

**Understanding the role of T follicular helper cells in the
development and maintenance of chronic allergic airway
disease**

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for the degree of Doctor of Philosophy

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Declaration of Originality

I hereby certify that all the scientific work outlined in this thesis was conducted or directly supervised by myself, apart from the following exceptions. Simone Walker carried out all the lung function assessments using the FlexiVent system. Lorraine Lawrence from the Imperial College London Histology facility, carried out all the paraffin embedded lung tissue sectioning and staining for histological analyses. In chapter 6, the confocal microscopy imaging was performed by Rohit Siroya, while some of the wide-field microscopy was performed by Artur Gerasimov. In chapter 6, the qPCR of switch circle and germline transcripts was performed by Catherine Spilsbury, while lung qPCR in IL-17A knockout experiments was performed by Chloe Pyle. The samples used for the mouse strain comparisons in chapter 5 were generated by Robert Snelgrove and Dhiren Patel. The sorting of cells for qPCR were performed by Catherine Simpson, Jessica Rowley and Jane Srivastava from the Imperial College London Flow Cytometry facility.

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Abstract

T follicular helper cells (T_{FH}) are critical regulators of antibody production and have been implicated in several diseases of abnormal antibody generation. However, the role that T_{FH} play in the pathogenesis of allergic asthma is little understood. Therefore the aim of this thesis was to further examine their contribution using clinical relevant mouse models of chronic allergic airway disease (AAD).

During chronic AAD T_{FH} were first found in the mediastinal lymph node (mLN), and with further allergen exposure in the spleen and the lung tissue. T_{FH} induced during chronic AAD were bona fide, tissue resident T_{FH} which expressed CXCR5, PD1, ICOS, Bcl-6 and produced IL-4 and IL-21. Interestingly, lung T_{FH} were more skewed towards a T_{FH17} phenotype and also expressed $Ror\gamma_t$, CCR6 and produced IL-17A.

T_{FH} were accompanied by germinal centre (GC) B cells, memory B cells and plasma cells, and the generation of these cellular populations and allergic specific antibodies was completely dependent on T_{FH} . Large tertiary lymphoid structures (TLS), complete with GCs were also found in lungs and their formation was partly dependent on cellular recruitment to the lungs, IL-17A driven B cell aggregation and T_{FH} . The lungs were an active site of class switching to IgA, in addition to IgE and IgG1, suggesting the lungs to be a local site of both protective and pathogenic antibody production.

T_{FH} could be therapeutically targeted during established chronic AAD by administration of ICOS-L blocking antibodies. This reduction in T_{FH} populations was accompanied by decreased GC B cells, allergen specific IgE, and improved hallmark AAD symptoms. Interestingly, T_{FH} were also found to be regulators of chronic AAD, and their absence at the inception of chronic AAD led to enhanced type 2 biased inflammation and worsened airway function.

Collectively, these findings show T_{FH} to be important regulators and drivers of chronic AAD.

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List of abbreviations

AAD	Allergic airway disease
AHR	Airway hyper-responsiveness
AID	Activation-induced cytidine deaminase
AIT	Allergen immunotherapy
ALT	<i>Alternaria alternata</i>
Alum	Aluminium hydroxide
AM	Alveolar macrophages
APC	Allophycocyanin
APRIL	A proliferation-inducing ligand
Ascl2	Achaete-scute homologue 2
ASM	Airway smooth muscle
BAFF	B-cell activator of the TNF- α family
BAL	Bronchoalveolar lavage
Bcl-6	B cell lymphoma 6
BCR	B cell receptor
Blimp1	Beta Interferon Gene Positive Regulatory Domain 1
BM	Bone marrow
bNAbs	Broadly neutralising antibodies
BSA	Bovine Serum Albumin
BV	Brilliant violet
C	Constant
CD	Cluster of differentiation
cDNA	Complementary DNA
CCL	C-C motif chemokine ligand
CCR	C-C Chemokine receptor
COPD	Chronic obstructive pulmonary disease

CSN2	alternate name: hypersensitivity region V
CSR	Class switch recombination
cT _{FH}	Circulating T follicular helper cells
CVID	Common variable immunodeficiency
CXCL	C-X-C motif chemokine ligand
CXCR	CXC-Chemokine Receptor
Cy	Cyanine
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DSS	Dextran Sulfate Sodium
DZ	Dark zone
EBI2	Epstein Barr Induced gene 2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoabsorbent assay
EMT	Epithelial mesenchymal transition
FACS	Fluorescent activated cell sorting
FBS	Fetal Bovine Serum
FcεR1	High affinity IgE receptor 1
FDC	Follicular dendritic cell
FEV ₁	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FTY720	Fingolimod
G	Gauge
GATA3	GATA binding protein 3
GC	Germinal centre
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
GLT	Germline transcript

HDM	House dust mite
H&E	Haematoxylin & eosin
HET	Heterozygous
HEVs	High endothelial venules
HIV	Human immunodeficiency virus
iBALT	Inducible bronchus associated lymphoid tissue
ICOS	Inducible T cell co-stimulator
ICOS-L	Inducible T cell co-stimulator ligand
α -ICOS-L	Anti-inducible T cell co-stimulator ligand
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
ILN	Inguinal lymph node
i.m	Intramuscular
i.n	Intranasal
i.p	Intraperitoneal
i.v	Intravenous
KO	Knockout
LN	Lymph node
LT	Lymphotoxin
LTi cells	Lymphoid tissue inducer cells
LZ	Light Zone
MCh	Methacholine
MCPT1	Mast cell protease 1
MHC	Major Histocompatibility Complex
mLN	Mediastinal lymph node
MMP9	Matrix metalloprotease 9

mRNA	Messenger ribonucleic acid
NAL	Nasal lavage
<i>N. bras</i>	<i>Nippostrongylus brasiliensis</i>
NK	Natural Killer
NKT cells	Natural Killer T cells
OVA	Ovalbumin
PAS	Periodic acid Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCLS	Precision Cut Lung Slices
PCR	Polymerase chain reaction
PE	Phycoerythrin
PERCP	Peridinin Chlorophyll Protein Complex
PFA	Paraformaldehyde
plgR	polymeric immunoglobulin receptor
PLO	Primary lymphoid organ
PMA	Phorbol 12-myristate 13-acetate
PPs	Peyer's Patches
PRR	Pattern recognition receptor
PSGL1	P selectin glycoprotein ligand 1
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RAG	Recombination activating gene
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROR	RAR related orphan nuclear receptor
RSV	Respiratory Syncytial Virus
RT	Room temperature

RT-PCR	Real time quantitative polymerase chain reaction
S	Switch
SCS	Subscapular sinus
SCT	Switch circle transcript
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
SLOs	Secondary lymphoid organs
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
STRA	Severe therapy resistant asthma
S1P	Sphingosine 1 phosphate
S1PR	Sphingosine 1 phosphate receptor
TB	Mycobacterium tuberculosis
Tbet	T-box transcription factor
TCR	T cell receptor
TD	T cell dependent
T _{EFF}	Non-T follicular helper T effector cells
T _{FH}	T follicular helper cells
T _{FR}	T follicular regulatory cells
Th	T helper
TI	T cell independent
TLR	Toll like receptor
TLS	Tertiary lymphoid structure
Tregs	Regulatory T cells
TRM	Tissue resident memory
V(D)J	Variable, diversity, joining
VEH	Vehicle
WT	Wild Type

XLP	X-linked lymphoproliferative disease
2A3	Isotype control antibody

Chapter 1: Introduction

1.1. Asthma

Asthma is a disease of chronic airway inflammation, remodeling, airway hyper-responsiveness (AHR) and reversible airway obstruction (Lambrecht and Hammad, 2015) that affects around 334 million people worldwide (Global Asthma Network, 2014). In the UK, 5.4 million people suffer from the disease and although prevalence has plateaued in recent years, it is still the most common chronic disease in children and resulted in 1410 UK deaths in 2016 (Asthma UK, 2017). Clinically the major asthma symptoms are chest tightness, wheezing, coughing and breathlessness and most asthmatics can control these symptoms with the use of inhaled and oral corticosteroids and/or long and short acting β_2 adrenergic agonists (Olin and Wechsler, 2014). The corticosteroids function to reduce the airway inflammation, while the β_2 adrenergic agonists act on the airway smooth muscle (ASM) cells to widen the airways and make it easier to breathe (Olin and Wechsler, 2014). Despite this, about 5% of asthmatics, known as severe asthmatics cannot control their asthma with current therapies and place a massive financial burden on the NHS, accounting for 50% of the UK's asthma expenditure (Adcock and Ito, 2004). Although, a great deal is understood about asthma, it is a complex and heterogeneous disorder, which can be subdivided into several endotypes based on the age of onset, whether the disease is allergic or non-allergic and what the cells appear to be involved in the pathogenesis (Wenzel, 2012). As there is no cure for asthma, a great deal of research effort is devoted to better understanding asthma pathogenesis.

1.1.1. Allergic asthma

Allergic asthma is the most common type of asthma and is characterised by a dysregulated type 2 immune response. Type 2 immune responses are thought to have initially developed to deal with large extracellular pathogens, such as helminthic parasites. However, during allergic asthma, the body over-reacts to normally innocuous inhaled environmental agents, termed allergens, such as house dust mite (HDM), fungi and animal dander, mimicking an anti-helminthic response. The allergic response is traditionally characterised by inflammation

consisting of the influx of eosinophils and T helper (Th) 2 cells into the lungs and airways, the abnormal production of allergen specific immunoglobulin E (IgE) and mast cell and basophil degranulation. Alongside mucus hyper-secretion, airway remodelling and AHR, allergic asthma is generally considered a Th2 cell disorder as they are strong producers of type 2 cytokines, Interleukin (IL)-4, -5, -9 and -13, which drive key components of the disorder (Lambrecht and Hammad, 2015). However, through years of research it is now clear that allergic inflammation is far more complex than previously described and is orchestrated by more than the action of Th2 cells alone. In fact, the disease is now known to involve the interplay of multiple cell types, including, innate lymphoid cells (ILCs), Th17 cells, T follicular helper cells (T_{FH}), dendritic cells (DCs), granulocytes, and alveolar macrophages (AM) (**Figure 1.1**).

1.1.2. Th2 cells and allergic asthma

Th2 cells are $CD4^+$ T cells which are critical for type 2 immune responses and have traditionally been considered the major driver of allergic asthma. Inhaled allergens are either recognised by pattern recognition receptors (PRR) on epithelial cells and DCs or are capable of crossing disrupted airway epithelium and are taken up by specialised local DC populations (Lambrecht and Hammad, 2012). The allergen is then processed by the DCs which travel via the lymphatic vessels to the lung draining lymph node (LN) to present via major histocompatibility complex (MHC) II molecules to naive $CD4^+$ T cells (Hammad et al., 2010). IL-4 signalling via signal transducer and activator of transcription (STAT) 6 and DC co-stimulation cause naïve $CD4^+$ T cells to upregulate the transcription factor, GATA binding protein 3 (GATA3) and commit to Th2 cell differentiation (Hammad et al., 2010, van Rijt et al., 2005, Zhu et al., 2010). Newly differentiated Th2 cells travel to the lungs where they secrete type 2 cytokines which drive allergic disease. Th2 cells secrete IL-4 which is critical for IgE production (Snapper and Paul, 1987); IL-5, for eosinophil survival and maintenance (Foster et al., 1996), IL-9, for mast cell survival (Kearley et al., 2011) and IL-13 which drives goblet cell hyperplasia and

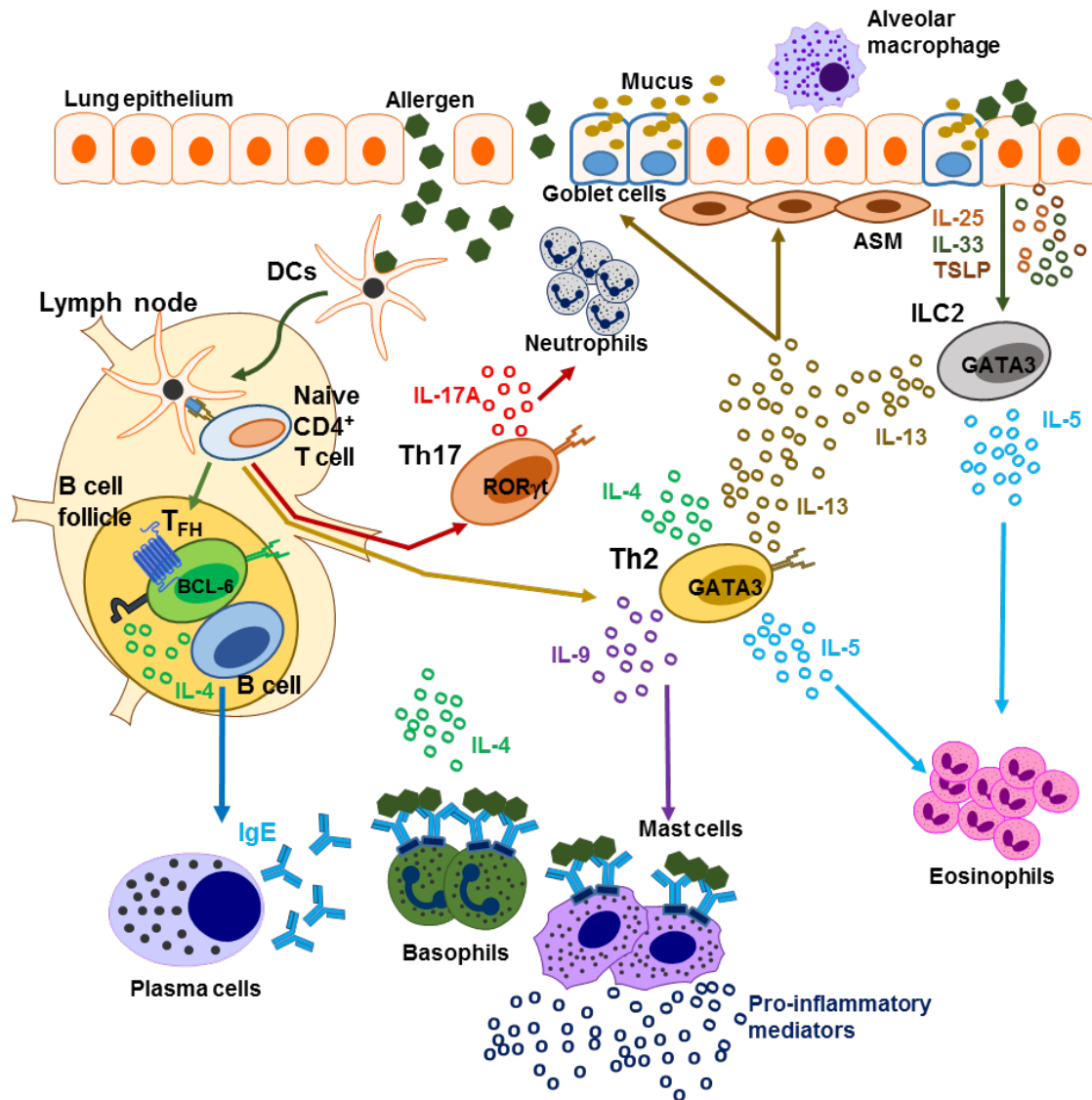


Figure 1.1: Allergic asthma schematic

The inflammatory response during allergic disease is initiated by the interaction of the allergen with the lung epithelial cells. Epithelial cells secrete pro-inflammatory cytokines, including IL-25, IL-33 and TSLP that recruit group 2 innate lymphoid cells (ILC2) which secrete the type 2 cytokines, IL-5 and IL-13. Epithelial cells can also secrete a number of mediators that recruit dendritic cells (DCs) into the lungs. Activated DCs take up allergen and travel to the lung draining lymph node to present the antigen via MHC II molecules to naïve CD4⁺ T cells. DCs priming drives the differentiation of Th2 cells, Th17 cells and the initiate steps of T_{FH} generation. Th2 cells secrete IL-4, IL-5, IL-9 and IL-13, while Th17 cells secrete IL-17A. In the B cell follicle T_{FH} secrete IL-4 and provide T cell help aiding the differentiation of B cells into IgE secreting plasma cells. IL-4 is critical for IgE generation, IL-5 for eosinophil survival, IL-9 for mast cell survival and IL-13 for mucus hypersecretion by goblet cells and acts on airway smooth muscle (ASM) cells driving airway hyperresponsiveness. IL-17A is associated with the recruitment on neutrophils into the lungs and the airways. IgE from plasma cells binds to FcεR1 receptors on the surface of basophils and mast cells, and cross-linking of the receptor and allergen causes degranulation of these cells, leading to the release of a number of pro-inflammatory mediators, which recruit cells into the lungs and airways. IL-4 production by basophils is thought to be the initiate source of IL-4 required for Th2 cell differentiation during allergic disease.

induces AHR (Grünig et al., 1998, Wills-Karp et al., 1998) (**Figure 1.1**). Th2 cells are found to be elevated in the airways of patients with allergic asthma (Robinson et al., 1992) and the adoptive transfer of *in vitro* polarised Ovalbumin (OVA)-specific Th2 cells into OVA aerolised animals is sufficient to drive allergic airway disease (AAD) (Cohn et al., 1999a, Cohn et al., 1997, Cohn et al., 1999b). However, Th2 cells are not the only cells that produce type 2 cytokines and recently another major producer, ILCs, have been highlighted for their role in allergic asthma.

