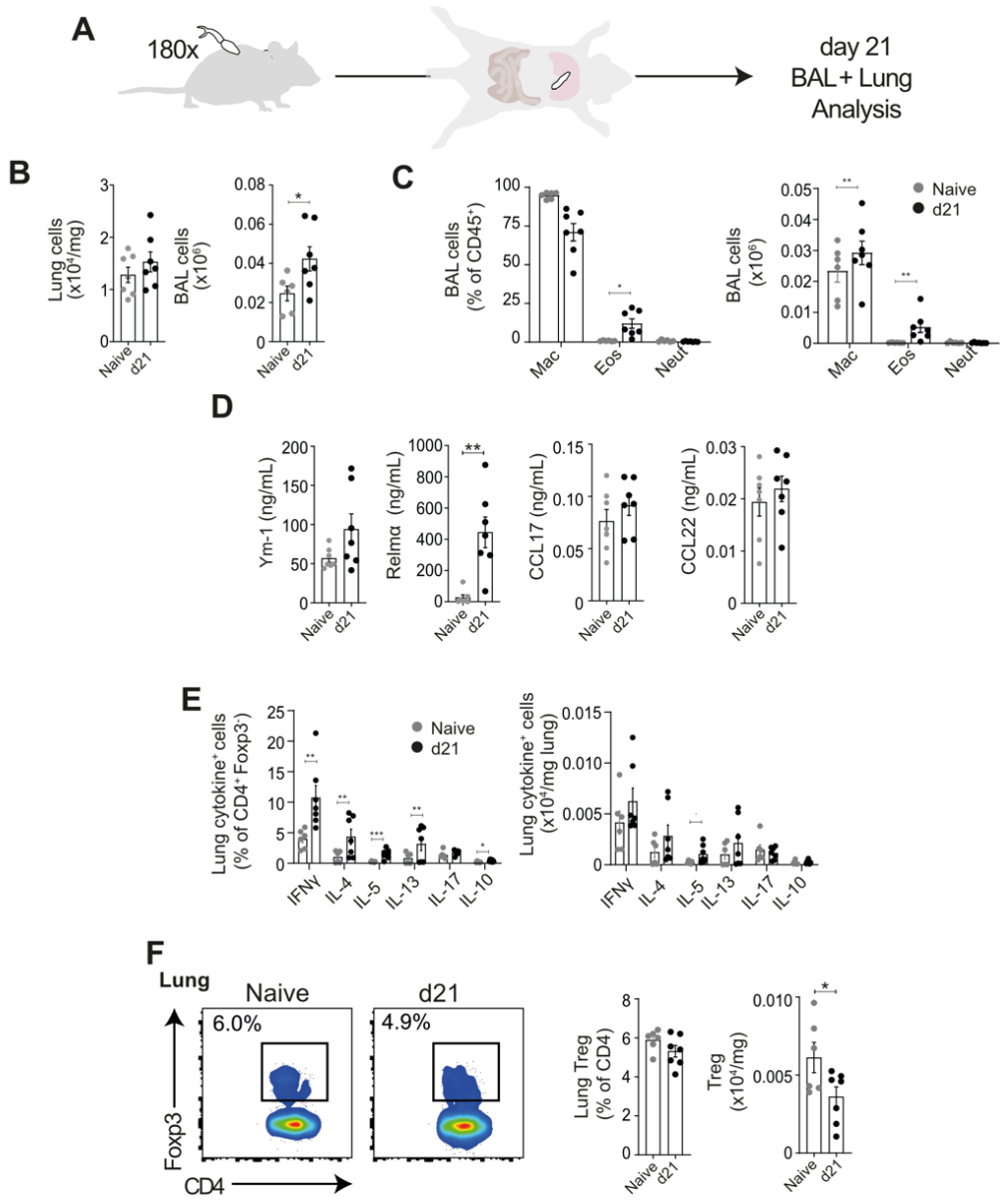

Figure 5.1. Lung migratory schistosomula provoke Th2/Th1 lung immune responses

A) C57BL/6 mice were percutaneously exposed to 180 cercariae, at day 21 lungs were taken. B) Lung and BAL cell counts C) BAL cell isolates were assessed by flow cytometry. D) BAL cytokines were assessed via ELISA. E) Lung cell isolates were stimulated with PMA/ionomycin, and $CD4^{+}Foxp3^{-}$ T cell intracellular cytokines assessed by flow cytometry. F) Representative flow cytometry plots depict gating of Tregs, post PMA/ionomycin stimulation. Gate frequencies show % of $CD4^{+}$ cells. Flow cytometry data is from 2 experiments (n= 6-7 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test was used. *=P <0.05, **=P <0.01, ***=P <0.001.

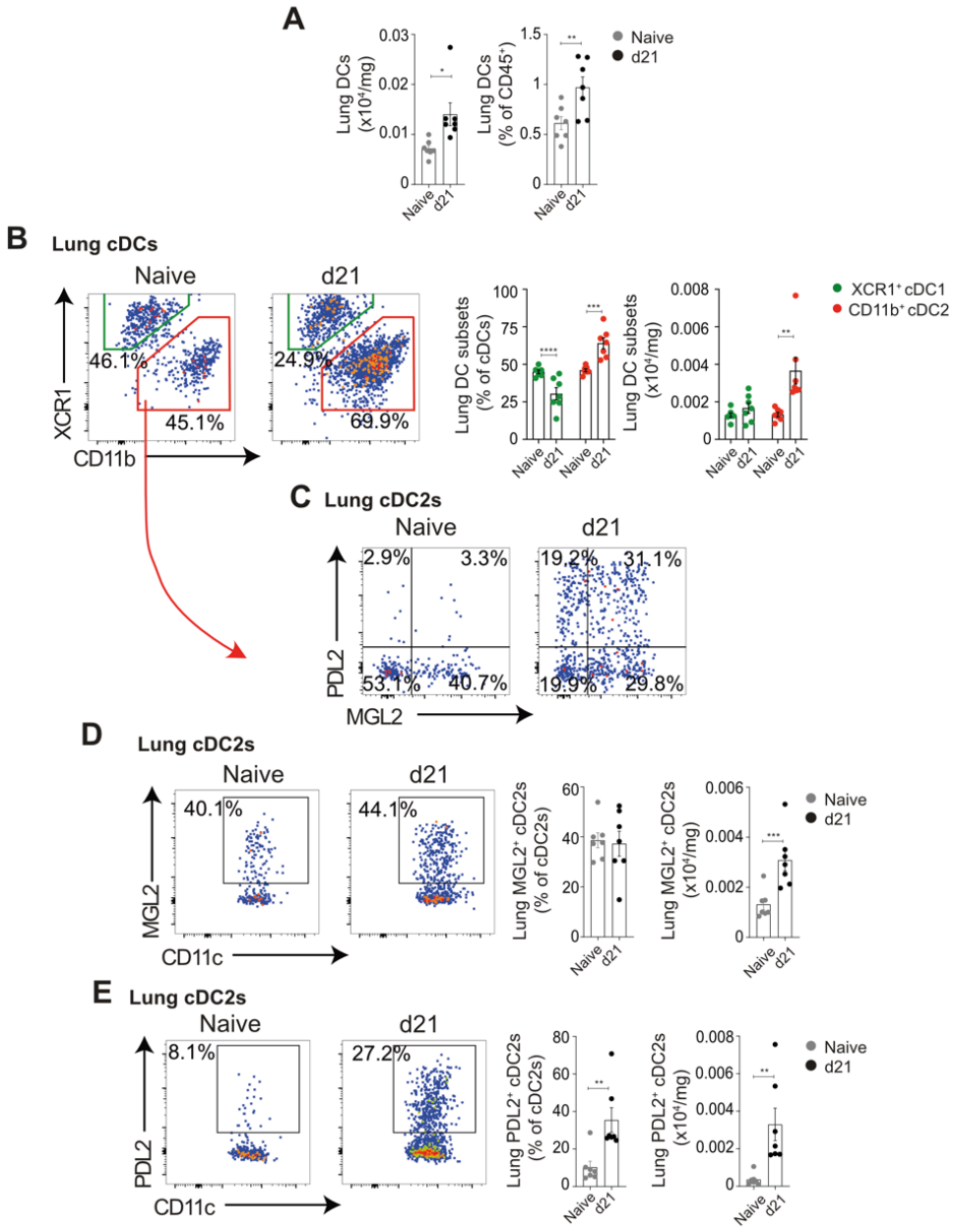

Figure 5.2. Lung migratory schistosomula provoke expansion and phenotypic changes in cDC2s

As in Fig. 5.1. C57BL/6 mice were percutaneously exposed to 180 cercariae, at day 21 lungs were taken. A) Lung cell isolates were assessed by flow cytometry, with cDCs identified as MHCII$^+$ CD11c$^+$, post exclusion of post exclusion of doublets, dead cells, Lineage$^+$ (CD3, CD19, Ly6G, Ter119, NK1.1) cells, macrophages, neutrophils, and eosinophils. B) Representative flow cytometry plots show changes in cDC populations. Gate frequencies show % of cDCs. C) Representative flow cytometry plots show MGL2 and PDL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. D) Representative flow cytometry plots show MGL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. E) Representative flow cytometry plots show PDL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. Flow cytometry data is from 2 experiments (n= 6-7 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test was used. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