1.1.3. ILCs and asthma

ILCs are cells of the innate immune system which have been shown to be important in the development of asthma. Unlike traditional immune cell subsets, ILCs do not appear to express a specific lineage defining cell surface marker, such as CD19, CD3, and CD4 (Artis and Spits, 2015). Instead ILCs are defined as lineage negative cells that express a series of common leukocyte markers including, CD45, CD90 and CD127 and have the capability to produce Th associated cytokines (Artis and Spits, 2015, Moro et al., 2010, Neill et al., 2010). Despite early studies speculating the existence of a non-T or B cell capable of secreting IL-5 and IL-13 in response to epithelial cytokines (Fort et al., 2001), the existence of ILCs was not confirmed until three landmark studies described them in 2010 using type 2 pathology models (Price et al., 2010, Moro et al., 2010, Neill et al., 2010). Human ILCs have also been identified (Mjösberg et al., 2011).

ILCs are categorised into three major subgroups based on transcription factor expression and cytokine secretion (Spits et al., 2013). Group 1 ILCs express T-box transcription factor (Tbet) and produce interferon (IFN)- γ in response to IL-12, IL-15 and IL-18. The group consists of ILC1s and natural killer cells (NK) cells (Bernink et al., 2013, Eberl et al., 2015). Group 2 ILCs (ILC2s) express the transcription factors, GATA3 and RAR related orphan nuclear receptor (ROR) α and produce IL-5 and IL-13 in response to the epithelially derived cytokines IL-25, IL-33 and TSLP. Finally, group 3 ILCs express ROR γ t and secrete IL-17A and/or IL-22 in

response to IL-1 β and IL-23. This group consists of ILC3s and Lymphoid tissue inducer (LTi) cells (Spits et al., 2013).

ILC2s, with their secretion of type 2 cytokines, have been heavily studied and implicated in AAD pathogenesis (**Figure 1.1**). ILC2s have been shown to be elevated in the bronchoalveolar lavage (BAL) and peripheral blood of paediatric and adult asthmatics (Bartemes et al., 2014, Christianson et al., 2015, Nagakumar et al., 2016), induced sputum in severe asthmatic children (Nagakumar et al., 2016) and in mouse models of AAD (Barlow et al., 2012, Bartemes et al., 2012). Pulmonary ILC2s express the IL-33 receptor, T1/ST2 and are strongly induced by IL-33 to produce IL-13 (Barlow et al., 2013). Adoptive transfer of ILC2s has been shown to be sufficient to drive allergic inflammation and AHR in T cell deficient animals (Barlow et al., 2012, Bartemes et al., 2012). ILCs are considered important for the initiation of AAD and ILC2 deficient animals have impaired allergen or helminth induced Th2 responses (Halim et al., 2014, Oliphant et al., 2014). This is primarily thought to be due to MHCII expressing ILCs directly presenting antigen to T cells (Oliphant et al., 2014, Mirchandani et al., 2014) and ILC2 derived IL-13 promoting DC migration to the mediastinal lymph nodes (mLN), thereby facilitating Th2 cell differentiation (Halim et al., 2014). Consequently, ILCs provide an early source of type 2 cytokines which initiate pathology and therefore bridge together the innate and adaptive immune response during AAD.

1.1.4. Th17 cells and asthma

Th17 cells are CD4⁺ T cells that are important for controlling fungal and bacterial infections at mucosal surfaces. They are characterised by production of IL-17 family cytokines, in particular IL-17A, IL-17F and IL-22 (Newcomb and Peebles, 2013). In mice Th17 differentiation is initiated by TGF- β and IL-6 or IL-21 (Zhou et al., 2007, Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006) and is maintained and further propagated by IL-23 signalling (McGeachy et al., 2009, Zhou et al., 2007). In humans, the role of TGF- β in Th17 differentiation is more controversial but appears to also require the interplay of IL-1 β , IL-6, and IL-23 (Acosta-

Rodriguez et al., 2007, Wilson et al., 2007, Manel et al., 2008). Together these cytokines function to drive the expression of the lineage defining transcription factor ROR γ t (Ivanov et al., 2006, Yang et al., 2008) and ROR α (Yang et al., 2008), therefore committing naïve cells to the Th17 lineage.

IL-17 family cytokines have been heavily implicated in allergic asthma. IL-17A expression has been detected in the blood, BAL, induced sputum and lung biopsies from asthmatic patients (Chakir et al., 2003, Molet et al., 2001, Al-Ramli et al., 2009). Peripheral blood mononuclear cells (PBMC) treated with anti-CD3 and IL-2 show cells from severe therapy resistant asthma (STRA) patients produce 5-fold more IL-17A than steroid sensitive severe asthmatics (Nanzer et al., 2013). While IL-17A⁺ and IL-17F⁺ cells have been identified in the sub-epithelial layer of bronchial airways and data shows IL-17A in the lung tissue positively correlates with asthma disease severity (Chakir et al., 2003).

In general, IL-17A has been generally associated with severe asthma characterised by the influx of neutrophils rather than eosinophils (Lambrecht and Hammad, 2015, Al-Ramli et al., 2009) (**Figure 1.1**). IL-17A is known to be a potent inducer of neutrophil recruitment by promoting the production of the neutrophil attractant IL-8 by a variety of stromal cells including, fibroblasts (Fossiez et al., 1996), epithelial cells (Laan et al., 1999) and ASM cells (Vanaudenaerde et al., 2003). This fits with data showing that severe asthmatics on high dose corticosteroids have greater numbers of BAL and lung neutrophils compared to mild-moderate asthmatics and healthy controls (Wenzel et al., 1997).

Given that Th17 cells are a major source of IL-17 family cytokines they have been studied using murine models of AAD. The transfer of *in vitro* generated Th17 into OVA challenged animals was sufficient to induce mucus secretion and AHR (McKinley et al., 2008), indicating these cells can drive AAD. Furthermore, these transferred Th17 cells were steroid-insensitive even when mice were treated with the steroid dexamethasone prior to OVA challenge (McKinley et al., 2008). Consistent with mouse studies, dexamethasone failed to suppress IL-17A production from PBMC cultures from severe asthmatics, however the addition of Vitamin

D sufficiently suppressed IL-17A and IL-22 production (Nanzer et al., 2013). Therefore, the role of vitamin D in asthma is also being explored (Lange et al., 2009).

However, despite growing understanding of the contribution of IL-17A family cytokines to asthma, it is much more complex than originally thought. IL-17 receptor and IL-17 knockout animals have improved OVA driven allergic disease compared to controls (Nakae et al., 2002, Schnyder-Candrian et al., 2006), but the exogenous addition of IL-17A also reduced AAD symptoms (Schnyder-Candrian et al., 2006). This regulatory role of IL-17 fits with data from a study showing IL-17RA⁺ cells to be increased in the epithelium and submucosa paediatric patients with STRA. This was associated with higher intraepithelial neutrophils and correlated with better disease control (Andersson et al., 2017). Therefore, IL-17A may have pro-inflammatory and pro-regulatory roles during asthma pathogenesis.

1.1.5. IgE and Allergic disease

The production of allergen specific IgE is one of the hallmark features of allergic asthma (**Figure 1.1**). IgE is found at very low concentrations in the serum in comparison to other isotypes (Galli and Tsai, 2012) and is elevated in atopic asthmatics compared to non-atopic asthmatics and non-asthmatics (Humbert et al., 1996, Burrows et al., 1989). Therefore, the presence of high IgE along with a positive allergen skin prick test is one of the diagnostic tools used to define allergic asthma in the clinic (NHS, 2016).

IgE production is driven by IL-4 secreted by CD4⁺ T cells leading to class switch recombination (CSR) in B cells (Snapper and Paul, 1987). IgE is generally found bound to the high affinity IgE receptor 1, FcεR1 expressed on the surface of mast cells and basophils rather than free in solution (Malveaux et al., 1978, Conroy et al., 1977, Froidure et al., 2016). Both mast cells and basophils are tissue resident cells found in the mucosa and connective tissue that contain large granules containing pre-formed pro-inflammatory mediators (Beaven, 2009). In asthmatics, mast cells are found in relatively high numbers in the airway epithelium, ASM layer

and airway mucosal glands and when bound with IgE are primed for release upon allergen challenge (Brightling et al., 2002, Bradding et al., 2006).

When asthmatic individuals encounter their allergen, FcεR1 bound IgE crosslinks the allergen, activating signalling via the FcεR1 β and γ chains leading to cellular degranulation and the rapid release of several pro-inflammatory mediators, including histamine (Stone et al., 2010). Furthermore, mast cells start to break down arachidonic acid using the cyclooxygenase and 5-lipoxygenase pathway to generate lipid mediators, including leukotrienes B₄, cysteinyl leukotrienes and prostaglandins D₂. These mediators can act on the ASM cells resulting in bronchoconstriction, mucus secretion from goblet cells and increase vascular permeability resulting in airway narrowing (Andersson et al., 2016). This part of the allergic response is termed the early phase reaction and occurs rapidly within minutes of allergen exposure being a critical component of acute asthma exacerbation.

The early phase reaction is followed by the late phase response which develops within a couple of hours and is characterised by the recruitment of immune cells including eosinophils, neutrophils, Th2 cells and ILCs into the lungs and airways. This phase is also facilitated by mast cells and basophils that secrete several cytokines and chemokines which create a pro-inflammatory environment driving the recruitment and activation of these recruited cells which further exacerbate allergic disease symptoms (Gould and Sutton, 2008).

1.1.6. Granulocytes and asthma

Eosinophils are granulocytes, heavily implicated in protection from helminth infections and contain cytotoxic granules filled with cationic proteins, including eosinophilic cationic protein and eosinophil peroxidase (Rothenberg and Hogan, 2006). Eosinophilia is a hallmark feature of allergic asthma and thus eosinophils are elevated in the peripheral blood and airways of asthmatic patients compared to healthy individuals (Bousquet et al., 1990). During allergic disease eosinophils are recruited into the lungs and airways by the chemokines, eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26 (Jose et al., 1994, Forssmann et al., 1997,

Ponath et al., 1996, Humbles et al., 1997), and are maintained by IL-5 signalling (Foster et al., 1996). The presence of eosinophils in the lungs and airways is generally associated with more traditional Th2 cell driven allergic asthma (Wenzel, 2012) and the level of eosinophilia has been shown to correlate with disease severity (Louis et al., 2000, Bousquet et al., 1990). Eosinophils were initially implicated in playing a role in airway remodelling during allergic disease, however, eosinophil deficient animals still develop allergen induced AHR (Fattouh et al., 2011), and patients treated with an anti-IL-5 monoclonal antibody (Mepolizumab) show reduced eosinophils and asthma exacerbations numbers but do not have improved airway function (Pavord et al., 2012). Nonetheless, it is clear that eosinophils are a prominent cell involved in the disorder, especially given high levels of degranulated eosinophil components are found in the airways of asthmatic patients (Rothenberg and Hogan, 2006).

Neutrophils have not traditionally been implicated as a key cell type involved in asthma pathogenesis, however, in recent years it has become clear that they could contribute. Increased neutrophils have been found in the airways of asthma patients and have generally been associated with more severe disease (Moore et al., 2014, Wenzel, 2012, Wenzel et al., 1999). As discussed above with Th17 cells neutrophils are associated with more severe asthma. Neutrophils function as phagocytic cells but also release a great deal of inflammatory mediators including matrix metalloprotease 9 (MMP9). Recently, it has been shown that the *in vitro* culture of isolated neutrophils with allergen causes MMP9 release by cells from sensitised asthmatic patients but not healthy controls (Ventura et al., 2014). Consistent with this MMP9 was found to be higher in the induced sputum of asthmatic following an asthma exacerbation (Lee et al., 2001). Given the increasing evidence of a link between MMP9 and airway remodelling this suggests neutrophils could be contributing to disease (Kelly and Jarjour, 2003). However the link between neutrophils and asthma could be more complex than originally thought, especially given that in paediatric patients with STRA, high intraepithelial neutrophil levels were associated with improved disease control (Andersson et al., 2017).

1.1.7. $\gamma\delta$ T cells and asthma

$\gamma\delta$ T cells are CD4⁺ T cells that express T cell receptor (TCR) molecules made up of γ and δ chains, rather than the more traditional α and β chains (Murphy et al., 2012). $\gamma\delta$ T cells in the lungs are generally found close to epithelial tissue where they play critical roles in immune surveillance (Wands et al., 2005, Lloyd and Hessel, 2010). $\gamma\delta$ T cells were found to be increased in the BAL of allergic asthma patients compared to healthy controls, suggesting them to play a role in disease (Spinozzi et al., 1996). However, the role of $\gamma\delta$ T cells during disease appears to be highly dependent on their cytokine repertoire and potentially the γ chain that they express. Transfer of IL-17 producing $\gamma\delta$ T cells into mice with OVA-driven allergic disease promoted disease resolution, suggesting a pro-regulatory role for the cells (Murdoch and Lloyd, 2010). Interestingly, a great deal of these IL-17A producing $\gamma\delta$ T cells were V γ 4⁺ (Murdoch and Lloyd, 2010), consistent with other studies showing pulmonary V γ 4⁺ $\gamma\delta$ T cells to negatively regulate allergen induced AHR (Hahn et al., 2003, Lahn et al., 2002). However, on the flipside V γ 1⁺ $\gamma\delta$ T cells are implicated as drivers of allergic disease as adoptive transfer of these cells into $\gamma\delta$ deficient animals which were then subsequently OVA sensitised and challenged led to increased IL-5 and IL-13 secretion, eosinophilic inflammation and allergen induced AHR (Hahn et al., 2004).

1.1.8. Airway remodelling and airway hyperresponsiveness

Airway remodelling refers to the large structural changes that occur to the small and large airways during AAD. The remodelled airway features an enlarged smooth muscle layer, increased mucus producing goblet cells, impaired epithelial integrity, thickened epithelial basement membrane and extracellular matrix deposition beneath the basement membrane collectively resulting in leaky, narrowed and stiffened airways (Naylor, 1962, Heard and Hossain, 1973, Aikawa et al., 1992, Bergeron et al., 2009). It has been suggested that these structural changes develop after weeks or years of chronic inflammation, resulting from persistent allergen exposure, but this has not been confirmed. Nonetheless the airway

remodelling is thought to be a major cause of the decline in lung function in asthmatic patients (Homer and Elias, 2000).

The epithelial layer is impaired by the protease activity of allergens, such as HDM which break down the tight junctions between cells (Wan et al., 2001, Wan et al., 1999). Furthermore, epithelial cells undergo epithelial mesenchymal transition (EMT) and differentiate into goblet cells which subsequently secrete thick mucus, containing the mucins, MUC5AC and MUC5B (Lambrecht and Hammad, 2015). There is also a thickening of the epithelium basement membrane resulting from the deposition of extracellular matrix components, including collagen I, III and V, tenascin and fibronectin by fibroblasts (Laitinen et al., 1997, Wilson and Li, 1997, Bergeron et al., 2009). ASM cells undergo hypertrophy and hyperplasia resulting in increased muscle mass around the airway (Heard and Hossain, 1973, Lambrecht and Hammad, 2012). Furthermore, IL-13 receptor (IL-13R α 1 and IL-4R α) and IL-13R α 2 subunit are expressed on human ASM cells and can signal via STAT6 driving cellular proliferation (Risse et al., 2011, Moynihan et al., 2008).

AHR is another hallmark feature of asthma and is characterised by the fact that the airways of asthmatic patients narrow far easier in response to a stimulus than healthy individuals (Lambrecht and Hammad, 2015). Thus, provocation tests have been utilised for years to assess asthma severity. Briefly, patients are exposed the muscarinic 3 receptor agonist, methacholine (MCh) which causes ASM contraction, and the concentration which causes a 20% fall in forced expiratory volume in 1 second (FEV₁) is recorded (Society, 2000). Using mouse models of AAD IL-13 has shown to be a potent driver of AHR (Grünig et al., 1998, Wills-Karp et al., 1998), thereby implicating airway inflammation in the development of AHR.

1.1.9. Mouse models of allergic airway disease (AAD)

To better understand allergic asthma, mouse models have been developed which mimic the pathophysiology of the disease. Original models were sensitisation and challenge models utilising the chicken antigen, OVA. Briefly, mice were sensitised peripherally with OVA in

aluminium hydroxide (Alum) via the intraperitoneal or subcutaneous route, then later challenged via the airways with solubilised OVA either intranasally, intratracheally or using an aerosol chamber to generate a local lung response (Kumar et al., 2008). OVA successfully drives a strong Th2-skewing allergic response, including allergen specific IgE, type 2 cytokines, eosinophilic inflammation and AHR (Foster et al., 1996, Kuperman et al., 1998). However, the model has many limitations, for example, patients are generally not allergic to OVA, OVA inhalation without sensitisation results in antigen tolerance and due to the acute nature of the model, mice do not develop chronic inflammation or remodelling features associated with the human disease (Nials and Uddin, 2008). Thus, researchers moved to chronic models using more clinically relevant allergens, such as HDM, to which 48-90% of asthmatics are sensitised (de Vries et al., 2005, Nelson et al., 1996, Smith et al., 1969).

Chronic intranasal exposure of mice to HDM extract was used to model AAD without the need of prior allergen sensitisation. 3 weeks of continuous inhaled HDM produces a strong Th2 cell polarised airway inflammation and humoral responses (Johnson et al., 2004, Gregory et al., 2009a, Saglani et al., 2009), while longer exposures cause airway remodelling, including collagen deposition, ASM layer thickening and goblet cell hyperplasia (Johnson et al., 2004, Saglani et al., 2009). An advantage of the HDM model is that allergic disease symptoms persist and do not result in the induction of tolerance and reduced inflammation over time which is observed with prolonged OVA exposure (Swirski et al., 2002).

Other clinically relevant allergens have now also been used to model chronic AAD, for example the fungal allergen, *Alternaria alternata* (ALT). Fungal allergen exposure has been linked to worsened asthma symptoms, reduced lung function, hospitalisation and asthma associated deaths in asthmatics (Agarwal, 2011). ALT is associated with severe asthma and a rapid increase in ALT spores following a thunderstorm has been linked to an asthma epidemic with multiple asthma related hospitalisations (Pulimood et al., 2007). Chronic exposure of inhaled ALT to mice models key features of allergic disease, including type 2 cytokines, eosinophilic inflammation, AHR and airway remodelling (Castanhinha et al., 2015,

Löser et al., 2017). ALT exposure to neonatal mice has been shown to drive more severe type 2 disease than HDM exposure (Castanhinha et al., 2015).

Lastly, allergen avoidance is a well-known strategy to help improve allergic symptoms such as inflammation and AHR (Cipriani et al., 2017, Platts-Mills et al., 1982). Analogous to humans following allergen cessation, the HDM driven allergic inflammation and AHR are dramatically reduced while structural changes persist (Kearley et al., 2009, Cates et al., 2004, Johnson et al., 2004). Furthermore, similar to human upon allergen re-exposure mice undergo a rapid allergic response characterised enhanced type 2 inflammation (Cates et al., 2004). Thus, the HDM model is a sufficient model to understand many features of the pathogenesis of allergic disease.

1.2. Humoral Immunity

The humoral antibody mediated immune response is a critical component of the adaptive immunity and allows the highly specific recognition and elimination of pathogens, such as bacteria and viruses from extracellular spaces. The humoral response involves the interplay of a multiple cells of the immune system, in particular CD4⁺ T cells and B cells, and is critically dependent on antibody production by plasma cells. Plasma cells secrete large quantities of high affinity antibody of multiple isotypes, which mediate protective immunity. Firstly, antibodies can specifically bind to epitopes on pathogens and/or the toxins they produce, thus neutralising and preventing their entry into cells or across barrier sites. Secondly, multiple antibodies can coat the pathogen surface, opsonising the microbe for recognition by phagocytic cells via Fc receptors. Thirdly, antibodies can bind and target cells leading to complement activation and their eventual lysis. Finally, antibodies are relatively stable and the cells that produce them are long lived allowing long term protection from evading pathogens. Taken together, this makes antibody responses critical for protection of extracellular surfaces and spaces (Murphy et al., 2012).

However, dysregulated or inappropriate humoral immunity can also contribute to disease. Firstly, the humoral response can be inefficient at handling the evading pathogen, as is seen in patients with primary immunodeficiency (Raje and Dinakar, 2015). Secondly, the humoral response can be inappropriately activated and result in the production of antibodies that recognise the host, termed autoantibodies as observed during autoimmune disease, or alternatively overact to normally innocuous agents, as observed during allergic disease (Lambrecht and Hammad, 2015, Rosenblum et al., 2015). Lastly, over-exuberant B cell responses can lead to malignancy, for example B cell lymphoma (Shaffer III et al., 2012). Taken together, it is clear that a fully functioning, well regulated, humoral response is critical to normal protective immunity and disease prevention.

1.2.1. Antibody functions in immunity

Antibodies are an essential component of humoral immunity, and come in 5 different isotypes, IgA, IgD, IgE, IgG and IgM. In humans IgA antibodies are sub-classed into IgA1 and IgA2, while IgG is sub-classed into 4 isotypes, (IgG1, IgG2, IgG3 and IgG4). In mice, only the IgG isotype is sub-classed, into IgG1, IgG2a, IgG2b, IgG2c and IgG3, however IgG2a and IgG2c are congenic and thus mice have a total of 7 antibody isotypes, while humans have 9. Antibody isotype is determined by the constant (C) region of the Ig heavy chain, termed the Fc region (Murphy et al., 2012).

IgG is the predominant antibody type within serum, and IgG1 is most prevalent of the isotypes. IgG subclasses mediate protection via multiple methods, including neutralisation, optimisation, complement activation (Murphy et al., 2012). However IgG4 antibodies cannot activate complement and has been associated with positive outcomes from immunotherapy (Scadding et al., 2010). IgM is generally of low affinity, but exists as a pentamer to increase its avidity, and is a strong activator of complement (Murphy et al., 2012). IgE is the least abundant isotype in the serum of healthy individuals, but increases in the serum of allergic patients (Galli and Tsai, 2012, Burrows et al., 1989, Humbert et al., 1996). IgE is generally found bound to Fc

receptors on basophils and mast cells and IgE surface cross-linking drives the activation of these cells (Malveaux et al., 1978, Conroy et al., 1977, Froidure et al., 2016). The role of IgD is the least understood of all the isotypes, but it is known to activate complement and the secreted form can confer protection by binding to virulent factors, such as diphtheria toxin (Chen and Cerutti, 2010).

IgA is the predominant antibody isotype found within secretion from mucosal sites, such as the respiratory epithelium and gastrointestinal tract (GIT) and thus plays a critical role in providing protection from inhaled and digested pathogens (Murphy et al., 2012). Secreted IgA is generally dimeric, and can be transcytosed to mucosal surfaces via interactions of the J chain with epithelial polymeric immunoglobulin receptor (pIgR) (Johansen et al., 2000, Brandtzaeg and Prydz, 1984). One of the major functions of IgA is to neutralise bacteria, viruses and toxins preventing their entry into cells or across barrier sites (Cerutti, 2008). IgA is also known to be important for maintaining commensal bacteria populations within the GIT (Suzuki et al., 2004).

1.2.2. Primary lymphoid organs

The critical cells involved in humoral immunity start their lives in primary lymphoid organs (PLOs) such as the bone marrow (BM) and thymus. Both, T and B cells develop from hematopoietic precursor cells in the BM, which are seeded from the fetal liver during embryogenesis (Schulz et al., 2016).

Early B cell development occurs within the BM, where B cells go through multiple stages of maturation. B cell development starts from the pro-B cell stage, where cells express no surface B cell receptor (BCR) and have germline Ig genes. Developing B cells are supported by a network of stromal cells which provide cytokine signals allowing cells to start undergoing recombination activating gene (RAG) 1 and 2 dependent rearrangement of their Ig heavy chain variable domain, variable (V), diversity (D) and Joining (J) segments. This is followed by light chain rearrangement and thus cells eventually become immature B cells expressing the

surface IgM as their BCR (Allman et al., 1999, Li et al., 1996, Hardy et al., 1991). These IgM bearing B cells then leave the BM via the central sinus and transition to the spleen where they develop into transitional B cells and subsequently become mature naïve B cells expressing both IgD and IgM (Chung et al., 2003, Loder et al., 1999, Allman et al., 2001). The initial V(D)J rearrangement is important for the generation of a large B cell repertoire able to recognise many specific epitopes and humans have a B cells expressing BCRs able to recognise 10^{15} different antigens (Schroeder, 2006). During development B cells also undergo positive and negative selection via BCR recognition to allow the generation of specific B cells that do not express BCRs that recognise self (Hartley et al., 1991, Goodnow et al., 1988, Nemazee and Buerki, 1989).

Similar to B cells, T cell development begins in the BM from haematopoietic precursor cells. However T cells must migrate to the thymus to complete their development. T cell differentiation is driven by a complex network of thymic stromal cells which provide multiple signals to developing thymocytes. The stages of $\alpha\beta$ T cell differentiation are defined by surface expression of the CD8 and CD4 co-receptors. T cells first transition from CD4⁻CD8⁻ double negative cells, to CD4⁺CD8⁺ double positive cells, before deciding to express either CD4 or CD8 and become either naïve CD4⁺ or CD8⁺ T cells respectively (Koch and Radtke, 2011). As developing T cells move through these stages they re-arrange the V(D)J regions of their TCR α and β chains in a RAG1 and 2 dependent manner and also undergo positive and negative selection to remove self-reactive clones (Klein et al., 2014, Murphy et al., 2012). At the end of this process mature naïve T cells can leave the thymus and enter the circulating pool along with mature B cells moving around the body visiting secondary lymphoid organs (SLOs) via blood vessels and lymphatics until they encounter their activating antigen.

1.2.3. Secondary lymphoid organs (SLOs)

SLOs are a critical component of the lymphatic system and are essential for the generation of adaptive immunity as they are major sites of lymphocyte activation and differentiation. SLOs

include the tonsils, spleen, Peyer's patches (PPs) and LNs and their development begins during embryogenesis with LN development starting at embryonic day 13.5 (Randall et al., 2008). SLO development is heavily dependent on lymphotoxin (LT) signalling and LT α cells (Eberl et al., 2003). Briefly, LT α β 2 expressing LT α cells interact with LT β R⁺ stromal cells, guiding these stromal cells to differentiate into lymphoid tissue organising cells which subsequently secrete chemokines and express adhesion molecules driving the growth of SLOs and the recruitment of cells (Randall et al., 2008).

Lymphocytes enter the LN via afferent lymphatic vessels or high endothelial venules (HEVs), while, the spleen does not have either afferent lymphatic vessels nor HEV and instead cells move into the white pulp region via the marginal zone. SLOs are then generally subdivided into distinct B cell and T cell zones. In the LN the B cell and T cell zones are located in the cortex and paracortex region respectively, while in the spleen they are located within the white pulp. The B cell zone is made up of primary follicles where B cells and specialised stromal cells called follicular dendritic cells (FDC) reside, while the T cell zone consists of T cells and DCs (Schulz et al., 2016). This separation of B and T cells is achieved by the homeostatic chemokine gradients of C-X-C-motif chemokine ligand (CXCL) 13, C-C motif chemokine ligand (CCL) 19 and CCL21 which guide the migration and positioning of circulating lymphocytes. CXCL13 is produced by FDCs in the B cell zone attracting mature B cells expressing high levels of CXC-chemokine (CXCR) 5. Thus, B cell homing is defective in CXCR5 or CXCL13 deficient animals (Ansel et al., 2000, Förster et al., 1996). CCL19 and CCL21 are produced by fibroblastic reticular cells, while CCL21 is expressed by HEVs, attracting CC-Chemokine receptor 7 (CCR7) expressing T cells and DCs (Gunn et al., 1998, Schulz et al., 2016). Therefore, *plt/plt* mice lacking CCL19 and CCL21 have disorganised T cell zone in their LNs, PPs and spleen (Nakano et al., 1997, Gunn et al., 1999, Nakano et al., 1998). LN organisation is summarised in **Figure 1.2**.

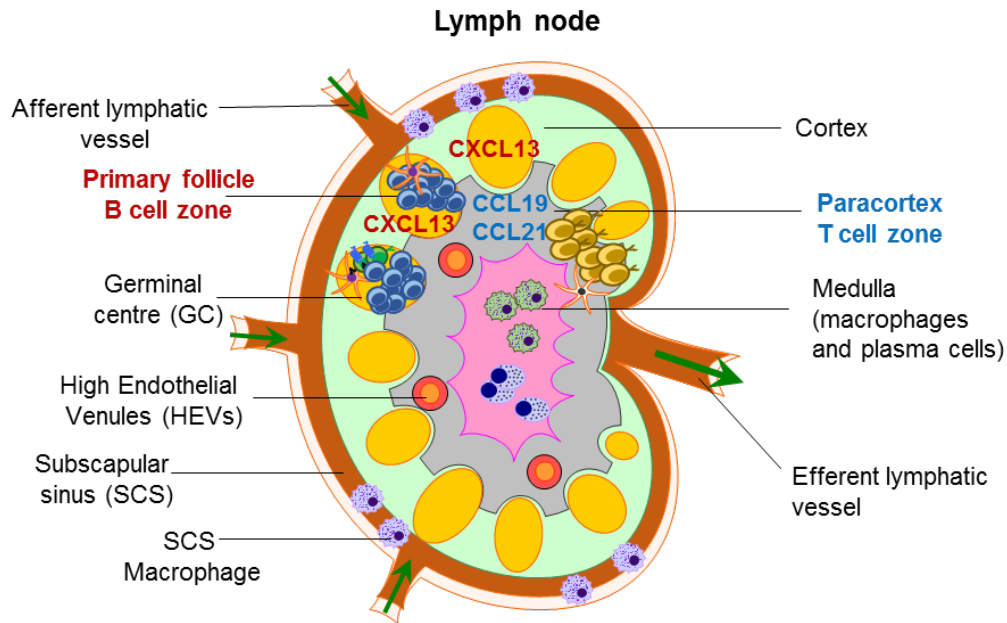


Figure 1.2: Lymph node organisation

Lymphocytes enter into the lymph node via the afferent lymphatic vessels or the high endothelial venules (HEVs) in the paracortex region, and are organised into distinct zones. B cells are found in primary B cell follicles within the cortex region, while T cells are found within the T cell zone in the paracortex region. This tight organisation is maintained by chemokine gradients, where CXCL13 is found at high levels in the B cell zone and CCL19 and CCL21 are found in the T cell zone. Primary B cell follicles can develop into germinal centres which are sites of B cell proliferation, differentiation and antibody diversification and B cells here are accompanied by follicular dendritic cells and T follicular cells (T_{FH}). T cells in the T cell zone are accompanied by dendritic cells. Subscapular sinus (SCS) macrophages are found in the SCS regions, while medullary macrophages are located in the medulla region, along with antibody secreting plasma cells. Lymphocytes leave the lymph node via the efferent lymphatic vessels.