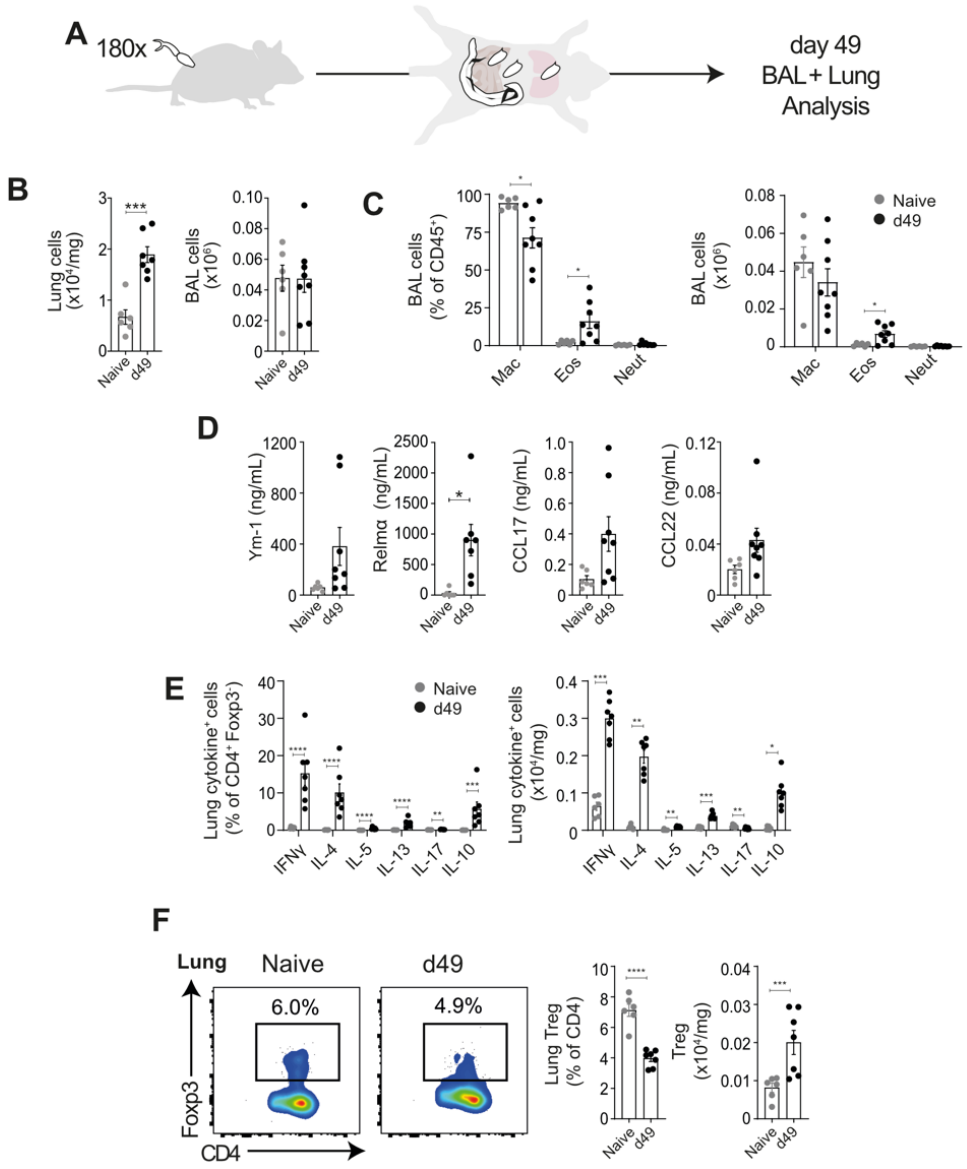

Figure 5.3. Patent schistosomiasis provokes enhanced Th2/Th1 lung immune responses
A) C57BL/6 mice were percutaneously exposed to 180 cercariae, at day 49 lungs were taken. B) Lung and BAL cell counts C) BAL cell isolates were assessed by flow cytometry. D) BAL cytokines were assessed via ELISA. E) Lung cell isolates were stimulated with PMA/ionomycin, and $CD4^{+}Foxp3^{-}$ T cell intracellular cytokines assessed by flow cytometry. F) Representative flow cytometry plots depict gating of Tregs, post PMA/ionomycin stimulation. Gate frequencies show % of $CD4^{+}$ cells. Flow cytometry data is from 2 experiments (n= 6-8 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test was used. *=P <0.05, **=P <0.01, ***=P <0.001.

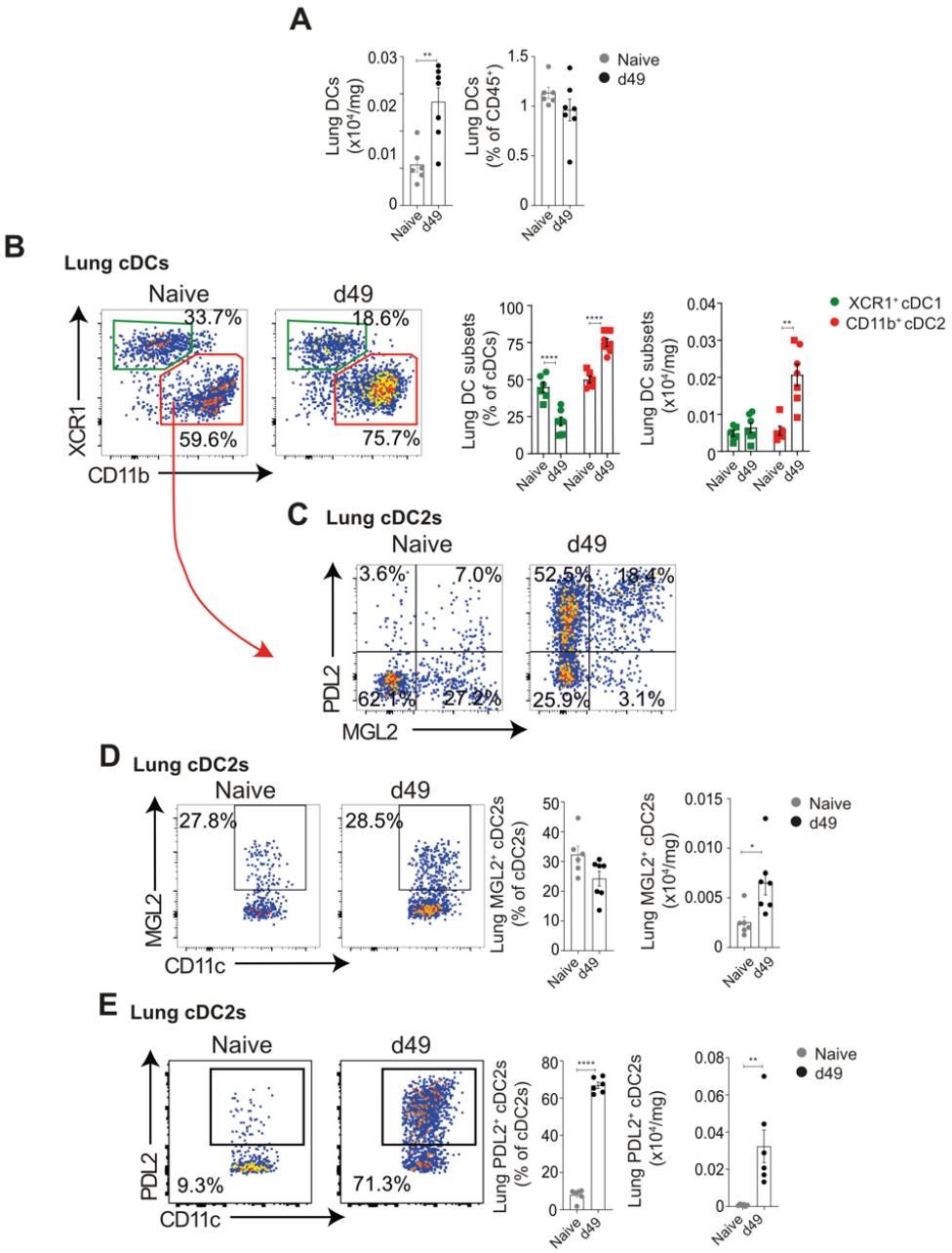

Figure 5.4. Patent schistosomiasis provokes expansion and phenotypic changes in lung cDC2s

As in Fig. 5.3. C57BL/6 mice were percutaneously exposed to 180 cercariae, at day 49 lungs were taken. A) Lung cell isolates were assessed by flow cytometry, with cDCs identified as MHCII$^+$ CD11c$^+$, post exclusion of post exclusion of doublets, dead cells, Lineage$^+$ (CD3, CD19, Ly6G, Ter119, NK1.1) cells, macrophages, neutrophils, and eosinophils. B) Representative flow cytometry plots show changes in cDC populations. Gate frequencies show % of cDCs. C) Representative flow cytometry plots show MGL2 and PDL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. D) Representative flow cytometry plots show MGL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. E) Representative flow cytometry plots show PDL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. Flow cytometry data is from 2 experiments (n= 6-7 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test was used. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

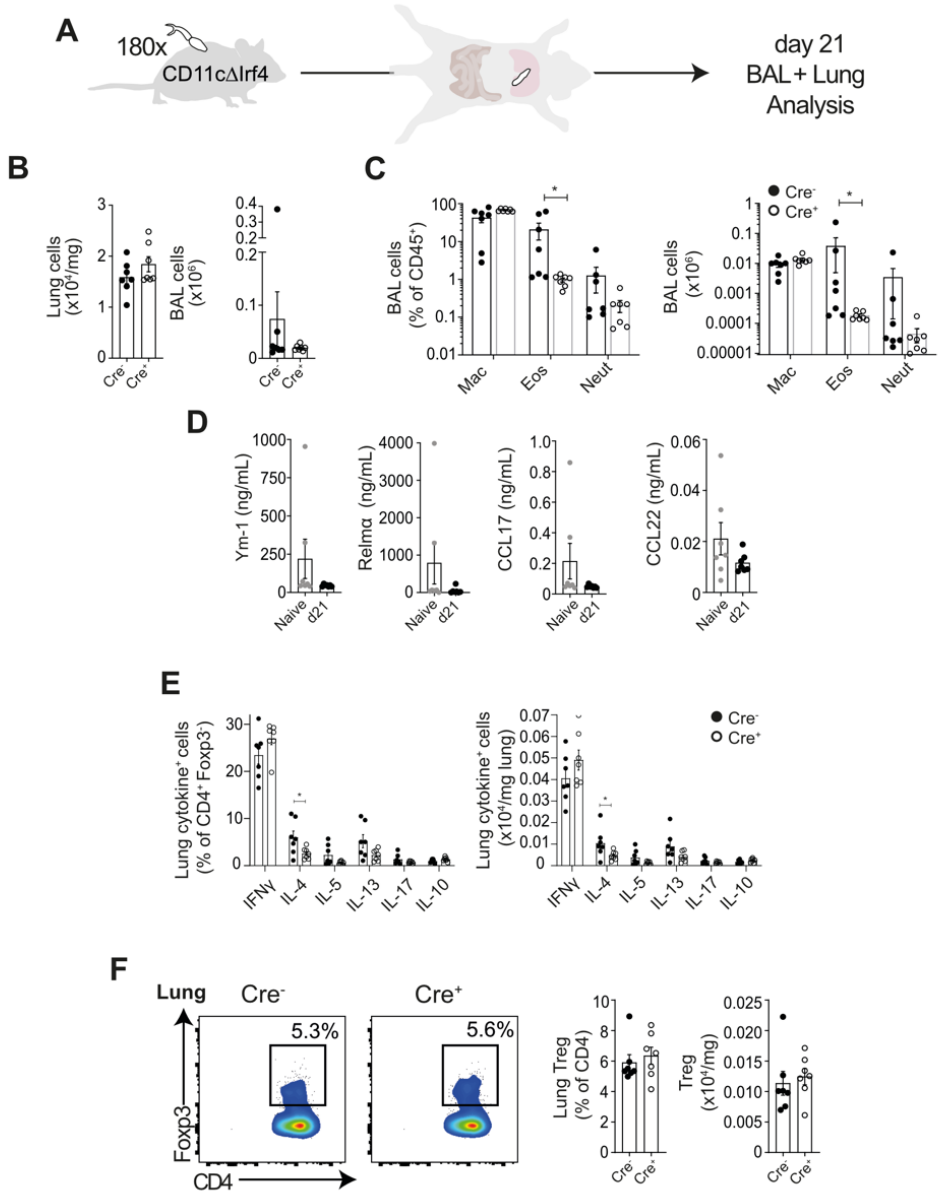

Figure 5.5. Reduced type-2 inflammation in CD11cΔIrf4 mice during lung migratory schistosomiasis.

A) CD11cΔIrf4 mice were percutaneously exposed to 180 cercariae, at day 21 lungs were taken. B) Lung and BAL cell counts C) BAL cell isolates were assessed by flow cytometry. D) BAL cytokines were assessed via ELISA. E) Lung cell isolates were stimulated with PMA/ionomycin, and $CD4^{+}Foxp3^{-}$ T cell intracellular cytokines assessed by flow cytometry. F) Representative flow cytometry plots depict gating of Tregs, post PMA/ionomycin stimulation. Gate frequencies show % of $CD4^{+}$ cells. Flow cytometry data is from 2 experiments (n= 7 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test was used. *=P <0.05

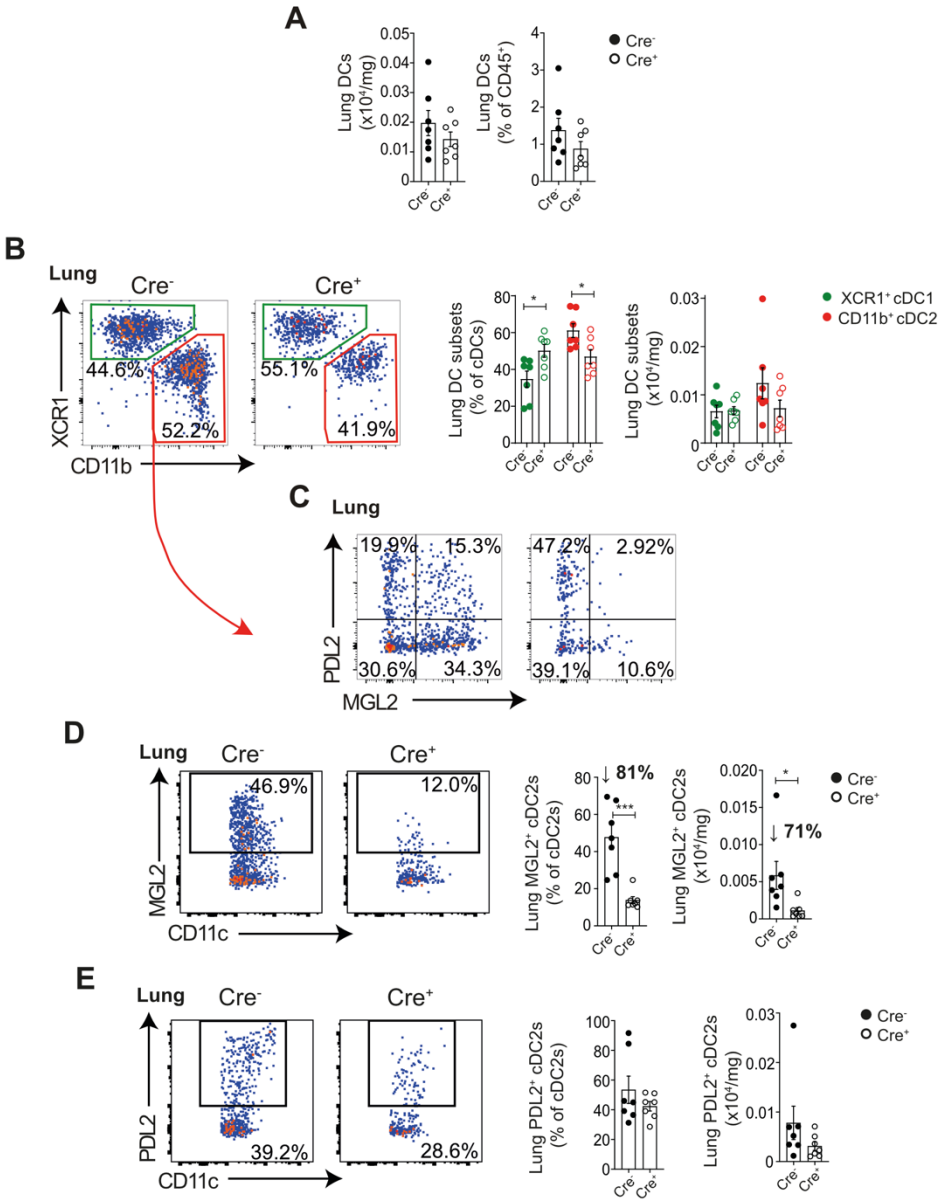

Figure 5.6. Specific depletion of MGL2⁺ cDC2s in CD11cΔIrf4 mice during lung migrating schistosomiasis.

As in Fig. 5.5. CD11cΔIrf4 mice were percutaneously exposed to 180 cercariae, at day 21 lungs were taken. A) Lung cell isolates were assessed by flow cytometry, with cDCs identified as MHCII⁺ CD11c⁺, post exclusion of post exclusion of doublets, dead cells, Lineage⁺ (CD3, CD19, Ly6G, Ter119, NK1.1) cells, macrophages, neutrophils, and eosinophils. B) Representative flow cytometry plots show changes in cDC populations. Gate frequencies show % of cDCs. C) Representative flow cytometry plots show MGL2 and PDL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. D) Representative flow cytometry plots show MGL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. E) Representative flow cytometry plots show PDL2 expression within the cDC2 population. Gate frequencies show % of cDC2s. Flow cytometry data is from 2 experiments (n= 6-7 per group). Linear mixed effect modelling applied, with experimental repeat as a random effect variable. To compare difference between groups LS means Students t test was used. *=P <0.05, **=P <0.01, ***=P <0.001.