1.2.4. T cell activation and differentiation

Naïve T cells circulate around the lymphatic system until they encounter migrating or resident DCs within SLOs presenting their cognate antigen via MHC molecules. In the case of $CD4^+$ T cells their surface TCR molecules interacts with DC peptide/MHC II complexes, while $CD8^+$ T cells interact with peptide/MHC II complexes, driving CD3 to provide the first signal of T cell activation. Co-stimulation is then provided to T cells by constitutively expressed CD28 interacting with CD80/86 on DCs (Acuto and Michel, 2003, Murphy et al., 2012). After T cell engagement T cells also upregulate inducible T cell co-stimulator (ICOS) which interacts with inducible T cell co-stimulator ligand (ICOS-L) on DCs providing further co-stimulation to

proliferate and take up effector functions (Dong et al., 2001, Hutloff et al., 1999). Co-stimulation can also be received via a number of molecules including OX40, 4-1BB and GITR with their respective ligands (Chen and Flies, 2013). Lastly, the final signal of T cell activation is cytokine signalling which can also be provided by DCs and/or other leukocytes. The specific cytokine signal provided helps determine the differentiation pathway of the CD4⁺ T cells. In mice Th1 differentiation is driven by IL-12 and IFN- γ (Hsieh et al., 1993, Lighvani et al., 2001), Th2 by IL-4 (Swain et al., 1990, Le Gros et al., 1990), T_{FH} by IL-6 (Nurieva et al., 2008) and Th17 by IL-6, TGF- β and IL-23 (Veldhoen et al., 2006, Mangan et al., 2006, Bettelli et al., 2006). After T cell priming the majority of activated T cells exit the SLOs via efferent lymphatic vessels in a Sphingosine 1 phosphate receptor (S1PR) dependent fashion and travel to the site of infection or inflammation where they fulfil their effector functions (Matloubian et al., 2004). T_{FH} however move towards the B cell zone to continue their differentiation process (T_{FH} differentiation will be discussed in section 1.3.2).

1.2.5. B cell activation

B cell activation occurs in the B cell zone of SLOs when B cells encounter their BCR specific antigen. In the LN B cells can acquire cognate immune complexes from subcapsular sinus (SCS) macrophages located below the SCS (Phan et al., 2007, Carrasco and Batista, 2007, Junt et al., 2007). Alternatively, B cells can acquire small soluble antigen that enter the LN via the SCS pores (Pape et al., 2007) or from FDCs immune complexes within the B cell follicle (Mandels et al., 1980). BCR signalling is then orchestrated via antigen crosslinking and phosphorylation of ITAM in the Ig α and Ig β chains, along with signalling of co-receptors including CD19 and CD21 driving B cell activation. These B cells can then internalise their antigen/BCR complexes into endosomes and process the antigen to be present on MHC II (Harwood and Batista, 2010). B cells then upregulate CCR7 and migrate to the T-B cell border to present their peptide/MHC II complexes to their cognate T_{FH} (Reif et al., 2002). T_{FH} then provide B cell help via CD40-L and cytokine mediated signalling driving full B cell activation.

This can then either generate an extra-follicular response driving rapid plasmablast differentiation and antibody responses or instead drive germinal centre (GC) initiation enabling the generation of long lasting, high affinity antibody production by plasma cells (MacLennan et al., 2003, Mesin et al., 2016). Expression of the receptor, Epstein Barr Induced gene 2 (EBI2) drives the localisation of B cells to the outer follicle while its deficiency allows activated B cells to move into follicles for GC responses (Pereira et al., 2009, Gatto et al., 2009).

1.2.6. Germinal centre (GC) organisation

Following antigen stimulation activated B cells and their cognate T_{FH} move into B cell follicles and drive the formation of anatomically distinct structures, termed GCs. GCs are critical for the generation of long lasting high affinity, isotype switched antibody as they are sites of rapid GC proliferation, secondary antibody diversification and differentiation into memory B cells and plasma cells (Mesin et al., 2016).

The GC is split into 2 distinct zones, the dark zone (DZ) and the light zone (LZ) and B cells within the GC are called GC B cells. GC B cells express high levels of the transcriptional repressor, B cell lymphoma 6 (Bcl-6) which is critical for their development and thus GCs do not form in Bcl-6 deficient animals (Cattoretti et al., 1995, Dent et al., 1997, Ye et al., 1997). GC B cells within the DZ undergo rapid proliferation, express high levels of CXCR4 and are called centroblasts (CXCR4^{hi}CD86^{lo}). While, GC B cells within the LZ are non-dividing cells expressing lower levels of CXCR4 and are termed centrocytes (CXCR4^{lo}CD86^{hi}). In the LZ centrocytes are found alongside T_{FH} and FDCs (Victora et al., 2010, Allen et al., 2004, De Silva and Klein, 2015). These zones are maintained by chemokine gradients, with CXCL12 being found at higher levels in the DZ, allowing GC B cells to move between the two zones based on their CXCR4 expression status. While LZ centrocytes express higher levels of CXCR5 (Allen et al., 2004, Victora et al., 2010). Similar LZ and DZ organisation is also observed in human GCs, being distinguished by CXCR4 in combination with CD83 (Victora et al., 2012) (**Figure 1.3**).

GC formation and maintenance is heavily dependent on FDCs and T_{FH} . FDCs are the major source of CXCL13, thereby facilitating the migration of B cells and their cognate T_{FH} into the follicle to generate a GC response (Vinuesa et al., 2010). FDCs are also critical for trapping antigen as immune complexes on their complement receptors, CD21 and CD35, or Fc receptor thus allowing long term antigen retention in the GC long after the initiate source disappears (Nossal et al., 1968, Szakal et al., 1989, Qin et al., 2000, Yoshida et al., 1993, Papamichail et al., 1975). Furthermore, T_{FH} are also critical for GC generation and thus T_{FH} deficient animals do not develop GC responses (Hollister et al., 2013).

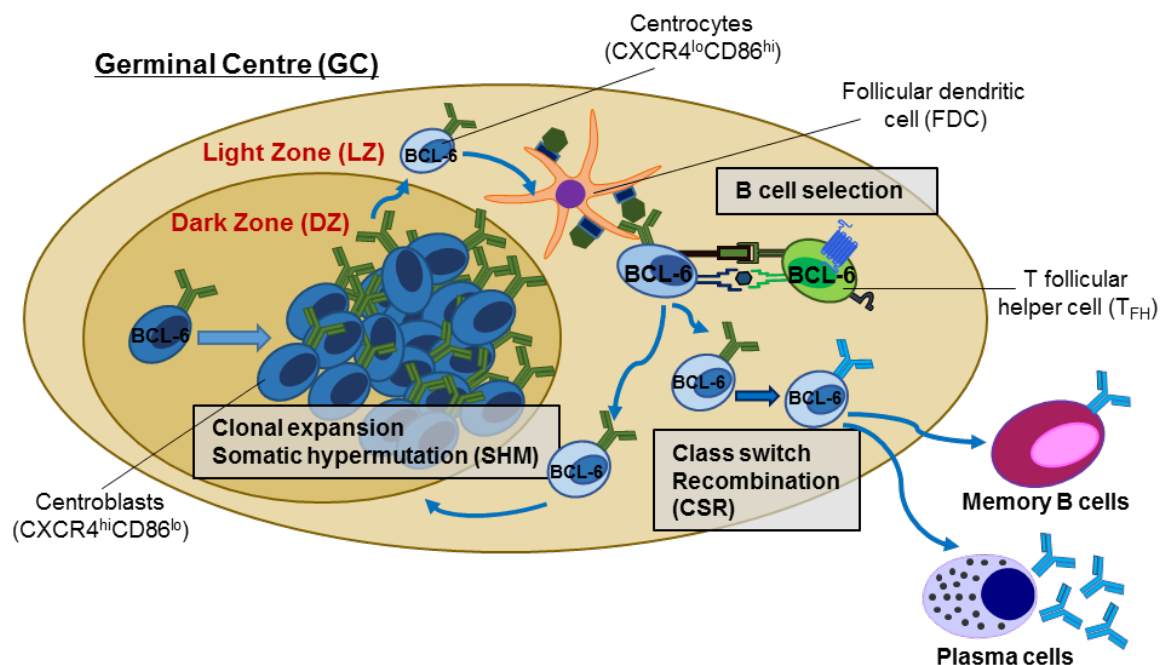


Figure 1.3: Germinal centre (GC) organisation

GCs are a site of antibody diversification, B cell proliferation and differentiation. GCs are split into 2 defined cells, the dark zone (DZ) and the light zone (LZ). The GC B cells within the DZ are termed centroblasts ($CXCR4^{hi}CD86^{lo}$). Centroblasts undergo rapid proliferation and go through the process of somatic hypermutation (SHM) allowing them to accumulate mutations in the variable region of their immunoglobulin gene locus. These mutations can increase, decrease or keep the affinity of B cell receptors (BCRs) at the same level. Centroblasts can exit the DZ, enter the LZ and become centrocytes ($CXCR4^{lo}CD86^{hi}$). Here centrocytes interact with follicular dendritic cells (FDCs) and present antigen via MHC II molecules to T follicular helper cells (T_{FH}) which provide survival signals to B cells with high affinity BCRs. Within the LZ centrocytes can also undergo the process of class switch recombination allowing them to change their antibody isotype. Centrocytes can then either re-enter the DZ to accumulate more mutations or differentiate into antibody secreting plasma cells or memory B cells.

1.2.7. Germinal centre events

The GC is essential for the generation of antibodies of higher affinity via the processes of somatic hyper mutation (SHM) and affinity maturation, and antibodies of multiple different isotypes via class switch recombination (CSR) (**Figure 1.3**). Both SHM and CSR are initiated by the enzyme activation induced cytidine deaminase (AID), which is expressed in GC B cells and converts deoxycytidine nucleotides to deoxyuracil (Muramatsu et al., 1999, Revy et al., 2000, Muramatsu et al., 2000, Chaudhuri et al., 2003, Pham et al., 2003). AID can only deaminase single stranded DNA therefore germline transcription must occur first to unwind DNA (Chaudhuri et al., 2003, Ramiro et al., 2003, Pham et al., 2003). As deoxyuracil is not normally found in DNA, its presence initiates repair processes that drive SHM and CSR. SHM occurs within DZ centroblasts and here AID introduces mutations into V region of the Ig heavy and light chains of the BCR. This is then repaired using error-prone polymerases resulting in point mutations, insertions or deletions that can increase, decrease or have no impact on BCR affinity (Wilson et al., 1998, Goossens et al., 1998, Teng and Papavasiliou, 2007), . These B cells then move to the LZ where B cells bearing high affinity BCRs are selected via cognate interactions with T_{FH} (Shulman et al., 2014, Gitlin et al., 2014, Victora et al., 2010, Liu et al., 1989).

In contrast, during CSR mutations are introduced into the Ig heavy chain constant (C) domain locus found downstream of the V(D)J genes (**Figure 1.4**). Briefly, germline transcripts (GLT) are produced by transcription starting at I promoters opening the DNA for AID action. To switch from IgD to IgE for example, mutations are introduced into the switch (S) regions upstream of the C_μ constant domain and the S_ε1 region upstream of the C_ε1 domain. Repair is mediated by base excision or mismatch repair mechanisms creating double stranded DNA breaks within both S regions, allowing the intervening DNA to be excised as a switch circle transcript (SCT) and the two S regions to be connected, bringing the C_ε1 domain downstream of the V(D)J genes (**Figure 1.4**) (Xu et al., 2012, Muramatsu et al., 2000, Muramatsu et al., 1999, Maul et al., 2010). CD40-L and cytokine signalling from T_{FH} is important for promoting CSR, with

specific cytokines determining the resulting Ig isotype, for example in mice IL-4 drives IgG1 and IgE switch, while IFN- γ drives IgG2a generation (Reinhardt et al., 2009, Toellner et al., 1998, Vinuesa et al., 2016). Overall, CSR thus allows antibodies to change their isotype from IgM and IgD to IgA, IgG and IgE while maintaining their BCR affinity and specificity.

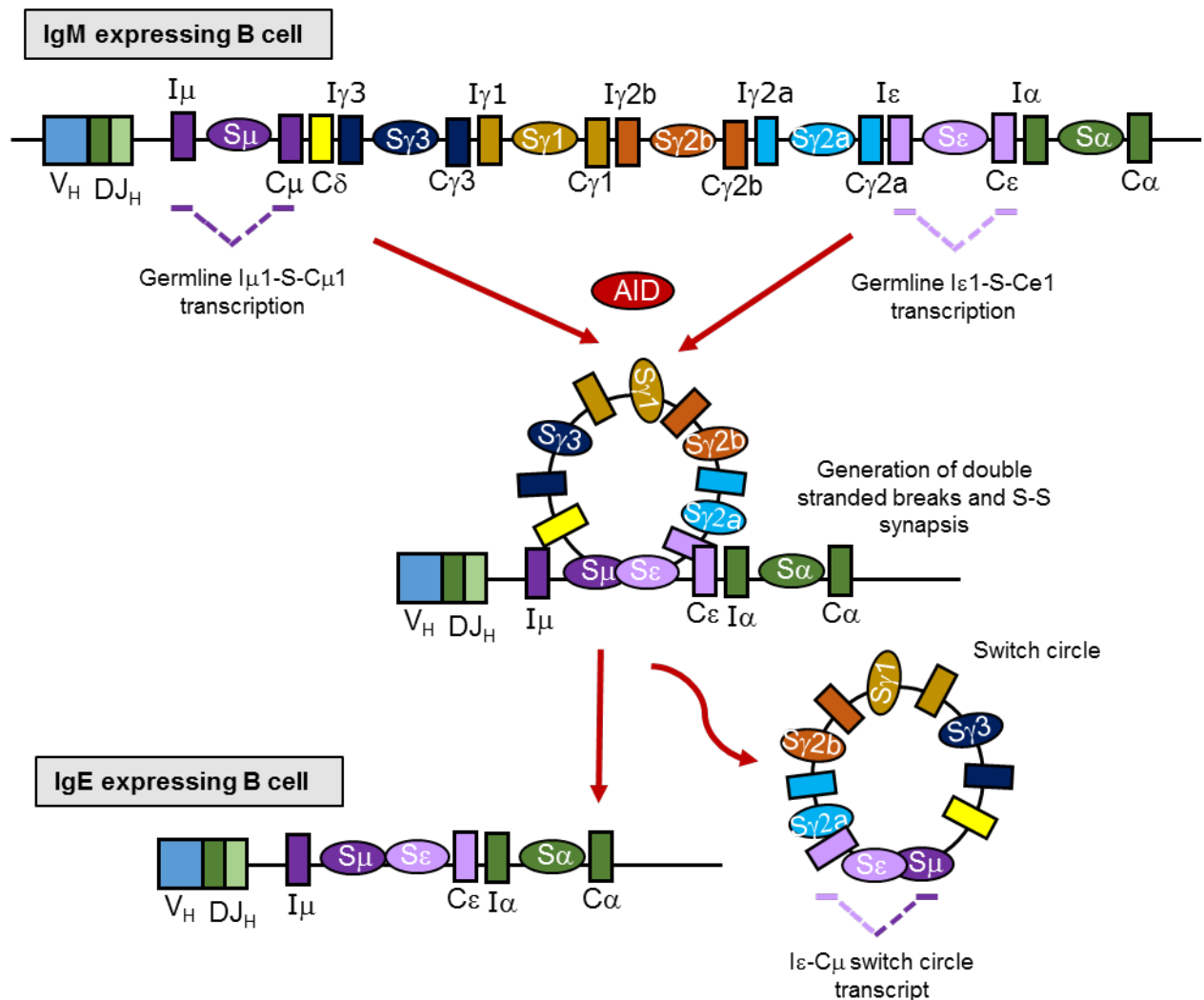


Figure 1.4: Class switch recombination

The mouse immunoglobulin heavy chain locus is displayed. The VDJ segments of the variable region are found upstream of the constant region, which contains multiple constant domains referring to different antibody isotypes. Each constant domain is preceded by an I promoter and switch (S) region. To switch from one isotype to another, germline transcription begins at the I promoter of the donor and acceptor constant domain. The enzyme activation induced cytidine deaminase (AID) creates mutations from cytidine to uracil in the donor and acceptor S regions. The mutations are then processed to create double stranded breaks that are joined by non-homologous end repairing. This brings the acceptor switch region downstream of the VDJ variable domains. The intervening DNA is removed as a switch circle and switch circle transcripts are produced.