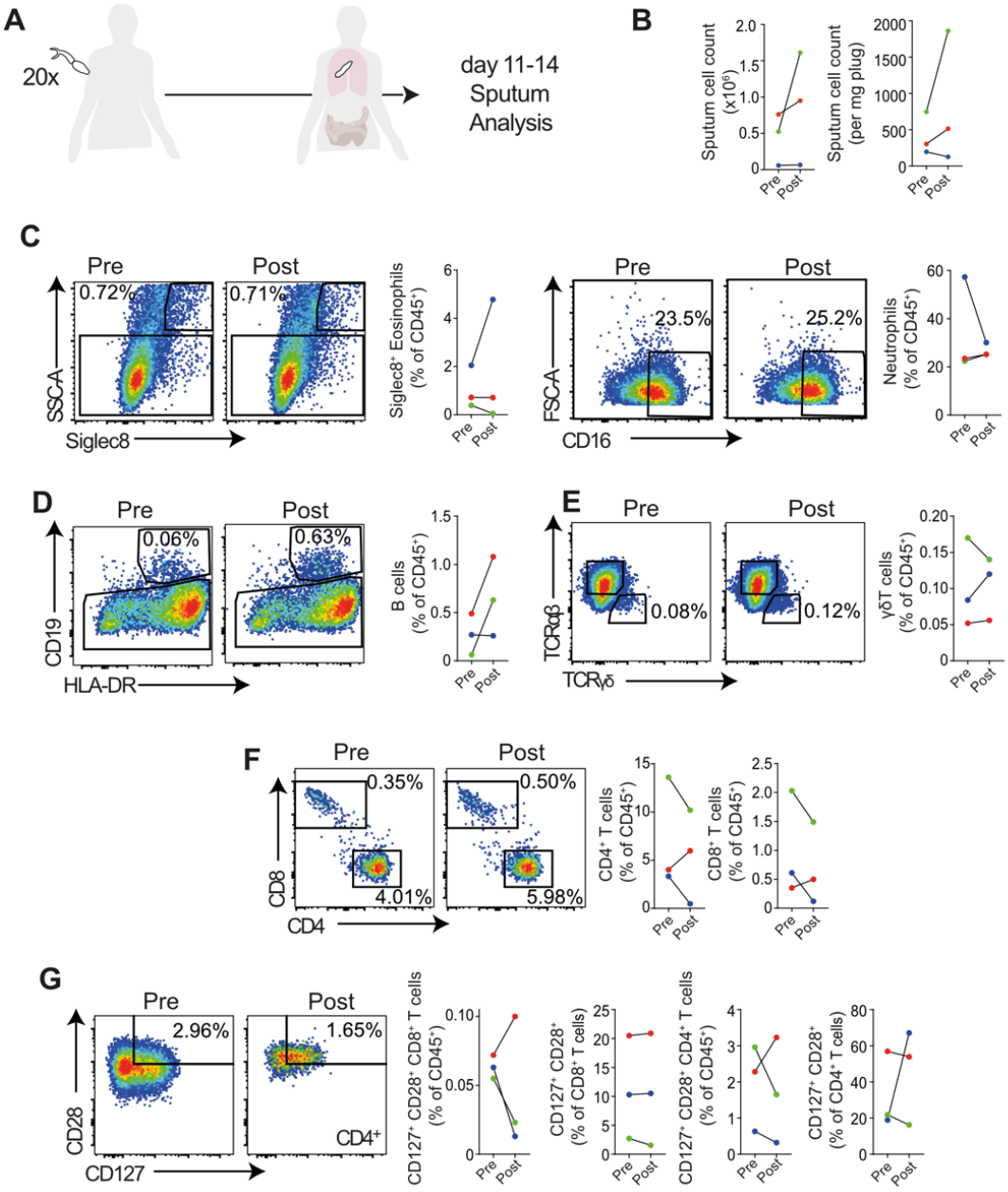

Figure 5.7. Human lung migratory *S. mansoni* infection does not lead to alterations in granulocyte or lymphocyte populations in sputum.

A) Non-endemic (dutch) participants were percutaneously exposed to 20 male cercariae, with samples taken pre, and 11-14 days post infection. B) Sputum cell counts, absolute or per mg of selected sputum plugs are shown. Sputum cell isolates were assessed by flow cytometry. Representative flow cytometry plots show changes in cells, as a proportion of total leukocytes ($CD45^+$) for C) eosinophils and neutrophils, D) B cells, E) γδT cells, F) $CD4^+$ and $CD8^+$ T cells and G) $CD127^+$ $CD28^+$ $CD4^+$ and $CD8^+$ T cells. Certain cell types are also shown as a proportion of their parental gate, namely G) $CD127^+$ $CD28^+$ cells as a proportion of $CD4^+$ and $CD8^+$ T cells. Data is from one experiment (n=3 per group). To compare differences between groups paired t tests were used. *=P <0.05, **=P <0.01, ***=P <0.001, ****=P<0.0001.

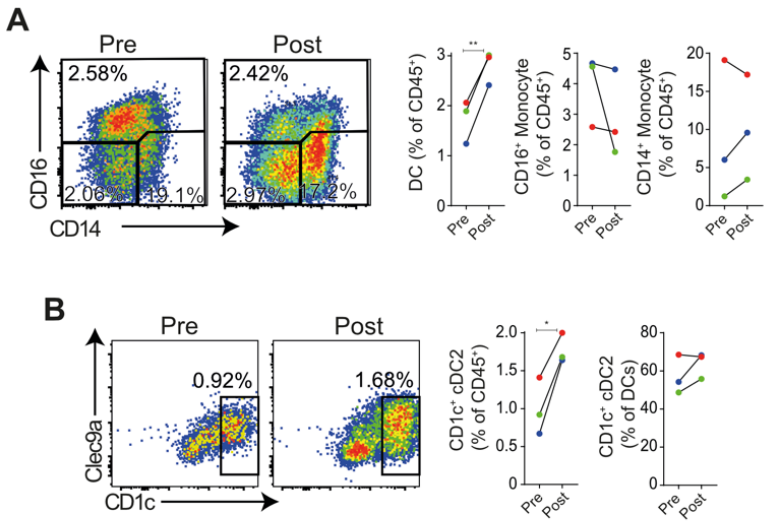

Figure 5.8. Human lung migratory *S. mansoni* infection leads to increased sputum cDC2s
As in Fig. 5.7 Non-endemic (dutch) participants were percutaneously exposed to 20 male cercariae, with samples taken pre, and 11-14 days post infection. Sputum cell isolates were assessed by flow cytometry. Representative flow cytometry plots show changes in cells, as a proportion of total leukocytes ($CD45^+$) for A) cDCs and monocytes and B) cDC2s. CDC2s are also shown as as a proportion of cDCs. Data is from one experiment (n=3 per group). To compare differences between groups paired t tests were used. *=P <0.05, **=P <0.01

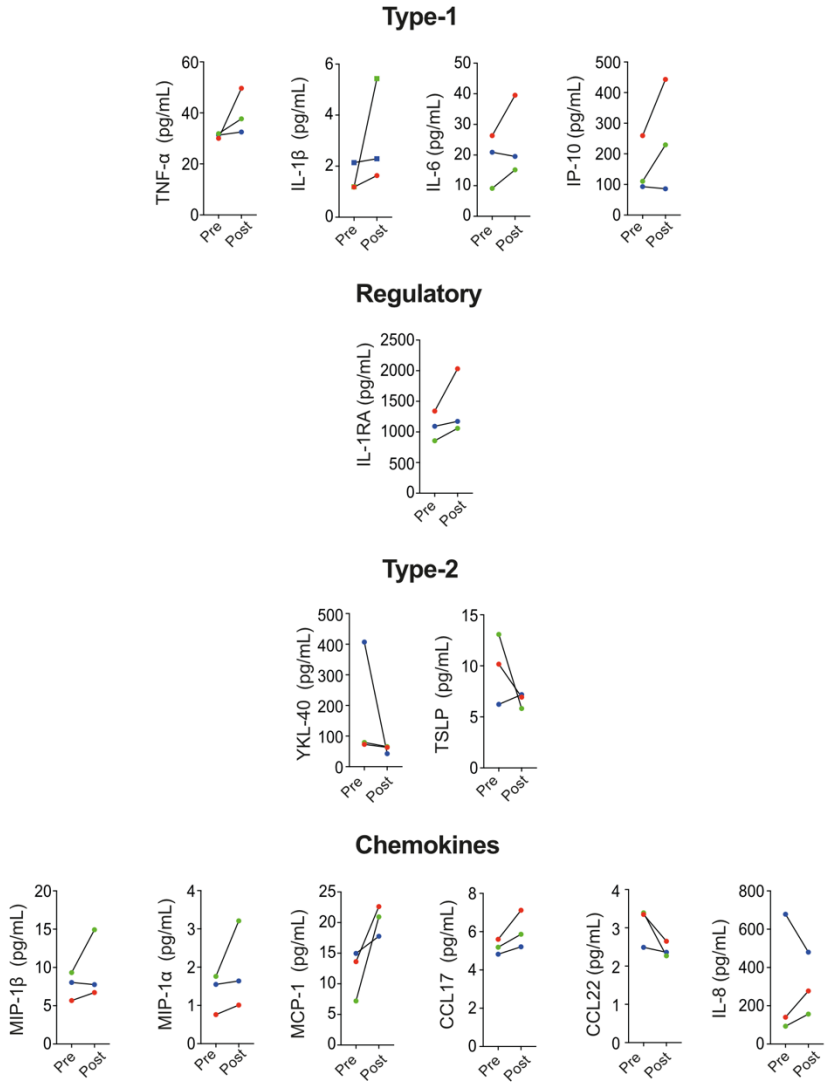

Figure 5.9. Human lung migratory *S. mansoni* infection leads to a trend for increased sputum cytokines

Non-endemic (dutch) participants were percutaneously exposed to 20 cercariae, with samples taken pre, and 11-14 days post infection. Sputum supernatants were assessed by luminex and ELISA for changes in soluble mediators. The following cytokines had levels below the lowest standard of the assay and so were not able to be accurately detected: IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, FGF-b, PDGF-BB, VEGF, IFNγ, G-CSF, GM-CSF. To compare differences between pre and post infection t tests were used, p values displayed. For fold changes a three colour scale (green, yellow, red) was used. Data is from one experiment (n=3 per group).

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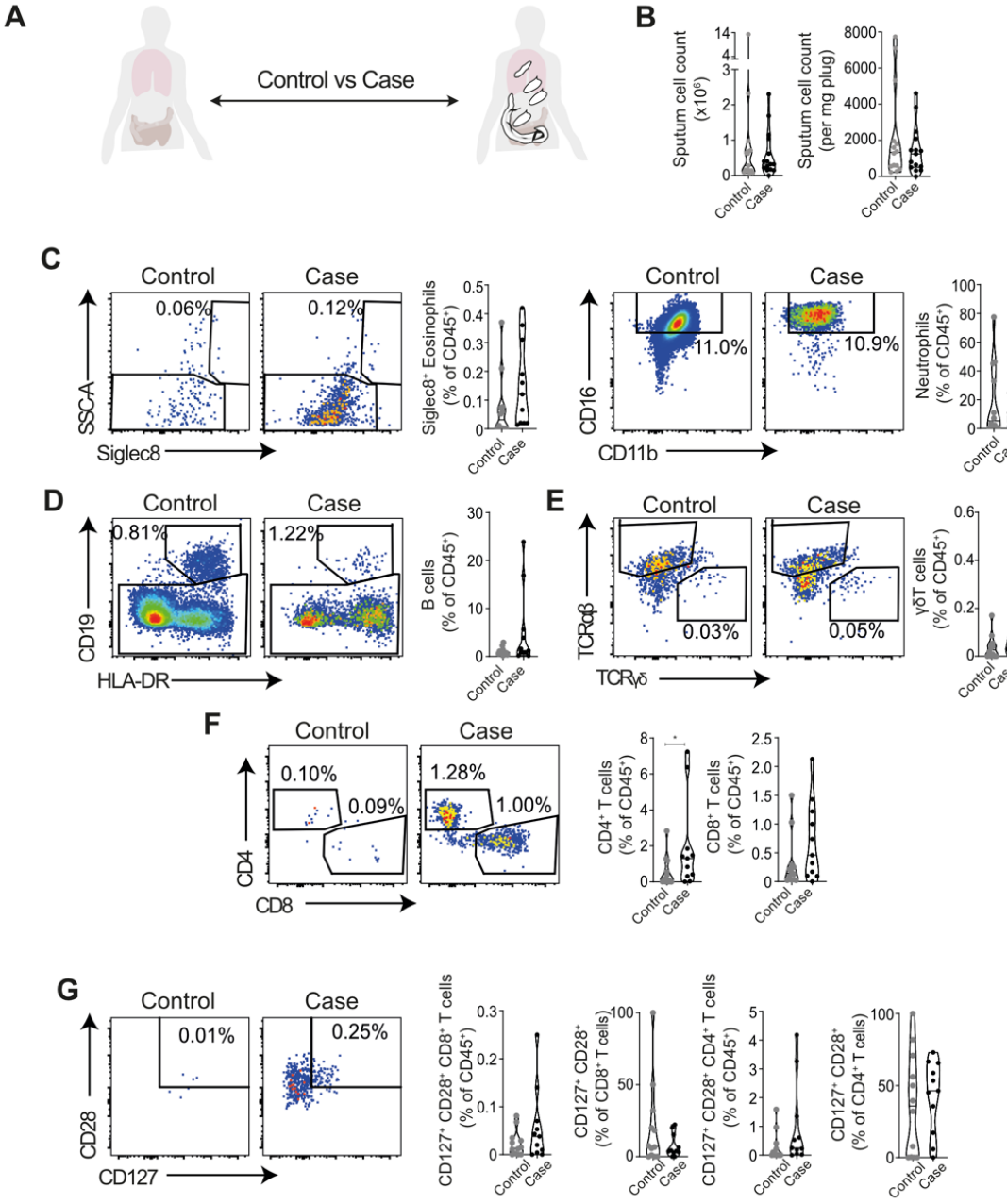

Figure 5.10. Human endemic patent *S. mansoni* infection is associated with an increase in sputum CD4⁺ T cells.

A) A case control study was performed on endemic individuals from Entebbe, Uganda. Active, egg producing *S. mansoni* infection was confirmed via CCA and Kato Katz, and individuals allocated into the case group. B) Sputum cell counts, absolute or per mg of selected sputum plugs are shown. Sputum cell isolates were assessed by flow cytometry. Representative flow cytometry plots show changes in cells, as a proportion of total leukocytes (CD45⁺) for C) eosinophils and neutrophils, D) B cells, E) γδT cells, F) CD4⁺ and CD8⁺ T cells and G) CD127⁺ CD28⁺ CD4⁺ and CD8⁺ T cells. Certain cell types are also shown as a proportion of their parental gate, namely G) CD127⁺ CD28⁺ cells as a proportion of CD4⁺ and CD8⁺ T cells. 7 samples with no convincing immune populations (less than 100 CD3⁺ cells, CD19⁺ cells and CD11c⁺HLA-DR⁺ cells) were excluded from flow cytometric analysis (3 control, 4 case), resulting in n=11-12 per group. To compare differences between groups Mann-Whitney tests were used. *=P <0.05.

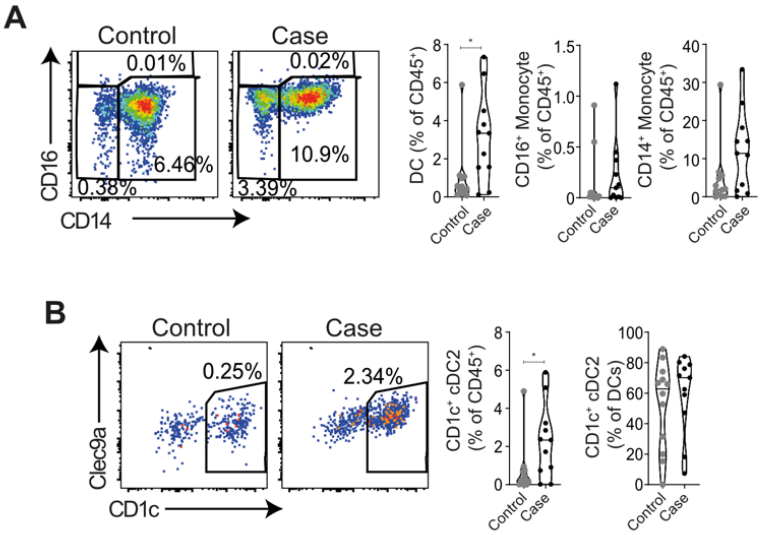

Figure 5.11. Human endemic patent *S. mansoni* infection is associated with increased sputum cDC2s

As in Fig. 5.10 a case control study was performed on endemic individuals from Entebbe, Uganda. Active, egg producing *S. mansoni* infection was confirmed via CCA and Kato Katz, and individuals allocated into the case group. Sputum cell isolates were assessed by flow cytometry. Representative flow cytometry plots show changes in cells, as a proportion of total leukocytes ($CD45^+$) for A) cDCs and monocytes and B) cDC2s. CDC2s are also shown as as a proportion of cDCs. 7 samples with no convincing immune populations (less than 100 $CD3^+$ cells, $CD19^+$ cells and $CD11c^+HLA\text{-}DR^+$ cells) were excluded from flow cytometric analysis (3 control, 4 case), resulting in n=11-12 per group. To compare differences between groups Mann-Whitney tests were used. *=P <0.05.

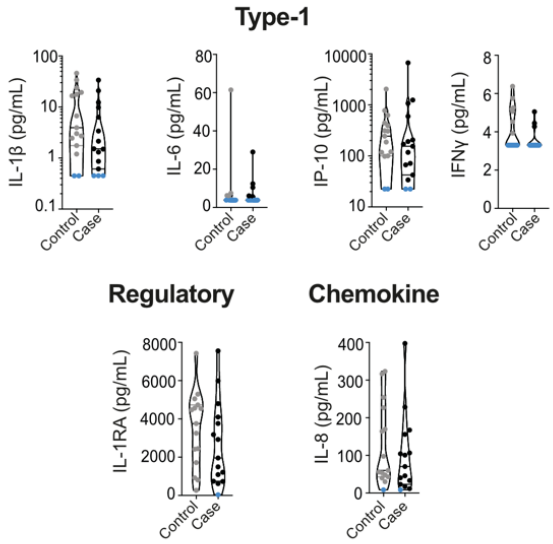

Figure 5.12. Human endemic patent *S. mansoni* infection is not associated with consistent changes in sputum cytokines.

A case control study was performed on endemic individuals from Entebbe, Uganda. Active, egg producing *S. mansoni* infection was confirmed via CCA and Kato Katz, and individuals allocated into the case group. Sputum supernatants were assessed by luminex for changes in soluble mediators. For the following cytokines over two thirds of the samples were below the lowest standard of the assay and so were not able to be accurately detected: IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, MCP-1, MIP-1α, MIP-1β, Eotaxin, FGF-b, G-CSF, GM-CSF, PDGF-BB, VEGF, RANTES, TNFα. For cytokines in which there was a mix of measurements above and below the detection limit samples below the detection limit were assigned the value of the lowest standard, shown in blue. To compare differences between control and case Mann-Whitney tests were used. Data is from one experiment (n=15 per group).