The GC is also the major site of B cell differentiation into long lasting plasma cells and circulating memory B cells allowing the production of long lasting antibody mediated immunity. GC B cells that have undergone SHM and/or CSR can then be driven to differentiation into effector cells. Plasma cell differentiation is initiated by high affinity antigen and finalised by signals from T_{FH} (Kräutler et al., 2017). T_{FH} can drive this differentiation by providing CD40L co-stimulation and IL-21 signalling to affinity matured GC B cells (Bryant et al., 2007, Ettinger et al., 2005, Kuchen et al., 2007, Vinuesa et al., 2016). Developing plasma cells can then express the master regulator Beta Interferon Gene Positive Regulatory Domain 1 (Blimp1) which commits cells to the lineage (Turner et al., 1994, Angelin-Duclos et al., 2000, Kallies et al., 2004). Blimp1 mediates the molecular re-programming of plasma cells, causing downregulation of Bcl-6, AID and PAX5 and upregulation of XBP1, to name a few (Reimold et al., 2001, Lin et al., 2002, Shaffer et al., 2002, Nutt et al., 2015). Together this turns off the GC program and allows them to phenotypically change into large B cells, no longer expressing surface BCR, CD19, B220 and MHC Class II molecules and are filled with endoplasmic reticulum ready for antibody production (Nutt et al., 2015). Plasma cells upregulate CXCR4 allowing them to home to bone marrow niches, but they can also be found in other bodily sites, both lymphoid and non-lymphoid organs (Hargreaves et al., 2001).

Unlike plasma cell differentiation, there is no known master regulator of memory B cell differentiation and thus their differentiation pathway is less well understood. Memory B cells are long lasting cells that circulate through lymphatic and blood vessels, until they encounter their antigen and differentiate into plasma cells allowing the rapid generation of secondary antibody responses (Kurosaki et al., 2015). Phenotypically memory B cells share many similarities with naïve B cells, as they retain their BCR and MHC II molecule and display similar gene expression patterns. However, memory B cells are of higher affinity and express higher levels of effector molecules, including CD80 and CD86 and survival molecules, such as Bcl-2 (Klein et al., 2003, Bhattacharya et al., 2007, Good et al., 2009). Recently, it was shown that memory B cells are produced prior to plasma cells from the GC reaction (Weisel et al., 2016), consistent with observations that these cells are of lower affinity and acquire less SHM

mediated mutations (Shinnakasu et al., 2016). Furthermore, expression of the transcription factor, ABF-1 appears to promote memory B cell generation and its expression is IL-21 and CD40-L responsive suggesting T_{FH} to also be important for memory B cell generation outside of simply driving GC formation (Chiu et al., 2014). Fitting with this, IL-9 production by T_{FH} has also recently been shown to be critical for memory B cell generation (Wang et al., 2017, Takatsuka et al., 2018).

1.2.8. Extrafollicular responses

Although the GC response is critical for the generation of high affinity memory B cells and antibody secreting long lived plasma, foreign antigens can also stimulate extrafollicular responses. These responses occur outside of B cell follicles and are initiated prior to GC formation to provide a rapid first wave source of antibody from short lived plasmablasts (MacLennan et al., 2003). Thus this antibody plays a critical role in limiting the spread of infection by neutralising dividing pathogens and viruses (Luther et al., 1997). In the LN, extrafollicular responses generally occur in the medullary cord area, while in the spleen they are found within the red pulp or the T cell area of the white pulp. As extrafollicular B cells do not go through a GC, the resulting antibody is of much lower affinity, but can be isotype switched as CSR can occur outside of a GC (Toellner et al., 1996, MacLennan et al., 2003). There is also evidence that memory B cell responses can also be generated independently of GCs (Inamine et al., 2005, Toyama et al., 2002).

Extrafollicular responses can be T cell dependent (TD) or T cell independent (TI). B cell activation for TD extrafollicular responses starts off similar to GC responses, occurring at the B cell zone and eliciting T cell help at the T and B cell border (MacLennan et al., 2003). However, instead of entering the follicle, they are thought to re-locate themselves to their extrafollicular locations in a CXCR4 mediated manner and rapidly proliferate and differentiate into plasmablasts (Hargreaves et al., 2001). During *Salmonella enterica* infection it has been

shown that T cell help can be provided by T_{FH}-like cells (CXCR5⁺Bcl-6⁺PD1^{lo}) during an extrafollicular response (Lee et al., 2011).

In contrast TI responses are generally driven by non-protein antigens and TI responses can be subdivided into TI-1, TI-2 and TI-3 antigen driven responses. TI-1 are B-cell mitogens that have an intrinsic ability to drive B cell proliferation and differentiation via toll-like receptor (TLR) mediated signalling (Mosier et al., 1977, Mond et al., 1995). TI-2 antigens themselves do not directly activate B cells but are generally multivalent antigens which cause extensive BCR cross-linking and require Btk dependent signalling (Mond et al., 1995). Lastly TI-3 antigens require help from cells of the myeloid lineage including neutrophils and mast cells. This help is generally cytokine driven for example with B cell activating factor (BAFF) and A proliferating inducing ligand (APRIL) and/or via CD40-L mediated co-stimulation (Puga et al., 2012, Vinuesa and Chang, 2013, Merluzzi et al., 2010).

1.2.9. Tertiary Lymphoid Structures

SLOs develop during normal embryonic development and are critical sites for lymphocyte activation and differentiation, enabling the generation of effective adaptive responses. However, in response to an inflammatory stimuli highly organised lymphoid like structures, termed tertiary lymphoid structure (TLS) can also develop within peripheral, non-lymphoid tissues (Neyt et al., 2012). TLS have been found in many bodily sites, including the lungs (Rangel-Moreno et al., 2006, Hogg et al., 2004, Dieu-Nosjean et al., 2008), liver (Shomer et al., 2003) and kidneys (Pei et al., 2014) and are generally associated with chronic infection, inflammation, autoimmunity or cancer (Dieu-Nosjean et al., 2008, Hogg et al., 2004, Rangel-Moreno et al., 2006, Neyt et al., 2012).

Structurally TLS resemble SLO and contain B and T cells organised in distinct zones. The level of organisation can vary extensively from forming distinct lymphocyte zones to simply containing B cells, and some TLS can also be associated with HEVs (Dieu-Nosjean et al., 2014). Some TLS can develop functional GCs containing GC B cells, T_{FH} and even FDC

networks (Moyron-Quiroz et al., 2004, Fleige et al., 2014, Slight et al., 2013, Takemura et al., 2001, Shi et al., 2001) and local CSR and antibody generation has been shown to occur within TLS (Slavin et al., 1992). TLS have therefore been suggested to be important contributors to protective response to Influenza A (Moyron-Quiroz et al., 2004) and *Mycobacterium tuberculosis* (TB) (Ulrichs et al., 2004) and this contribution has been shown to sufficiently confer protection from both insults even in the absence of SLOs (Day et al., 2010, Lund et al., 2002). However, TLS have also been implicated in the pathology of a number of diseases including rheumatoid arthritis (RA) (Rangel-Moreno et al., 2006) and diabetes mellitus (Lee et al., 2006).

How TLS form however is not completely understood but it is known to critically involve the ectopic expression of the homeostatic cytokines, CXCL13, CCL19 and CCL21 which actively recruit T and B cells to the site of inflammation and organise these cells into their specific areas (Rangel-Moreno et al., 2007, Wengner et al., 2007, Moyron-Quiroz et al., 2004, Winter et al., 2010). However, many other mediators have now been implicated to play a role in the formation of TLS, including IL-1, IL-17A, IL-17F, IL-22 and IFN- α (Neyt et al., 2016, Rangel-Moreno et al., 2011a, Barone et al., 2015). However, whether TLS formation is critically dependent on LT_i cell function similar to SLO is up for debate. On one hand forced pancreatic expression of CXCL13 drives TLS formation in a LT dependent manner (Luther et al., 2000) but, in the absence of LT_i cells, TLS can still form in the lung following Influenza A infection (Rangel-Moreno et al., 2011a) or in the colon after Dextran Sulfate Sodium (DSS) treatment (Lochner et al., 2011). Multiple other cells including DCs, B cells, Th17 cells and T_{FH} have been implicated as important for TLS formation and maintenance (Lochner et al., 2011, Rangel-Moreno et al., 2011a, GeurtsvanKessel et al., 2009, Halle et al., 2009). Therefore, the way that TLS form and their eventual function is most likely dependent on their initiating inflammatory stimulus.

1.3. T follicular helper cells (T_{FH})

T_{FH} are a specialised subset of CD4⁺ T cells that are critical for the generation of GCs and high affinity, isotype antibody via the GC. They are essential for providing T cell help to B cells to allow the generation of both follicular and extrafollicular T dependent antibody production (Vinuesa et al., 2016).

1.3.1. Defining T_{FH}

Although, T cell help to B cells was one of the earliest observations in the field immunology (Mitchison, 1971), defining the specific T cell subset that fulfilled this role was more complicated to understand. However, in the early 2000s, CXCR5⁺ CD4⁺ T cells were found to exist in the B cell follicles of human tonsils and these cells were found to preferentially provide T cell help to B cells (Schaerli et al., 2000, Breitfeld et al., 2000, Kim et al., 2001). This made sense as CXCR5 expression would allow T cells to re-locate themselves in the B cell zone of SLOs alongside CXCR5⁺ B cells (Haynes et al., 2007). These CXCR5⁺ cells were thus termed T_{FH} and were shown to have distinct gene expression profile to other CD4⁺ T cells, and uniquely upregulated *Bcl-6*, *Achaete-scute homologue 2 (Ascl2)* and expressed *ICOS* and *pcdh1* (Chtanova et al., 2004, Kim et al., 2004, Vinuesa et al., 2005). However despite suggestions that T_{FH} were a separate Th lineage (Nurieva et al., 2008), this was not confirmed until 3 landmark studies were published in 2009 showing T_{FH} differentiation to be dependent on Bcl-6 (Yu et al., 2009, Nurieva et al., 2009, Johnston et al., 2009). Thereby, confirming T_{FH} possess a lineage defining master regulator similar to other CD4⁺ T cell. Since this finding, knowledge on T_{FH} has grown exponentially and now T_{FH} are defined both by their location within B cell zones of SLOs but also by expression markers, including CXCR5, PD1, Bcl-6 and ICOS (Vinuesa et al., 2016). T_{FH} have been identified in multiple SLOs, including the spleen and LNs. While, CXCR5⁺ CD4⁺ T cells which share some phenotypical similarities with tissue resident T_{FH} cells have been found in the blood and are termed circulating T_{FH} (cT_{FH}) (Forster et al., 1994, Morita et al., 2011).

1.3.2. T_{FH} differentiation

T_{FH} differentiation is a complex, multiple step process, involving both DCs and B cells and multiple signalling pathways (Vinuesa et al., 2016) (**Figure 1.5**). T_{FH} differentiation generally begins in the T cell zone of SLOs and similar to the differentiation of other Th subsets is reliant on DC mediated priming. Here DCs provide antigen, CD28 and ICOS co-stimulation along with IL-6 signalling to naïve T cells driving Bcl-6 expression and commitment to the T_{FH} lineage (Goenka et al., 2011, Krishnaswamy et al., 2017). After DC mediated priming, the developing T_{FH} upregulates CXCR5 and downregulates CCR7, allowing them to migrate to the T-B cell border (Haynes et al., 2007, Choi et al., 2011). Commitment to the T_{FH} lineage occurs early and Bcl-6 expression can be detected in developing T_{FH} as early as day 2 after the initiation of an inflammatory response (Kitano et al., 2011, Kerfoot et al., 2011). At the T-B cell border, T_{FH} interact with their cognate activated B cells which continues to provide antigen and ICOS co-stimulation to the developing T_{FH} (Deenick et al., 2010, Choi et al., 2011, Nurieva et al., 2008). Firm interactions between T_{FH} and B cells are mediated by SAP and SLAM family member interactions (Deenick et al., 2010, Qi et al., 2008). In the final stage of T_{FH} differentiation, T_{FH} further upregulate CXCR5 expression and move into the follicle along with their cognate B cells and establish a GC. These T_{FH} are called GC T_{FH} and possess the highest expression of key T_{FH} markers, including CXCR5, PD1 and Bcl-6 (Crotty, 2014). Bone fide T_{FH} express CXCR5, PD1, BCL-6, SAP, BTLA-4 and low levels of CCR7 and PSGL1, and secrete IL-4 and IL-21 (Poholek et al., 2010, Luthje et al., 2012, Vinuesa et al., 2016). These T_{FH} are critical for the generation of GC B cells and provide IL-21 and CD40-L signalling to B cells to promote their proliferation and differentiation (Linterman et al., 2010, Zotos et al., 2010, Vinuesa et al., 2016). The differentiation of T_{FH} is a highly regulated process. Similar to other CD4⁺ T cells cytokine signalling is critical for determining the differentiation fate for the naïve T cells. In mice IL-6 has been proven to be the critical driver of T_{FH} via STAT3 activation (Nurieva et al., 2008, Harker et al., 2011), while co-ordination with other cytokines including IL-21 and TGF- β have also been implicated (Nurieva et al., 2008, Eto et al., 2011, Marshall et

al., 2015). However, in humans the combination of IL-12 and Activin A has been shown to be important (Locci et al., 2016, Ma et al., 2009, Schmitt et al., 2009).

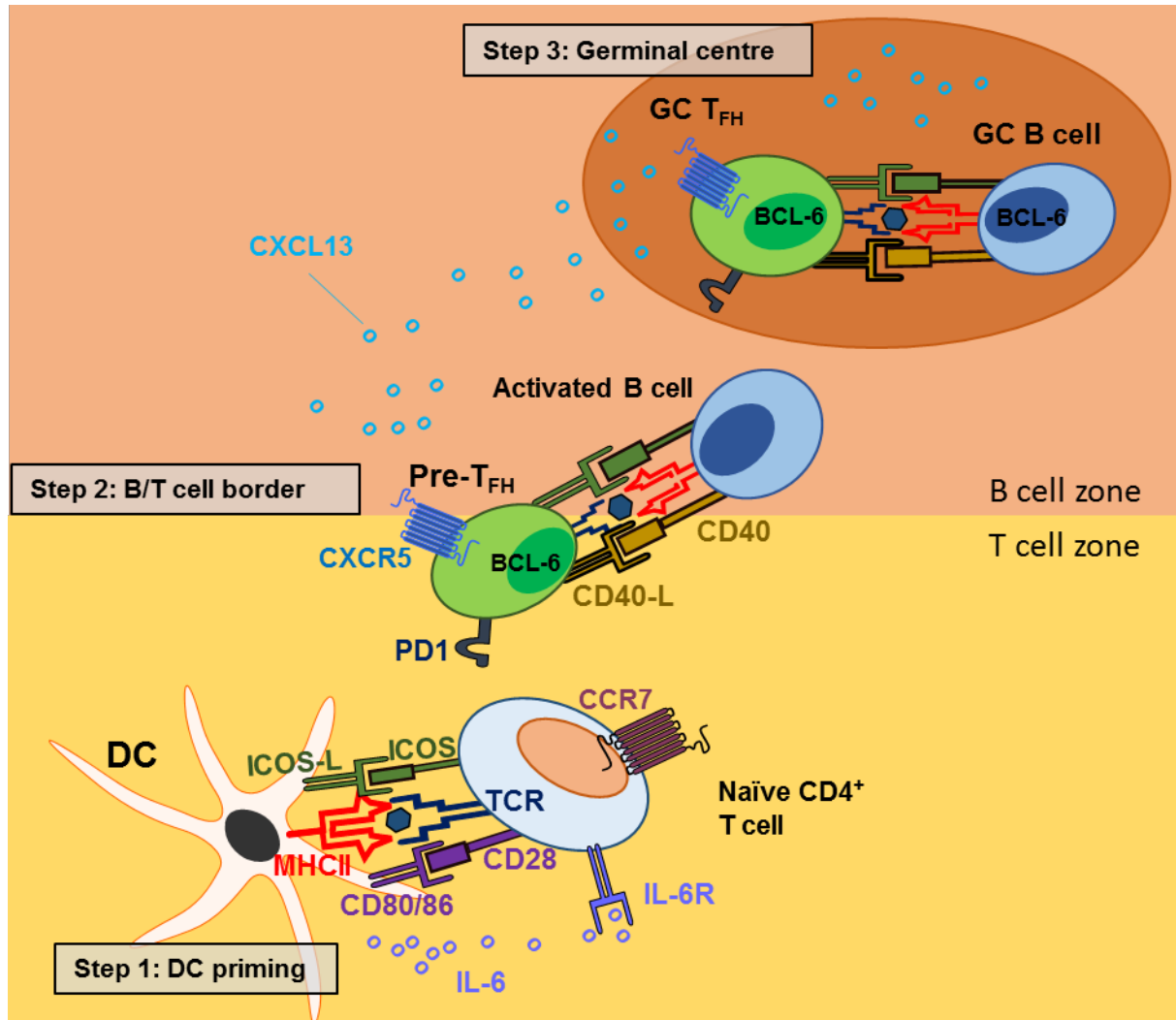


Figure 1.5: T follicular helper cell differentiation

T follicular cell (T_{FH}) differentiation is a multi-step process beginning within the T cell zone. Here naïve CD4⁺ T cells receive antigen stimulation via MHC II molecules, ICOS and CD28 co-stimulation and IL-6 stimulation from dendritic cells (DC). Following this stimulation, developing T_{FH} upregulate Bcl-6, and CXCR5, and downregulate CCR7, thus allowing the T cell to migrate towards the B cell zone via a CXCL13 chemokine gradient. At the T/B cell zone border, pre-T_{FH} expressing Bcl-6 interact with activated B cells, allowing pre-T_{FH} to further upregulate CXCR5 and move into the B cell zone and drive germinal centre (GC) formation. Fully differentiated T_{FH} within GCs are termed GC T_{FH} and express higher levels of several molecules, including CXCR5, PD1, Bcl-6, ICOS, CD40L, SAP, BTLA4 and many more. They provide T cell help to GC B cells via CD40-L mediated co-stimulation and IL-4 and IL-21 production, thereby driving B cell proliferation and their eventual differentiation into memory B cells and plasma cells.