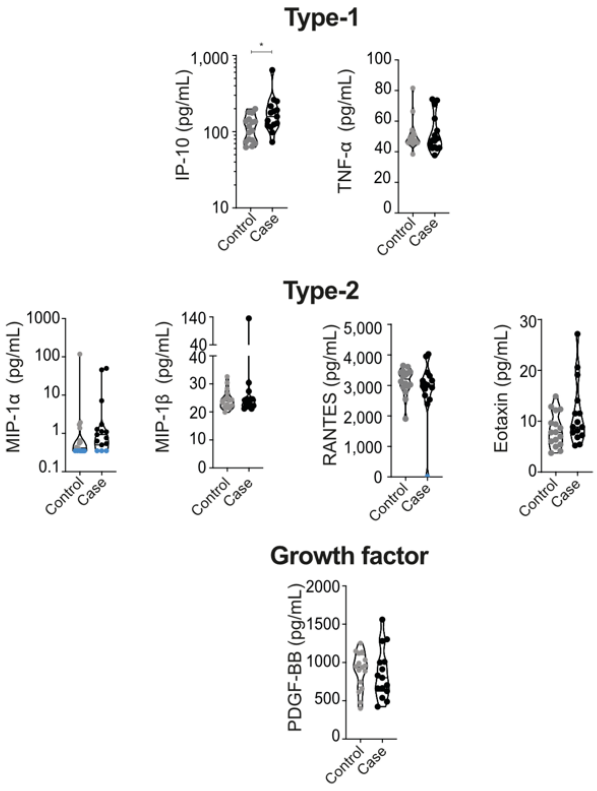

Figure 5.13. Human endemic patent *S. mansoni* infection is associated with a significant increase in the cytokine IP-10 in serum.

A case control study was performed on endemic individuals from Entebbe, Uganda. Active, egg producing *S. mansoni* infection was confirmed via CCA and Kato Katz, and individuals allocated into the case group. Sputum supernatants were assessed by luminex for changes in soluble mediators. For the following cytokines over two thirds of the samples were below the lowest standard of the assay and so were not able to be accurately detected: IFNγ, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, MCP-1, FGF-b, G-CSF, GM-CSF, VEGF. For cytokines in which there was a mix of measurements above and below the detection limit samples below the detection limit were assigned the value of the lowest standard, shown in blue. To compare differences between control and case Mann-Whitney tests were used. Data is from one experiment (n=15 per group).

Table 5.1 Participant demographics in lung migratory *S.mansoni* infection

	Participants (n=3)
Study design	Experimental, clinical trial
Cercarial dose	20
Time post exposure	11 – 14 days
Study location	Leiden, Netherlands
Age – median (min, max)	35 (18, 35)
Sex - male, number (% of total)	2 (66.7%)

Table 5.2 Participant demographics in endemic patent *S. mansoni* infection

	Control (n=16)	Case (n=15)
Study design	Observational, case control	
Infectious cercarial dose and time post first infection unknown		
Study location	Kigungu, Entebbe, Uganda	
Participants were recruited within a 2km radius of the Kigungu landing site controls from colleges, cases from the community		
Age – median (min, max)	19.5 (18, 23)	19 (18, 25)
Sex - male, number (% of total)	8 (50%)	7 (47%)
Student - yes, number (% of total)	12 (75%)	1 (6.7%)
Ever done work activities involving regular lake contact?		
Yes	6 (37.5%)	14 (93.3%)
currently/last month	2 (12.5%)	11 (73.3%)
two-six months ago	3 (18.8%)	1 (6.7%)
seven-twelve months ago	1 (6.3%)	0 (0%)
more than a year ago	0 (0%)	1 (6.7%)
unknown	0 (0%)	1 (6.7%)
No	9 (56.3%)	1 (6.7%)
Unknown	1 (6.3%)	0 (0%)
Kato Katz - positive, number (% of total)		
S. mansoni	0% (0±0)	16 (100%)
Hookworm	0% (0±0)	3 (20%)
No other worm eggs (Ascaris, Trichuris, Strongyloides, Trichostrongylus) found		
Last praziquantel dose - number (% of total)		
in the last month	1 (6.3%)	0 (0%)
two to six months ago	0 (0%)	0 (0%)
seven to twelve months ago	0 (0%)	1 (6.7%)
more than a year ago	6 (37.5%)	3 (20%)
not at all	2 (12.5%)	9 (60%)
do not remember/not recorded	6 (37.5%)	3 (20%)

Chapter 6

General discussion

Chapter 6 - General discussion

At the outset of this work we were aware that Tregs were critical to regulate AAI (Baru *et al.*, 2010), but there were conflicting reports on the functional stability of Tregs during inflammation (Rubtsov *et al.*, 2010; Chen *et al.*, 2017). The role of Tregs in fungal AAI had not been determined. Different subsets of DCs had been shown to be critical for directing Th2 and Treg expansion (Worthington *et al.*, 2011; Lombardi *et al.*, 2012; Nutsch *et al.*, 2016), although their role was not clear in ongoing AAI. Our understanding of lung immune responses in schistosomiasis was basic in mouse models, and almost non-existent in humans (Crabtree and Wilson, 1986; Schwartz, Rozenman and Perelman, 2000; Redpath *et al.*, 2015).

In this thesis we have revealed Tregs to expand during fungal AAI, remaining a stable and suppressive cell subset despite an increasingly Th2-like phenotype. The expansion of Tregs and Th2 cells was closely linked, both dependent upon $MGL2^{+}$ cDC2s and no other DC subsets. The lung immune response in schistosomiasis was shown to be type-2 dominated in murine models, with an expansion of cDC2s consistently observed in lung migrating and patent infection in mice and humans.

The first aim of this thesis was to establish the role of Tregs during *A.f.* driven AAI. Tregs have been shown to have regulatory roles at steady state, and in a number of inflammatory models (Brunkow *et al.*, 2001; Sawant and Vignali, 2014; Lu, Barbi and Pan, 2017; Sakaguchi *et al.*, 2020). However, how Treg numbers and phenotype changed during fungal AAI, whether they were stable, and whether they were required to suppress inflammation was still unknown. Understanding these questions is critical to design Treg targeted therapeutic approaches, for instance Treg adoptive transfer or *in vivo* expansion, and to evaluate if they are applicable to treatment of asthma in general, and fungal asthma in particular (Rana and Biswas, 2020).

In chapter 3, we demonstrated an expansion of Tregs during *A.f.* driven AAI in the lungs and lung dLN in mice. Lung Tregs were preferentially expanded in the tissue and expressed markers associated with residency (ST2/KLRG1), suggestive that these cells may provide long-term local regulatory and repair responses at the site of AAI (Delacher *et al.*, 2017). We were able to reveal the true extent of lung tissue Treg expansion by *i.v.* labelling and excluding circulatory Tregs which represent the majority of Tregs in naïve mice, a finding that must be taken into account when evaluating the dynamics of whole lung isolated Tregs in future work. The factors which dictated Treg expansion in this model are not clear, with proliferation at site, recruitment, enhanced survival and increased *de novo* Treg induction all potential mechanisms, and avenues for future research.

We then moved on to investigate the suppressive function of Tregs during AAI, starting with IL-10, a regulatory cytokine critical previously shown to be critical for Treg suppressive activity in the lung at steady state and during AAI (Rubtsov *et al.*, 2008). Utilising transcriptional

reporter mice, we identified expression of the regulatory cytokine IL-10 expression by lung Tregs, as well as Foxp3$^-$ CD4$^+$ T cells. Expression of IL-10 was elevated at the site of inflammation, namely in the lung tissue and airways, suggestive of active regulation by this cytokine of AAI. To understand the role of Treg IL-10 in this model Foxp3ΔIL-10 models could be used, or mixed bone marrow chimeras of DEREG and *Il10*$^{-/-}$ mice could be established (Kühn *et al.*, 1993; Lahl *et al.*, 2007; Rubtsov *et al.*, 2008). These suggested experiments would also allow us to distinguish the relative importance of Treg vs Foxp3$^-$ CD4$^+$ T cell derived IL-10, with recent work showing Foxp3$^-$ CD4$^+$ T cell production of IL-10 was critical to regulate pathogenic Th1 responses in the lung, via regulation of myeloid cells (Branchett *et al.*, 2020). There is evidence that IL-10 may have differing roles in fungal AAI, with work in a model of high dose (2x10^6 spores) *A.f.* exposure finding that IL-10 was required to promote the Th2 response, without affecting Th1 responses (Shreiner *et al.*, 2012).

The suppressive function of Tregs in this model was conclusively demonstrated utilising Treg depletion. Depletion of Tregs during AAI resulted in an increase in type-2 inflammation, for instance Th2 cytokine production and eosinophilia. The precise role of Tregs in AAI has been disputed, with some studies showing a suppressive role, while others found Treg depletion had no effect (Baru *et al.*, 2010, 2012). This is the first time Tregs have been shown to be required to suppress fungal AAI. Surprisingly, despite the well documented abilities of Tregs to inhibit Th17 cells (Chaudhry *et al.*, 2009) we observed no increase in the percentage of lung CD4$^+$ T cells able to express IL-17. There are numerous possible explanations for this, for instance Tregs can act as a sink for IL-2, a cytokine that has been shown to suppress Th17 differentiation (Laurence *et al.*, 2007), but increase Th2 differentiation (Cote-Sierra *et al.*, 2004). Upon Treg depletion, local IL-2 levels could therefore increase, leading to a suppression of Th17 and increase in Th2 priming. The balance between suppressive and permissive effects of Tregs on Th17 cells could lead to the observed equivalence in Th17 responses between Treg depleted and WT mice. Alternatively, Th17 cells could be less responsive to suppressive mechanisms used by Tregs during AAI. For instance, it could be determined whether Th2 and Th17 cells have differential IL-10R expression in this setting.