T_{FH} differentiation is potently inhibited by IL-2 signalling which has been shown to limit T_{FH} by activating STAT5 and driving the expression of Blimp1 (Johnston et al., 2012, Ballesteros-Tato et al., 2012). IL-2 limits T_{FH} differentiation as Blimp1 expression actively antagonises Bcl-6 (Johnston et al., 2009) and activated STAT5 can outcompete STAT3 for binding Bcl-6 and thus directly limit Bcl-6 expression (Walker et al., 2013). Thus, limiting IL-2 from T_{FH} is a critical component of the differentiation process and it is known that the DCs that prime T_{FH} actively quench IL-2 by producing soluble CD25 (Li et al., 2016). Interestingly, IL-7 has also been shown to inhibit T_{FH} differentiation by preventing both Bcl-6 and CXCR5 expression via activation of STAT5 (McDonald et al., 2016).

ICOS signalling has also been shown to be critical for T_{FH} differentiation, as it can drive CXCR5 and Bcl-6 expression (Choi et al., 2011). Furthermore, ICOS signalling via PI3K and the exclusion of FOXO-1 from the nucleus can subsequently limit the expression of genes that negatively regulate T_{FH} localisation. For example S1PR which is required by lymphocytes to exit lymphoid tissues is downregulated by ICOS signalling, thus enabling T_{FH} retention within SLOs (Stone et al., 2015, Weber et al., 2015, Lee et al., 2015). However, unlike other CD4⁺ T cells ICOS is also required for the maintenance of T_{FH} responses and thus T_{FH} populations can be depleted by blocking ICOS mediated signalling long after initiate DC priming (Weber et al., 2015). As a result, ICOS is critical for T_{FH} responses and thus ICOS deficient animals fail to generate T_{FH} (Choi et al., 2011, Akiba et al., 2005, Bossaller et al., 2006) and common variable immunodeficiency (CVID) patients with genetic defects in ICOS lack T_{FH} and have defective antibody responses (Salzer et al., 2004, Grimbacher et al., 2003). In contrast, Roquin 1 (*sanroquin*) mutants that overexpress ICOS accumulate T_{FH} populations (Vinuesa et al., 2005, Pratama et al., 2013, Vogel et al., 2013).

Bcl-6 functions as the master regulator of T_{FH} differentiation (Johnston et al., 2009, Nurieva et al., 2009, Yu et al., 2009) and consistent with this Bcl-6 deficient T cells fail to generate T_{FH} (Hollister et al., 2013). Bcl-6 functions as a transcriptional repressor and is thought to promote T_{FH} differentiation by actively suppressing the ability of the developing T_{FH} to choose an alternative route (Nurieva et al., 2009, Johnston et al., 2009, Yu et al., 2009). Therefore, Bcl-

6 targets genes associated with other Th lineages, such as *Tbx21*, *Gata3* and *Il-17a* (Hatzi et al., 2015). Bcl-6 actively represses *Prdm1* which encodes Blimp1, which is critical as *Prdm1* overexpression prevents T_{FH} differentiation (Johnston et al., 2009). Furthermore, Bcl-6 restrains T_{FH} within the SLOs and ensures normal T_{FH} mobility within SLOs by directly targeting migration associated genes such as *S1pr1* and by directly targeting a transcription factor called *Klf2*, which is a negative regulator of T_{FH} differentiation (Weber et al., 2015, Lee et al., 2015, Vinuesa et al., 2016).

The many transcription factors other than Bcl-6 and the signalling molecules involved in T_{FH} differentiation have been extensively reviewed in (Vinuesa et al., 2016). Briefly, *Ascl2* is critical for the initiation of the T_{FH} differentiation program (Liu et al., 2014), while both LEF-1 and TCF-1 have been shown to act upstream of Bcl-6 expression (Choi et al., 2015b, Xu et al., 2015). c-maf has also been shown to be important for T_{FH} production of IL-4 and IL-21 (Bauquet et al., 2009, Sahoo et al., 2015).

1.3.3. T_{FH} function

The fundamental role of the T_{FH} is to provide help to B cells and this encompasses many different tasks, including signals to promote B cell survival, proliferation, differentiation, CSR and SHM. T_{FH} mediate help via CD40-L co-stimulation and cytokine secretion, including IL-4 and IL-21 (Vinuesa et al., 2016).

To start with T_{FH} are absolutely critical for the generation of GC, providing IL-21 signals that drive Bcl-6 expression in B cells (Zotos et al., 2010, Linterman et al., 2010). Furthermore, T_{FH} provide IL-21 and CD40-L signals to drive B cell proliferation (Han et al., 1995, Ozaki et al., 2004) and these same signals can also drive B cells to differentiate into plasma cells (Bryant et al., 2007, Kuchen et al., 2007, Han et al., 1995, Ozaki et al., 2004). T cell help via IL-21 and CD40-L is also critical for memory B cell differentiation (Crotty, 2011) and recently IL-9 production by T_{FH} has also been implicated in memory B cell development (Wang et al., 2017, Takatsuka et al., 2018). T_{FH} also provide B cell help via the production of IL-4, which has been

shown to be provided by GC T_{FH} and required for optimal B cell help (Reinhardt et al., 2009, King and Mohrs, 2009, Zaretsky et al., 2009). IL-4 has been shown to mediate B cell survival via upregulation of the anti-apoptotic factor Bcl-XL (Wurster et al., 2002, Yusuf et al., 2010).. T_{FH} can also promote CSR via CD40-L mediated help and direct the switching outcome via antibody production, for example T_{FH} derived IFN- γ can direct switch to IgG2a (Reinhardt et al., 2009). IL-4 and IL-21, both produced by T_{FH} appear to co-ordinate to mediate successful CSR and thus IL-4/IL-21R double knockout mice display severe hypogammaglobulinemia to switched isotypes and reduced IgM (Ozaki et al., 2002). T_{FH} also are critical for driving SHM and the selection process that follows in the DZ (Mesin et al., 2016). Lastly, human T_{FH} have also been shown to produce CXCL13 thus implying that they actively recruit B cells towards themselves to facilitate T_{FH} mediated help (Kim et al., 2004),.

1.3.4. Circulating T_{FH} (cT_{FH})

T_{FH} -like cells have also been found in the peripheral blood of humans, as CXCR5⁺ CD4⁺ T cells and have been termed cT_{FH} (Forster et al., 1994, Morita et al., 2011). Although these cells express CXCR5 their expression of other marker such as PD1, ICOS and CCR7 can vary extensively, for example CCR7^{lo}PD1^{hi}, CCR7^{hi}PD1^{lo} and ICOS⁺PD1⁺ to name a few (Schmitt et al., 2014). These cells differ from bone fide T_{FH} within SLOs and do not express Bcl-6 (He et al., 2013b, Locci et al., 2016, Morita et al., 2011, Bentebibel et al., 2013), however they are still dependent on Bcl-6 for their development (He et al., 2013b). cT_{FH} appear to have a memory-like phenotype and still possess the capacity to provide B cell help (Morita et al., 2011, He et al., 2013b). However, it is not known how cT_{FH} relate to bona fide tissue resident T_{FH} populations.

Although cT_{FH} are a heterogeneous population in humans they can be subdivided in 3 distinct subsets based on expression of the chemokine receptors, CXCR3 and CCR6. T_{FH1} are CXCR3⁺CCR6⁻, T_{FH2} , CXCR3⁻CCR6⁻ and T_{FH17} , CXCR3⁻CCR6⁺ (Morita et al., 2011). These subsets mimic Th1, Th2 and Th17 cells in their gene expression and cytokine production with

cT_{FH1} expressing *Tbet* and secreting IFN- γ , cT_{FH2} expressing *Gata3* and secreting IL-5 and IL-13, while cT_{FH17} cells express *Rorc* and secrete IL-17A. Consistent with their varied cytokine repertoire they drive B cell CSR to specific Ig isotypes (Morita et al., 2011). The cT_{FH} population induced appears to vary depending on the inflammatory stimuli. Following Influenza A vaccination, T_{FH1} were found to rapidly increase in Influenza A vaccinated humans and this increase positively correlated with viral specific antibody generation (Bentebibel et al., 2013). cT_{FH17} cells meanwhile have been shown to increase in the blood of patients with Sjögren's syndrome and their frequencies correlate with disease severity and auto-antibody production (Li et al., 2012).

1.3.5. T follicular regulatory cells (T_{FR})

T_{FR} were discovered in 2011 as a subset of CD4⁺ T cells that expressed Foxp3 in combination with key T_{FH} associated markers such as CXCR5, PD1, Bcl-6 and ICOS. They were found to resemble T regulatory (Treg) cells and expressed high levels of GITR, CTLA-4 and IL-10 (Linterman et al., 2011, Chung et al., 2011, Wollenberg et al., 2011). T_{FR} actually differentiate from thymic derived Treg cells (Linterman et al., 2011, Wollenberg et al., 2011) but have also been shown to differentiate from naïve CD4⁺ T cells (Aloulou et al., 2016). Similar to T_{FH}, T_{FR} were found to be dependent on Bcl-6 expression and B cell help for their differentiation (Linterman et al., 2011, Chung et al., 2011), however, they also express Blimp1 (Sage et al., 2013, Linterman et al., 2011). T_{FR} are generally found at lower frequency than T_{FH} (Linterman et al., 2011) and are located in lymphoid organs, mostly at the T and B cell border but can also be found with GCs (Sayin et al., 2018, Linterman et al., 2011). Circulating T_{FR} can also be found in the blood of mice and humans (Sage et al., 2013, Fonseca et al., 2017).

T_{FR} do not produce IL-4 or IL-21 or express CD40-L, and instead function as negative regulators of the GC response. As a result, T_{FR} deficient animals have increased GC responses, characterised by increased T_{FH} and GC responses and elevated antibody production (Linterman et al., 2011, Chung et al., 2011, Wollenberg et al., 2011). T_{FR} can limit

T_{FH} and B cells responses via CTLA-4 ligand mediated co-inhibition (Sage et al., 2014b). T_{FR} mediated suppression can alter gene expression in T_{FH}, limiting their expression of *Il4* and *Il21* and act on B cells to reduce expression of *Xbp1*, *Aicda* and switched *Ig* genes (Sage et al., 2016). Furthermore they can alter the B cell metabolic program to limit GC responses and antibody production (Sage et al., 2016). Given the importance of GC responses, limiting T_{FR} responses are also important and this has been shown occur via PD1 (Sage et al., 2013) and CTLA-4 mediated inhibition (Sage et al., 2014b).

1.3.6. T_{FH} and disease

Dysregulated T_{FH} responses have been implicated in a number of diseases, in particular those associated with dysregulated humoral responses. T_{FH} have been linked to a number of immunodeficiency disorders, for example X-linked lymphoproliferative (XLP) disease. XLP patients have a genetic defect in *Shdh1*, the gene which encodes SAP and therefore fail to sustain the long lasting synapses between B cells and T_{FH} during T_{FH} differentiation (Coffey et al., 1998, Sayos et al., 1998, Nichols et al., 1998, Qi et al., 2008). As a result these patients fail to generate T_{FH} and experience hypogammaglobulinemia and increased susceptibility to infection (Ma et al., 2005, Panchal et al., 2018, Yin et al., 2003, Qi et al., 2008). Additionally, tissue resident T_{FH} and/or cT_{FH} populations are reduced in a number of other primary immunodeficiency disorders caused by mutations in STAT3 (Ma et al., 2012), ICOS (Bossaller et al., 2006, Mazerolles et al., 2013) and CD40-L (Bossaller et al., 2006) and again these are associated with impaired humoral immune responses (Mazerolles et al., 2013, Ma et al., 2012, Bossaller et al., 2006, Crotty, 2011).

T_{FH} have also been heavily implicated in autoimmune disease. *Roquin* (*sanroquin*) mutant animals accumulate T_{FH} and develop lupus-like symptoms, and both the inflammation and auto-antibody production can be attenuated by limiting T_{FH} function, suggesting T_{FH} drive the pathology (Linterman et al., 2009, Vinuesa et al., 2005). Increased frequencies of cT_{FH} are also observed in the blood of patients with a number of autoimmune disorders, including SLE,

Sjörgen syndrome and RA (Simpson et al., 2010, Choi et al., 2015a, Szabo et al., 2013, Li et al., 2012) and cT_{FH} frequencies also correlate with auto-antibody production and disease severity in a number of autoimmune diseases (Szabo et al., 2013, Choi et al., 2015a). Moreover, some studies show a skewing towards T_{FH2} and/or T_{FH17} in some autoimmune patients which again correlates with disease severity (Li et al., 2012, Le Coz et al., 2013). It has thus been suggested that overwhelming T_{FH} populations or defective T_{FH} populations could result in a breakdown of B cell selection within the GC, allowing auto-reactive B cells to escape and contribute to disease (Mesquita et al., 2016).

T_{FH} responses have also been shown to accumulate and become dysregulated during chronic infection. In mice chronic LCMV infection leads to the late accumulation of T_{FH} populations, driven by late IL-6 production and this response was critical for viral clearance (Harker et al., 2011). This T_{FH} response was much greater than observed with acute LCMV infection (Fahey et al., 2011). Similarly OVA immunised mice show higher splenic T_{FH} populations following chronic treatment compared to acute (Baumjohann et al., 2013). These chronic T_{FH} populations were found to be dysregulated in their differentiation, no longer requiring B cell help (Fahey et al., 2011), consistent with other studies showing excessive or high dose antigen can overcome the need for B cell or DC help in T_{FH} differentiation (Deenick et al., 2010, Dahlgren et al., 2015). Furthermore, T_{FH} were found to express *Tbx21* and *Gata3* in addition to *Bcl-6* suggesting that during chronic disease, T_{FH} can share properties associated with other Th cell lineages (Fahey et al., 2011).

Furthermore, T_{FH} populations are also increased in the LNs of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infected patients and macaques respectively (Perreau et al., 2013, Petrovas et al., 2012, Lindqvist et al., 2012). Consistent with this, T_{FH} populations decrease following combined anti-retroviral therapy suggests the viral load drives the T_{FH} increase (Perreau et al., 2013). Interestingly, increased T_{FH} populations and the subsequent GC response are not linked to production of effective broadly neutralising antibodies (bNAbs) and instead were associated with hypergammaglobulinemia (Lindqvist et al., 2012). T_{FH} have been shown to be a major reservoir for HIV infection (Perreau et al., 2013)

and T_{FH} from infected individuals have been shown to be defective and less capable of providing help to B than those from healthy individuals (Cubas et al., 2013). Conversely increased frequencies of functioning cT_{FH} have been shown to correlate with production of bNAbs (Locci et al., 2013), thereby further suggesting T_{FH} dysfunction to be pathogenic.

1.3.7. T_{FH} and allergic disease

Until fairly recently not much was known about the role of T_{FH} during type 2 pathology let alone during allergic disease. The original studies examining type 2 disease studied T_{FH} populations induced after helminth infections. These early studies clearly showed T_{FH} to be induced in the mesenteric LNs of helminth infected animals and actually found T_{FH} to be the major IL-4⁺ cell within the draining LNs of these mice (Liang et al., 2011, Zaretsky et al., 2009, King and Mohrs, 2009). Consistent with this it was found that following infection with the helminth *Nippostrongylus brasiliensis* (*N.bras*) there appeared to be a division of labour with Th2 cells being the major IL-4 producer in the lung tissue (Liang et al., 2011). Therefore, given the close proximity of IL-4 producing T_{FH} to B cells within the LN it was easy to suggest that T_{FH} would be the major driver of IgE switch during type 2 disease (Liang et al., 2011, Zaretsky et al., 2009, King and Mohrs, 2009).

However, T_{FH} produce both IL-4 and IL-21, which brought about a great deal of questions especially in the context of type 2 disease. While, IL-4 is the critical switch factor for IgE generation (Snapper and Paul, 1987), IL-21 has been shown to inhibit IgE switching and as a result IL-21R KO animals actually have increased total and antigen specific serum IgE (Ozaki et al., 2002). Furthermore, the *in vitro* culture of human splenic B cells with IL-21 showed the cytokine to drive B cells to produce IgG1 and IgG3, suggesting IL-21 to promote switching away from IgE (Pène et al., 2004). One potential explanation for the dichotomy in T_{FH} effector functions is that T_{FH} have the ability to produce IL-21 with or without IL-4, as was shown using an IL-21 reporter mouse (Luthje et al., 2012). Thus aligning with more recent work from Weinstein and colleagues showing the existence of 3 distinct T_{FH} subsets defined as IL-4⁺IL-

21⁻, IL-4⁺IL-21⁺ and IL-4⁻IL-21⁺ (Weinstein et al., 2016). Taken together this implied that during type 2 pathology IgE generation could result from an increase in the ratio of IL-4 producing T_{FH} to IL-21 producing cells.

Dysregulation of T_{FH} responses has also been suggested to occur during type 2 pathology. Following *Schistosoma masoni* infection it was demonstrated that T_{FH} induced in the mesenteric LN expressed both *Bcl-6* and *Gata3* messenger ribonucleic acid (mRNA) and it was suggested that Th2 cells could differentiate into T_{FH}, linking the two cell types (Zaretsky et al., 2009). However, this was not observed in all helminth studies and following *N. bras* infection Liang and colleagues showed Bcl-6 and GATA3 positive cells to be mutually exclusive (Liang et al., 2011). Additionally, they showed T_{FH} and Th2 cells to be functionally distinct with T_{FH} being incapable of producing the type 2 cytokines IL-5 and IL-13 (Liang et al., 2011). Thus, these studies suggested a complex relationship between T_{FH} and Th2 cells during type 2 pathology.

Recently, a hand full of studies have now started to use animal models to examine T_{FH} in the context of AAD. These studies have shown T_{FH} to be induced in the lung draining mLN after allergen sensitisation and challenge (Ballesteros-Tato et al., 2016, Coquet et al., 2015, Kobayashi et al., 2017, Noble and Zhao, 2016) and in the lungs following chronic allergen exposure (Vroman et al., 2017). Both IL-4 and IL-21⁺ T_{FH} populations have been reported in the mLN during disease (Coquet et al., 2015, Ballesteros-Tato et al., 2016) and T_{FH} have also been shown to be critical for IgE generation during AAD (Noble and Zhao, 2016, Kobayashi et al., 2017).

Although, T_{FH} are clearly important to the humoral response during AAD their contribution to other components of the disorder appears to be more controversial. Coquet and colleagues show IL-21 production during AAD to be critical for the generation of AAD phenotype. They show IL-21⁺ T_{FH} to exist in the mLN of HDM exposed animals and the adoptive transfer of these cells into non-sensitised hosts to drive airway eosinophilia (Coquet et al., 2015). Consistent with this Ballesteros and colleagues also show the adoptive transfer of IL-4⁺ or IL-21⁺ T_{FH} into previously sensitised hosts to drive type 2 biased inflammation upon HDM

challenge. However, controversially they showed this to be due to T_{FH} being precursor cells to Th2 cells and thus T_{FH} transfer resulted in lung Th2 cells that went on to drive disease (Ballesteros-Tato et al., 2016). This is different to the work by Coquet where they show $IL-21^+$ T_{FH} to not differentiate into Th2 cells (Coquet et al., 2015) or the work of Hondowicz and colleagues that show T_{FH} loss to be associated with an increase in tissue resident memory (TRM) Th2 cells upon allergen rechallenge (Hondowicz et al., 2016). Thus, although both the Coquet and Ballesteros studies implicate T_{FH} in driving disease their relationship to Th2 is unclear. Lastly, another group also examined T_{FH} and Th2 cells during type 2 disease and found the two cells to represent separate cellular lineages with distinct gene expression. Furthermore, they found only the adoptive transfer of Th2 cells but not T_{FH} to drive BAL IL-5 and IL-13 secretion and eosinophilia in an OVA and IL-33 (i.n) sensitisation and OVA challenge model. Consistent with this they observed following OVA and ALT sensitisation and OVA challenge that T_{FH} deficient animals had comparable levels of type 2 cytokines and eosinophilia, further implicating T_{FH} to solely act as regulators of allergen humoral responses (Kobayashi et al., 2017). To date most of the animal studies examining T_{FH} in the context of AAD have either been sensitisation and challenge models or utilised clinically irrelevant allergens such as OVA, thus more work needs to be done using more clinically relevant chronic models of AAD or human studies.

In the context of human allergic disease very little work on T_{FH} biology has been done, most likely due to the relatively recent identification of T_{FH} combined with their localisation within SLOs which makes them a difficult to examine in asthmatic patients. However, in patients with chronic rhinosinusitis with nasal polyps, $IL-4^+$, $IL-21^+$ and $IL-4^+IL-21^+$ T_{FH} were found in nasal polyps with eosinophilic inflammation and the proportions of all 3 correlated with local IgE production (Zhang et al., 2016). Consistent with this cT_{FH} populations have now been examined in the blood of allergic asthma patients, where they are shown to be elevated compared to healthy controls and their proportions again correlate with serum IgE levels (Gong et al., 2018) thereby, implying T_{FH} to play a role in IgE production in humans. With the exception of this very recent study in allergic asthma patients most work examining cT_{FH} has

been done in patients with allergic rhinitis. In this context, cT_{FH} patients have been shown to either be increased in allergic patients compared to controls (Schulten et al., 2017), decrease (Kim et al., 2016) or remain at the same frequency but become skewed towards a cT_{FH2} phenotype (Kamekura et al., 2015, Yao et al., 2018). Given that cT_{FH2} are associated with GATA3 expression and production of type 2 cytokines, including IL-4, -5 and -13, this implies T_{FH} could be contributing to other hallmark features of allergic disease in humans (Morita et al., 2011). One study examining cT_{FH} in allergic rhinitis patients showed that these patients also possessed IL-4⁺ cT_{FH} populations that were specific for one of the common HDM allergen components, Derp1 and showed that these allergen specific cells correlated with Derp 1 specific IgE levels, further implicating T_{FH} in promoting disease (Yao et al., 2018).

Lastly, a few studies have now started to examine the impact of allergen immunotherapy (AIT) on cT_{FH} populations in the blood of patients of allergic asthma. AIT involves the administration of low doses of an allergen to an allergic patient either subcutaneously or sublingually to induce tolerance (Akdis and Akdis, 2015). One of the hallmark features of successful AIT is a decrease in serum IgE and an increase in IgG4 and IgA2 (Scadding et al., 2010). Following AIT, one study showed total cT_{FH} populations to decrease (Schulten et al., 2017), while another demonstrated a fall in allergen specific cT_{FH} proportions to correlate with disease improvement scores (Yao et al., 2018). Taken together this implies AIT to alter T_{FH} populations and suggests T_{FH} to play a role in driving the dysregulated humoral response seen in allergic disease

1.4. Hypothesis

The overall hypothesis of the thesis is that dysregulated T_{FH} are induced during chronic AAD which contribute to pathology. It was hypothesised that T_{FH} would be critical for the abnormal IgE production observed during chronic AAD. Furthermore, it was hypothesised that T_{FH} induced in the heavy type 2 environment of chronic AAD would be dysregulated to take on features associated with Th2 cells and directly drive disease.

1.4.1. Aims

- Evaluate the timing and magnitude of the T_{FH} response during chronic AAD
- Phenotype T_{FH} populations induced during chronic AAD
- Determine the contribution of T_{FH} to chronic AAD

Chapter 2: Materials and methods

2.1 Mice

Both male and female BALB/c and C57BL/6J mice were purchased from Charles River Laboratories (UK). *Il-13gfp/gfp*, *il-17a^{cre/cre}-rosa26-rfp^{fl/fl}*, *cd4^{cre}bcl-6^{fl/fl}* and CD45.1 wild type (WT) mice were on a C57BL/6 background. *Il-13gfp/gfp* animals were on a BALB/c background. *bcl-6^{fl/fl}* and *cd4cre* mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) (Hollister et al., 2013). *Il-13gfp/gfp* mice were gifted from the MacKenzie lab (Cambridge University, UK) (Neill et al., 2010). *il-17a^{cre/cre}-rosa26-rfp^{fl/fl}* mice were gifted from Michelle Linterman (The Babraham Institute, UK) (Hirota et al., 2011). CD45.1 mice were gifted by the Lo Celso lab (Imperial College London, UK). All experiments were performed in accordance with the UK Home Office guidelines for animal welfare based on the Animal (Scientific Procedures) Act 1986. Mice were housed at Imperial College London Animal Facility and all work was performed under the animal licenses of Professor Clare M Lloyd (PPL70/7463 and P996A24E1) and Dr Fiona Culley (PPL70/7554).

2.2 Induction of allergen induced allergic airway disease

2.2.1. Preparation of house dust mite (HDM)

Whole HDM extract was prepared for *in vivo* use, as originally reported by (Gregory et al., 2009a). Briefly, Lyophilised HDM extract (*Dermatophagoides pteronyssinus*) was reconstituted by the addition of sterile phosphate buffered saline (PBS) (Gibco, UK) to a total protein concentration of 1mg/ml. 3 different HDM batches were used during this PhD. The details are listed below:

- 1) Batch Number: 15J02
- 2) Batch Number: 228282
- 3) Batch Number: 290903

HDM Batch 1 was purchased from Citeq Biologics, Groningen, The Netherlands, while batch 2 and 3 were from Greer Laboratories, Lenoir, USA.

2.2.2. Preparation of alternaria alternata (ALT)

Lyophilized ALT extract was reconstituted by the addition of sterile PBS to a total protein concentration of 0.4mg/ml. ALT was purchased from Greer Laboratories and batch #227751 was used throughout this PhD study.

2.2.3. Induction of allergic airway disease

Mice were intranasal (i.n) administered 25 µg HDM, 10 µg ALT in 25 µl PBS or a control of 25 µl PBS. BALB/c mice were exposed three times a week for up to 5 weeks. Mice on a C57BL/6 background were given 5 doses per week of HDM, or exposed to ALT 3 times a week. Repeated intranasal administrations were performed under isoflurane anaesthesia (Zoetis Limited, UK). Mice were culled by aesthetic overdose using intraperitoneal injection of Pentobarbital (Sigma-Aldrich, Dorset, UK), unless they were used to assess lung function as described in 2.4. Mice were culled 18 hours after the final allergen dose, unless otherwise stated.

2.2.4. Resolution of allergic disease and re-challenge

Following HDM exposure, mice were rested without allergen exposure for between 4 and 5 weeks to allow full disease resolution. To re-challenge animals, BALB/c mice were re-exposed (i.n) to 2 alternate day doses of either 25 µg HDM or 25 µl PBS. Mice were culled 18 hours after the final dose.

2.3. Manipulation of the HDM driven allergic disease model

2.3.1. Therapeutic anti-ICOS-L antibody administration during established AAD

Mice were exposed to HDM for 5 weeks. From week 4 of allergen exposure onwards mice were co-administered 150 µg ICOS-L targeted antibody (α -ICOS-L) (Clone: HK5.3, BioXCell, NH, USA) or an isotype control (Clone: 2A3, BioXCell, NH, USA) antibody in 200 µl PBS via

intraperitoneal (i.p.) injection 3 times a week for 2 weeks. Mice were culled at the end of week 5. Mice were culled 18 hours after the final allergen dose.

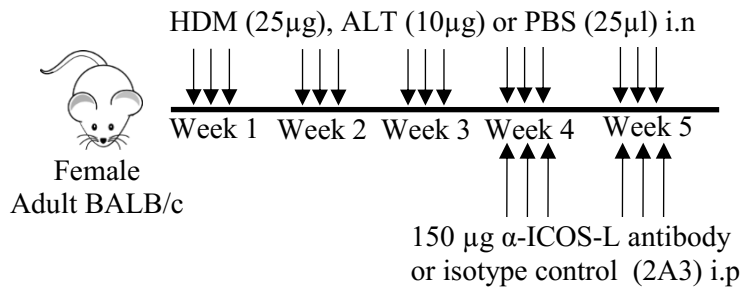


Figure 2.1: Therapeutic ICOS-L antibody administration protocol

2.3.2. Lymphocyte egress block using FTY720 administration

Mice were exposed to HDM for 3 weeks and after a 5 week rest period, animals were intraperitoneally injected with 5 doses of 25 µg FTY720 (Enzo Life Sciences, NY, USA) or a vehicle (VEH) control of 5% DMSO in 250 µl sterile deionised water. After the first 2 doses of FTY720 or VEH, mice were then rechallenged (i.n) with 2 alternate day doses of 50 µg HDM or 50 µl PBS. HDM re-challenge was performed 30 mins after the FTY720 or VEH administration. Mice were culled 18 hours after the final allergen dose.

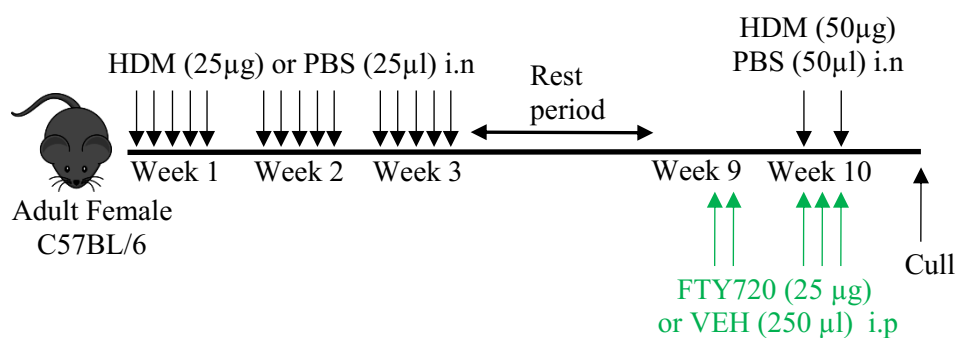


Figure 2.2: FTY720 mediated block of lymphoid organ egress dosing protocol

2.3.2. Mixed bone marrow chimera

Bone marrow cells were isolated from CD45.1 and *cd4^{cre}bcl6^{fl/fl}* CD45.2 or *bcl6^{fl/fl}* CD45.2 mice. Recipient C57BL/6 mice were irradiated using 2 doses of 550 rads gamma radiation to deplete the bone marrow. Bone marrow cells were a 1:1 mix of CD45.1 and *bcl6^{fl/fl}* CD45.2 cells or CD45.1 and *cd4^{cre}bcl6^{fl/fl}* CD45.2 cells. 5×10^6 freshly isolated bone marrow cells were then transferred into recipient mice by intravenous injection via the tail vein. Mice were then given 9 weeks to reconstitute their bone marrow. To prevent infection mice were given autoclaved water supplemented with diluted Baytril 2.5 % oral solution (Bayer, Berkshire, UK). Mice were then administered 25 μ g HDM extract or a control of PBS (i.n) 5 times a week for 3 weeks and culled 18 hours after the final dose.