In addition, there was a disconnect between two elements of the type-17 response in this model, with increased airway neutrophilia upon Treg depletion even though CD4$^+$ T cell IL-17 production was unaffected. This may suggest the Th17 response is not the main driver of neutrophilia in our *A.f.* model, and that upregulation of other cytokines or chemokines could be more important in this fungal AAI setting (Petri and Sanz, 2018). This is reminiscent of recent studies of human asthma, in which unsupervised clustering of asthmatic sputum transcriptomics revealed three disease endotypes, with asthmatics showing high IL-17 expression found in a different cluster to those displaying enhanced neutrophilia (Kuo *et al.*, 2017). Our results showing differential regulation of IL-17 and neutrophils by Tregs may support this finding.

Although we had revealed that Tregs played a critical suppressive role in fungal AAI, there was still the potential that the inflammatory response could be driven in part by Treg instability,

as has been suggested in other models of type-2 inflammation (Pelly *et al.*, 2017). Although we could identify a small proportion of Th2 cells that were derived from Tregs, this did not increase during *A.f.* driven AAI. There was a numeric increase in Th2 exTregs, but we were not able to determine if this was driven by an increased rate of Treg instability, or instead proliferation of existing Th2 exTregs. Transfer studies could be used to confirm our findings with respect to Treg stability. Specifically, fate marked Tregs could be transferred into a WT mouse, prior to *A.f.* driven AAI, and the number of exTregs measured. In this latter situation we would not expect to see any fate marked exTregs at baseline, allowing us to better interpret any appearance of new exTregs. This would require careful planning to ensure cell phenotypes are not changed by isolation and cell sorting prior to transfer, and that sufficient numbers of transferred cells are able to be detected. Our finding of Treg stability has translational relevance, showing that Tregs are generally stable during ongoing fungal AAI, and therefore suggesting that therapies designed to expand Tregs to control ongoing inflammation will not be condemned by the conversion Tregs to pro-inflammatory cells.

Although the data in the thesis suggested that Tregs were stable, we found a subset (6-12% of cells at d26) also expressed IL-4, leading us to question if these Tregs had altered suppressive capacity. Unexpectedly, there was no difference in suppressive capacity when pulmonary IL-4$^-$ Tregs from *A.f.* mice were isolated *ex vivo* and compared to those from PBS treated controls, in *in vitro* suppression assays. Notably, however, IL-4 expressing Tregs isolated from *A.f.* exposed mice displayed a small but significant reduction in suppressive capacity vs those isolated from naïve mice. It is possible that the IL-4 produced could be directly acting as a T cell growth factor (Ben-Sasson *et al.*, 2000). It is surprising that IL-4$^-$ Tregs isolated from *A.f.* driven AAI had comparable suppressive activity to those isolated from PBS treated mice as, during *A.f.* induced AAI, Tregs increased expression of factors associated with Treg function, such as IL-10, CD69 and PD1 (Rubtsov *et al.*, 2008; Francisco *et al.*, 2009; Cortés *et al.*, 2014). Expression of these factors often overlapped, potentially suggesting the generation of a subset of Tregs with enhanced suppressive capability. It is possible that the theorised enhanced suppressive capacity of this small population was not detectable in Treg suppression assays utilising the entire Treg population. To confirm our results, *in vivo* assays of Treg suppression could be performed, for instance different Treg populations (IL-4$^+$ vs IL-4$^-$, or naïve vs AAI) could be sorted and transferred into the airways of mice undergoing AAI, with inflammation assayed in the days following transfer (Xu *et al.*, 2012).

Having established expansion of a stable, suppressive population of Tregs during AAI, the next aim of this thesis, investigated in chapter 4, was to evaluate a potential role for DCs in driving Treg expansion. It is now appreciated that DCs are not one homogenous cell type, but instead consist of multiple subsets, specialised for particular functions (Durai and Murphy, 2016). Notably, many of these subsets, including pDCs and cDC1s, have been suggested to be required for the development of airway tolerance in naïve animals (de Heer *et al.*, 2004; Lombardi *et al.*, 2012; Khare *et al.*, 2013). Our question differed from these previous studies

in that we were interested in the role of DCs in Treg expansion during ongoing AAI, in line with the development of an inflammatory response.

To investigate this, we systematically ablated CCR2-dependent DCs, pDCs, cDC1s and pDCs utilising a variety of transgenic models. Unsurprisingly, CCR2-dependent DCs were not required for the expansion of Tregs during *A.f.* driven AAI. CCR2-dependent cells, including monocytes and some DCs, have been previously implicated in contributing to AAI responses (Plantinga *et al.*, 2013; Tashiro *et al.*, 2016), results that we were able to reproduce here, with a partial reduction in Th2 cytokine expression observed in *Ccr2*$^{-/-}$ mice undergoing *A.f.* driven AAI. Although CCR2 dependency was once thought of as specific to monocyte derived cells, we now know this to be oversimplified, with CCR2 dependent cDC subsets having also been shown to exist (Bosteels *et al.*, 2020). In this thesis we were not able to specifically identify a population of CCR2 dependent DCs, instead observing a slight reduction in cDC2s in *Ccr2*$^{-/-}$ mice. The most comprehensive way to understand which myeloid subsets are reduced in *Ccr2*$^{-/-}$ mice, and in all our depletion models, would be to carry out ssRNAseq of the myeloid compartment. This experiment may have to wait for advancements that reduce the prohibitive cost of ssRNAseq. In the meantime, addition of extra cell surface markers, such as MAR-1 (which detects FcεRIα, proposed to demarcate CCR2 dependent DCs) and CD26 (which is specific to DC and not monocyte populations) (Bosteels *et al.*, 2020), to our multicolour flow cytometry panels could suffice.

In this thesis we found that neither pDCs nor cDC1s were required for the induction of Tregs during *A.f.* driven AAI. As stated previously, these subsets have been shown to be required for the induction of Tregs in models of airway allergen tolerance (Lombardi *et al.*, 2012; Khare *et al.*, 2013). A key assumption in our hypothesis, that a particular DC subtype is responsible for Treg expansion, was that all DC subsets were not equally capable of inducing Tregs. This was not a certainty. The ability of DCs to induce Tregs has long been proposed to be a function of DC maturation status, with immature DCs able to induce or expand Tregs, whilst mature DCs provoke inflammatory responses (Banchereau and Steinman, 1998). There is a large body of research into “tolerogenic DCs”, that does not take in to account differential function of DC subsets, instead focusing on the critical importance of certain stimuli to promote a tolerogenic phenotypes in bulk DCs *in vivo* or *in vitro* (Raker, Domogalla and Steinbrink, 2015; J. H. Lee *et al.*, 2017). Further work is needed to synthesise these two areas of research. By sequentially depleting different DC subsets, this thesis has shown that Treg expansion is not a function equally shared by DCs as a whole during *A.f.* driven AAI, but instead is a function of a particular subset, as discussed later. We cannot claim to have depleted all DC subsets in this work, as we have not investigated the CCR7^{+} DCs that have been recently reported (Bosteels *et al.*, 2020; Maier *et al.*, 2020). Transgenic models to deplete CCR7^{+} DCs *in vivo* do not currently exist, and this is a key avenue for future research. In the context of previous reports, showing for instance critical roles for cDC1s in promoting Tregs during Th1 mediated inflammation (Jones *et al.*, 2016), it can be hypothesised that during inflammation the DC subset required to promote inflammatory responses will also

promote Treg responses. This hypothesis could be formally challenged by systematically depleting cDC subsets in different inflammatory contexts.

In this thesis, we have revealed a requirement for MGL2$^+$ cDC2s to drive Treg expansion during *A.f.* driven AAI. As indicated previously, and confirmed in this thesis, MGL2$^+$ DCs are a distinct developmental subset, dependent upon expression of the transcription factor IRF4 (Gao *et al.*, 2013). Upon depletion of MGL2$^+$ cDC2s, we observed a reduction in lung Treg frequency as well as number during *A.f.* driven AAI. This result was complicated by a corresponding decrease in type-2 inflammation, revealing that this DC subset is required to maintain *both* inflammatory and regulatory responses. A number of hypotheses could explain this:

1) MGL2$^+$ cDC2s could directly and simultaneously promote Th2 and Treg responses during *A.f.* driven AAI, using similar mechanisms triggered in these DCs following *A.f.* exposure
2) MGL2$^+$ cDC2s could express different levels or combinations of tolerogenic vs immunogenic molecules at different phases of *A.f.* driven AAI, directly promoting Treg, then Th2 responses (or vice versa)
3) MGL2$^+$ cDC2s could directly induce Th2 responses, with resulting downstream mechanisms feeding back to promote Treg expansion indirectly

We used an *in vitro* approach to try to understand if MGL2$^+$ cDC2s were directly responsible for Treg expansion, sorting pulmonary DC subsets *ex vivo* and comparing their ability to induce Treg induction from naïve T cells *in vitro*. We did not find that lung MGL2$^+$ cDC2s were specialised at promoting Treg induction, when compared to other sorted DC subsets. However, there were a number of technical issues with this approach that could underly these results. For instance, optimisation of the ratio of DCs and T cells could be required, or the *in vitro* assay could lack a substrate such as retinal, latent TGFβ, or tryptophan, that are present in the tissue environment (Kool *et al.*, 2009; Worthington *et al.*, 2011; Khare *et al.*, 2013) and required for the enhanced suppressive potential of cDC2s. Alternatively, this result could in fact reveal that MGL2$^+$ cDC2s do not have an intrinsically superior ability to promote Treg induction compared to other DCs. This could link to hypothesis 3, detailed above. Effector T cells are the main source of IL-2 required for Treg survival and proliferation in naïve mice (Owen *et al.*, 2018; Whyte *et al.*, 2020). To test if effector T cell derived survival signals are sufficient to provoke Treg proliferation in the absence of MGL2$^+$ cDC2s, future work could deplete MGL2$^+$ cDC2s alongside adoptive transfer of Th2 cells. If MGL2$^+$ cDC2s are directly responsible for Treg expansion, then Treg expansion would not be observed in the aforementioned experiment. If MGL2$^+$ cDC2s provoke Treg expansion via expansion of Th2 cells, and the resulting inflammatory milieu, then Treg numbers would be expected to be rescued, via Th2 driven inflammation and expression of factors such as IL-2.

The critical importance of DCs in driving Treg expansion during *A.f.* induced AAI was revealed by use of mice deficient in the integrin $\alpha v\beta 8$ on CD11c$^+$ cells. In this model, there was a significant decrease in Tregs in the lung during *A.f.* driven AAI, suggesting that activation of

TGFβ was critical to promote Treg expansion in this setting. Future work identifying which DC subset(s) express αvβ8 in the lung, utilising qPCR or reporter mice (Nakawesi *et al.*, 2020), is required to understand how this phenotype relates to our other DC subset related results. Notably, after depletion of MGL2$^+$ cDC2s, Treg numbers were reduced in both the lung and lung dLN, whereas only lung Tregs showed dependency on DC αvβ8 expression. This suggests that, at least in the lung dLN, DC αvβ8 expression cannot mechanistically explain the reduction in Tregs observed MGL2$^+$ cDC2 depleted mice. One interesting hypothesis could be that Tregs in the lung and lung dLN show different dependencies on TGFβ signalling for induction, proliferation or survival.

The first two aims of this thesis allowed us to identify the expansion of Tregs during *A.f.* driven AAI, with this observed expansion dependent upon the presence of MGL2$^+$ cDC2s, a DC subtype that was also required for the Th2 response induction in this setting. Tregs expanded during *A.f.* driven AAI were stable and suppressive, but began to develop a phenotype more traditionally characteristic of Th2 cells, with expression of IL-4 and the IL-33R ST2. Together this builds a picture of interrelated inflammatory (Th2) and regulatory (Treg) responses, that are dependent upon the same DC subtype for expansion and show similar phenotypes, for instance expression of ST2. Similar observations have been seen previously, with Tregs expressing Tbet observed in type-1 inflammatory models of bacterial infection, dependent upon the presence of cDC1s (Arnold *et al.*, 2019).

In comparison to AAI, few previous studies have focused on type-2 pulmonary immune responses during helminth infection in mouse or human systems, even though many helminths have a lung-migratory phase. The final aim of this thesis, described in chapter 5, was to elucidate pulmonary inflammatory responses during *S. mansoni* infection. Increasing our understanding of lung immunity in schistosomiasis is needed to improve our basic immunological understanding of parasite infections, while also being relevant on a number of translational levels. First, lung migrating schistosomula have long been a key target in vaccine development (Mountford and Harrop, 1998), but this has been hampered by a lack of basic understanding of what constitutes a protective or natural pulmonary immune response during schistosomiasis. Secondly, lung pathology is present in both lung migratory and patent stages of human disease, but the aetiology of this is largely unknown (Shaw and Ghareeb, 1938; Ross *et al.*, 2007; Knafl *et al.*, 2020).

Our work on this topic in this thesis is the first to describe pulmonary T cell responses to lung migrating and patent schistosomiasis. We found a low-level inflammatory response is promoted by lung migrating schistosomula in mice, with an increase in Th2 cytokines, as well as the Th1 cytokine IFNγ. At later stages of infection, after egg production, this Th2/Th1 lung immune response was enhanced. It is not clear why the pulmonary immune response in schistosomiasis increased after the parasites have left the lung. Pulmonary immune responses to intestinal parasites have been observed previously, with one study demonstrating that the purely intestinal parasite *H. polygyrus* induced lung Th2 responses, that were protective against subsequent infection with the lung migrating parasite *N.*

brasiliensis (Filbey *et al.*, 2019). It is possible to conceive a similar model whereby adult *S. mansoni* infection leads to protective immunity in the lung, reducing new migration of schistosomula, and therefore maintaining a low burden of infection in the host, reducing immunopathology. Unfortunately, formally testing this hypothesis in the murine model of *S. mansoni* infection may not be possible, as patent *S. mansoni* infection results in vascular changes in the murine liver that independently reduce the survival prospects of secondary *S. mansoni* infections (Wilson, 1990).

In this thesis, we also report the first characterisation of pulmonary DCs during murine schistosomiasis, revealing an expansion of pulmonary cDC2s with increased PDL2 expression in both lung migrating and patent schistosomiasis. We have also shown that IRF4 dependent cDC2s, characterised by expression of MGL2, are required for full development of the type-2 response to lung migrating schistosomula. In comparison to previous reports (Gao *et al.*, 2013), we did not find that $PDL2^+$ cDC2s were dependent upon IRF4, with no changes in the frequency or number of PDL2 expressing cDC2s in mice with IRF4 deleted in DCs. It is possible that the remaining $PDL2^+$ cDC2s may promote certain aspects of the type-2 immune response, potentially explaining the only partial reduction in type-2 responses we have observed in mice lacking IRF4 dependent DCs. Application of the DC depletion models used in our work in fungal AAI to pulmonary schistosomiasis would be informative to allow us to compare and contrast regulation of immune responses in each system. Of particular interest would be assessment of PDL2 expression on cDC2s during *A.f.* driven AAI. It is possible that $PDL2^+$ cDC2s during AAI co-express MGL2, and therefore would have been depleted in experiments using $MGL2^{DTr}$ mice, potentially explaining the greater reduction in the type-2 response in this model.

To investigate the translational relevance of our murine results, we next performed two complementary studies on pulmonary immune responses in people with schistosomiasis. Samples were taken from controlled human infection studies to investigate lung migrating schistosomiasis, with samples from endemic infected individuals used to investigate patent schistosomiasis. In both of these studies, induced sputum was used as an accessible proxy for lung immune responses, with changes in sputum immune cells observed. Before discussing the specifics of these changes, it must be pointed out that it was extraordinary that changes were observed at all in human lung migratory infection, where individuals were exposed to only 20 cercariae, a dose that is likely to be insufficient to promote consistent immune responses in the (much smaller) lungs of experimentally infected mice. In the human studies it was not possible to sample the lung tissue itself. Further, timepoints chosen had to be extrapolated from primate models, as the timing of lung migration in humans is not precisely known. In the case of endemic infection with patent *S. mansoni,* there were a number of factors increasing the inherent variability of the samples, for instance previous infections, genetics, duration and load of *S. mansoni* infection.

Despite these practical challenges, we observed that sputum DCs, and in particular cDC2s, were significantly increased in individuals with either lung migratory or patent *S. mansoni*

infection. Although the function of these DCs during human *S. mansoni* infection is not yet clear, our murine studies suggest that these cDC2s may be responsible for promoting type-2 responses in infected individuals. This suggestion could be further supported by follow up work interrogating T cell responses in human pulmonary schistosomiasis, to identify whether Th2 responses are evident as cDC2s increase. To do this, it may be necessary to utilise systemic samples (PBMCs) or to rely upon *in vitro* models, as T cells are rare in sputum samples. The early expansion of cDC2s in the airways of *S. mansoni* infected individuals may have particular relevance with respect to interactions with allergic airway conditions such as asthma. Patent *S. mansoni* infection has been shown to be protective against AAI, as well as atopy in general, in both human and murine systems (Van Den Biggelaar *et al.*, 2000; Smits *et al.*, 2007). It remains to be determined whether cDC2s in the airways of humans with *S. mansoni* infection play an immunogenic and/or a tolerogenic role. However, a regulatory role has been shown in blood or skin isolated DCs post schistosome infection (Everts *et al.*, 2010; Lopes *et al.*, 2018; Winkel *et al.*, 2018).

These results raise a number of major questions with respect to the specific role(s) of cDC2s during type-2 inflammation. In particular, it is not yet clear how cDC2s are able to promote both inflammatory and regulatory responses during AAI, a result that seems contradictory in the face of the traditional divide between tolerogenic and inflammatory DCs. In the case of AAI, it remains to be seen if our results are translatable to human asthma. In the case of *S. mansoni* infection, we have observed conserved expansions of pulmonary cDC2s in both murine and human models. This comparability is encouraging, opening up many future questions, particularly with respect to the specific function of human lung cDC2s during larval *S. mansoni* infection. With the current revolution in single cell technologies, including multi-parameter flow cytometry, our ability to identify DC subsets and their activation state is dramatically increasing. However, a critical next step is to better understand DC function. For murine studies, this could be the development of new transgenic models, allowing targeting of DC subsets with increased specificity at key timepoints (Jiali Wu *et al.*, 2020), whereas utilisation of humanised mouse models has great potential to allow us to mechanistically dissect the function of human DC cell subsets *in vivo* (Skelton, Ortega-Prieto and Dorner, 2018).

Together, the work in this thesis has identified a critical role for murine and human DCs, and in particular cDC2s, in both fungal allergen and helminth induced type-2 inflammation. In addition to provoking Th2 responses, we have identified a central role for cDC2s in promoting Treg responses in the lungs, as well as revealing that Tregs remain stable in fungal AAI, whilst taking on characteristics associated with Th2 cells. Together, these data should be invaluable as a platform for future work aiming to target the key cellular players and the mechanisms they employ in the coordination of pulmonary type-2 inflammation, to inform rational development of next-generation therapies for the range of diseases associated with such responses.

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