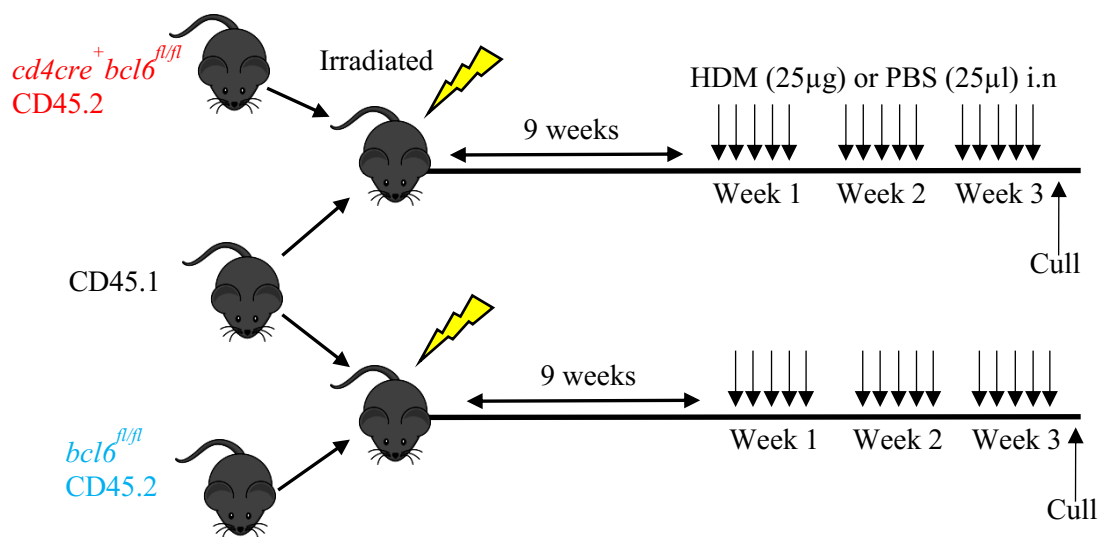


Figure 2.3: Mixed bone marrow chimera dosing protocol

2.4. Measurement of lung function

Airway resistance, elastance and compliance was measured using the FlexiVent™ small-animal ventilator (SCIREQ, Montreal, Canada) as previously reported in (Sagani et al., 2009). Briefly, mice were anesthetized with 50 mg/kg pentobarbital (i.p) (Sigma-Aldrich) and intramuscular (i.m) 200 mg/kg ketamine (Fortdodge Animal Health Ltd, Southampton, UK). After checking for a pedal reflex, mice were tracheotomised, cannulated and connected to the FlexiVent ventilator via a 19-gauge (G) blunt-ended needle. Mice were ventilated with an

average breathing frequency of 150 breaths/min, a tidal volume of 10 mL/kg body weight and a positive end-expiratory pressure of approximately 2 cm H₂O. To determine airway hyper-reactivity mice were exposed to increasing concentrations of nebulized methacholine (MCh) (Sigma-Aldrich). BALB/c mice were exposed to between 3-100 mg/ml MCh, while C57BL/6 mice were exposed to between 10-300 mg/ml MCh. Changes in airway resistance, elastance and compliance were determined from a user-defined protocol using the snapshot-150 perturbation, which is a single-frequency sinusoidal wave at a frequency equivalent to the ventilation rate (2.5 Hz). The resulting data were fitted by using multiple linear regressions to the single-compartment model in the following form:

$$\text{Pressure} = (\text{Resistance} \times \text{Flow}) + (\text{Elastance} \times \text{Volume}) + \text{Fitting Constant}$$

Airway resistance was used to assess the levels of bronchoconstriction and is associated with the narrowing of the airways. Compliance was used to assess the ease at which the lungs can be extended and is associated with stiffness and rigidity of the lungs. Elastance is used to measure the elastic rigidity of the lungs and is the inverse of Compliance (Elastance = 1/Compliance). Airway resistance was expressed as cmH₂O.s/ml, elastance as cmH₂O/ml and compliance as mL/cmH₂O. The lung function procedure was a non-recovery surgical procedure, and upon removal from the machine, mice were bled under terminal anaesthesia via a peripheral artery.

2.5. Cell Recovery

2.5.1. Bronchoalveolar lavage (BAL)

Mice were tracheotomised, cannulated and the BAL fluid was acquired by washing the airways 3 times with 0.4ml PBS via the cannula. To pellet cells the BAL fluid was centrifuged at 4000 x g for 5 mins for 2 mins. Supernatant was collected and stored at -80 °C, while cells were re-suspended in 500µl complete R10 media (RPMI 1640 media supplemented with 10 % fetal bovine serum (FBS) 100 U/ml Penicillin-Streptomycin (Thermo Fisher Scientific, UK) and 2 mM L-glutamine (Gibco).

2.5.2. Lung Parenchyma

The left lung lobe was excised, chopped and incubated with approximately 5 ml complete R10 media, 0.15mg/ml Collagenase D (Roche Diagnostics, West Sussex, UK) and 25 µg/ml DNase I (Roche Diagnostics) for 45 mins at 37° C. Digested lung tissue was then mashed and rinsed through a 100 µm mesh sized cell strainer (BD Pharmingen, UK). Recovered cells were centrifuged at 495 x *g* for 5 mins and subjected to ACK lysis (150 mM ammonium chloride, 10mM potassium bicarbonate, 0.1mM Ethylenediaminetetraacetic acid (EDTA) for 5 mins. The reaction was quenched by the addition of the equivalent volume of R10. Finally, the remaining cells were resuspended in 1 ml R10 media.

2.5.3. Spleen, inguinal lymph node (ILN) and mediastinal lymph node (mLN)

Spleen, ILN or mLN were mashed and rinsed through a 100 µm mesh sized cell strainer and centrifuged as described above. Splenic cells were subjected to ACK lysis. All recovered cells were resuspended in R10 media. 1 ml for mLN and ILN cells, while splenic cells were resuspended in 4 ml. When half a spleen was used, cells were resuspended in 2 ml and cell counts were normalised according to tissue weight.

2.5.4. Bone marrow cell isolation

Legs were removed from mice by cutting animals from the hips downwards. Harvested legs were placed into R10 until required. Muscle tissue was removed from the legs and the resulting bone was sterilised in 70 % ethanol for 5 mins and washed twice in PBS. Bones were then cut into smaller pieces and bone marrow was washed out with PBS into a petri dish using a 27 G needle and 1 ml syringe. Bone marrow cells were then resuspended into a single cell suspension, centrifuged at 495 x *g* for 5 mins and resuspended to a final volume of 1 ml R10 media.

2.5.5. Nasal lavage (NAL)

NAL was acquired by cannulating mice via the nasopharynx and washing the nasal cavity with 1 ml PBS. Cells were removed by centrifugation at 4000 x g for 5 mins and the supernatant was stored at -80 °C.

2.5.6. Blood

Mice were bled by cardiac puncture or severing the jugular artery and the blood collected by capillary action using 280 µl sodium heparinized coated capillaries (Hirschmann Laborgeräte, Eberstadt, Germany). Serum was acquired by centrifugation at 12,000 x g for 15 mins at 4 °C and stored at -80 °C. The cellular pellet was subjected to up to 3 rounds of ACK lysis to remove red blood cells. The remaining cells were kept in residual R10 volume.

2.5.7. Cell counts

Trypan blue exclusion was used to assess cell viability and the number of viable cells were counted using a disposable multiwall haemocytometer (Immune Systems, UK).

2.6. Lung histological assessment

2.6.1. Tissue preparation for histopathology analysis

After removal from animals the superior lung lobe were inflated with PBS then fixed overnight in 10 % formalin before being transferred into 70 % ethanol and stored at 4 °C until required. Tissues were then paraffin-embedded, sliced into 4 µm sections and stained with Haematoxylin & eosin (H&E), periodic acid-Schiff (PAS) or PicoSirus Red by Lorraine Lawrence (Histology Service, Imperial College London). All histological assessments were blinded and performed in a single sitting for each experiment by Faith Uwadiae.

2.6.2. Inflammation assessment

Paraffin embedded sections were stained with H&E to determine the amount of cellular inflammation around the airways. Lung were imaged at 20 x magnification and the scale of the inflammation was determined using a numerical scoring system. The level of lung infiltrate was scored from 0-5, where 5 indicated the greatest level of inflammation. 1 = small pocket of infiltrate, 2 = small pocket (<3 cells deep) in > 1 airway or vessel, 3= 1>, less than 50 % of airways and vessels have large infiltrates (>3 cells deep), 4= most airways and vessels have large infiltrates (>50 % < 75 %), 5= majority of airways and vessels have large infiltrates and cells present in alveolar bed (> 75 % airways). 4 airways were sampled per mouse and averaged. Data was displayed as arbitrary units.

2.6.3. Goblet cell hyperplasia assessment

Paraffin embedded sections were stained with PAS. Images were visualised at 20 x magnification and PAS stained goblet cells were assessed using a numerical scoring system from 0-4. The extent for mucus production was assessed as the proportion of airways with PAS positive cells as following: 0 = no staining, 1= 5-25 % of airways, 2 = 25-50 % of airways, 3=50-75 % of airways and 4= >75 % of airways. Scores were divided by the number of airways examined (10-20 per mouse) and displayed in arbitrary units.

2.6.4. Airway collagen deposition assessment

Collagen deposition around the airways was assessed using PicoSirus Red staining. Images were captured with the Leica application suite software (Leica Microsystems, Wetzlar, Germany) using a polarising lens to create a monochrome photo. Collagen fluoresced yellow, orange and green, depending on the type of collagen deposited. Type I Collagen appeared yellow-orange while type III collagen was green. For each tissue section, 4 airways were captured at 40 x magnification. The sum of red fluorescence was measured and divided by the number of airways assessed. .

2.6.5. Airway Smooth Muscle (ASM) thickness assessment

Paraffin embedded formalin fixed lung slices were immunohistochemically stained with rabbit anti-mouse smooth muscle actin (Abcam, Cambridge, UK) as previously described (Gregory et al., 2010). Lung sections were imaged at 20 x magnification and the size of the ASM layer was measured. Briefly, the thickness of 10 airways were measured per mouse and 10 measurements were taken per airway. The data is presented as an average. Data was blinded for analysis.

2.7. Total collagen quantification by Sircol assay:

Total collagen was assessed in lung homogenate using the Sircol™ Soluble Collagen assay (Biocolor, Carrickfergus, UK) according to manufacturer's instructions.

2.8. Flow cytometry

2.8.1. Cell samples:

Lung, mLN, ILN, blood and splenic cells were processed as described above in section 2.5 and flow cytometry was performed. Fluorochrome conjugated antibodies were purchased from Biolegend (UK), Thermo Fischer Scientific (UK) or BD Pharmingen™ (UK). Flow cytometry antibodies used in this thesis are outlined in **Table 1**.

2.8.2. Extracellular staining:

1-2 x 10⁶ cells/well were plated in 96-well plates. Firstly, cell viability staining was performed using the Zombie UV™ Fixable Viability Kit (Biolegend) or Alexa-eFluoro780 Fixable Viability Kit (Thermo Fisher Scientific) per manufacturer's instructions. Cells were incubated with fluorescently conjugated antibodies diluted in FACS buffer (PBS supplemented with 2 % FBS and 2mM EDTA). Extracellular staining was performed at 4 °C for 30 mins apart from anti-mouse CXCR5 antibody (L138D7) containing stains where cells were incubated for 1 hour at room temperature (RT). When biotinylated antibodies were required, cells were stained for 30

mins at 4 °C with biotin conjugated primary antibodies, followed by fluorescently tagged streptavidin. Anti-mouse TruStain-FcX (Biolegend) was added to all antibody staining cocktails at a 1:100 dilution to prevent unspecific binding. For intranuclear staining cells were fixed using the Foxp3 Staining Buffer Set (Thermo Fisher Scientific) following manufacturers' instructions. All other cells were fixed using 1 % paraformaldehyde (PFA).

2.8.3. Intracellular staining:

For intracellular cytokine staining, prior to extracellular staining cells were first stimulated for 5 hours with 10 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 0.5 µg/ml ionomycin (Sigma-Aldrich) in the presence of 10 µg/ml Brefeldin A (Sigma-Aldrich). After extracellular staining and fixation, the cells were washed, then stained with fluorescent conjugated antibodies diluted in permeabilization buffer (Biolegend) for 30 mins at 4° C. For intranuclear staining cells were stained with fluorescently labelled antibodies specific to transcription factors in permeabilization buffer for 1 hour at 4° C.

2.8.4. Data acquisition and analysis:

Single stained Ultracomp beads (BD Bioscience) were used for compensation controls. When required mCherry or AcGFP flow cytometry calibration beads (Clonetech, USA) were additionally used for compensation. Cells were acquired using the BD LSR Fortessa (Becton Dickinson Bioscience, UK) and flow cytometry data was analysed using the Flow Jo Software (FlowJo LLR, OR, USA).

2.8.5. Gating Strategies for flow cytometry

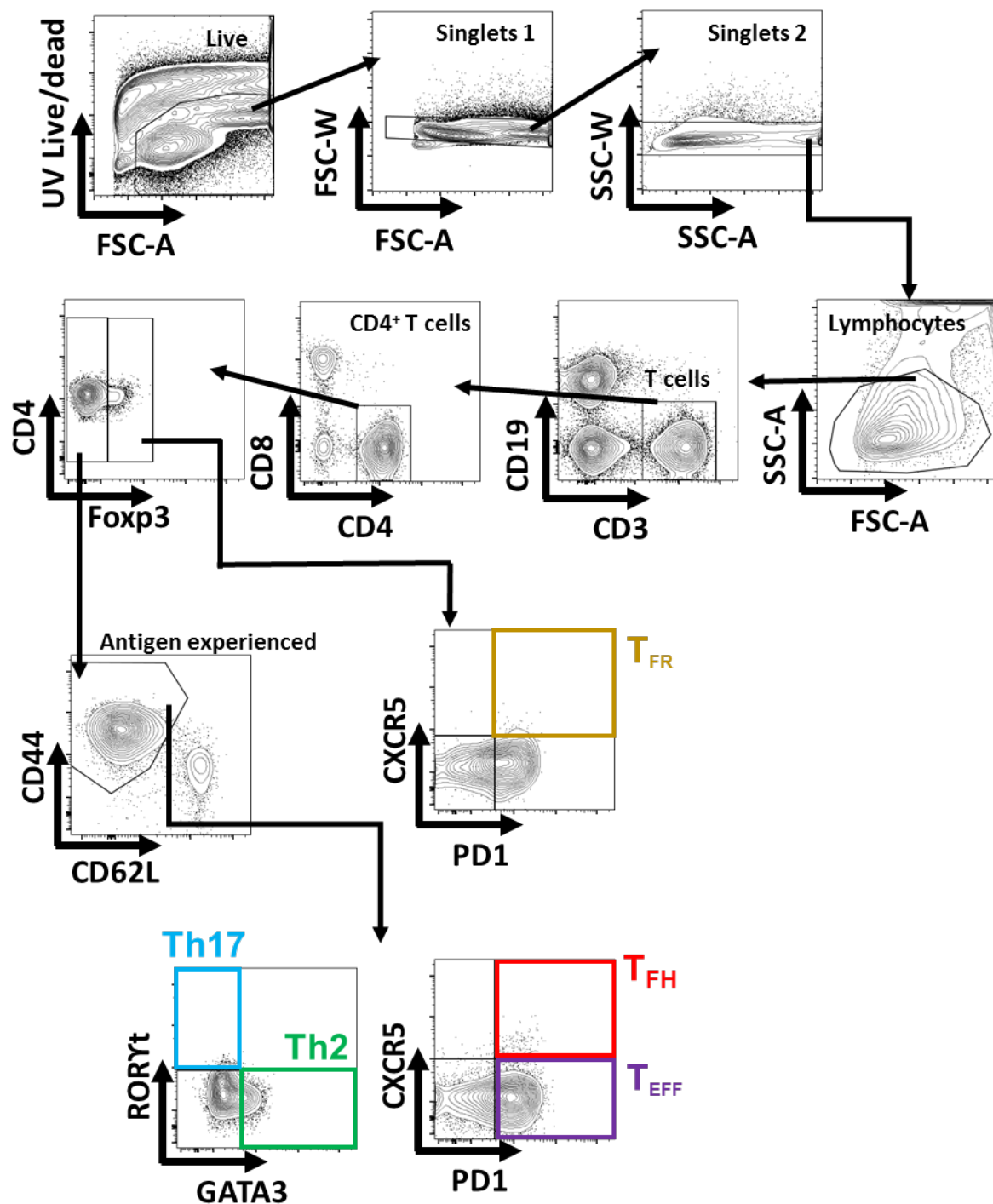


Figure 2.4: Gating strategy for T cell populations.

Live cells were selected as UV live dead negative cells. Doublet cells were excluded and lymphocytes were selected. T_{FR} were defined as CD3⁺CD4⁺Foxp3⁻CD44^{hi}CD62L⁻CXCR5⁺PD1⁺, T_{EF} as CD3⁺CD4⁺Foxp3⁻CD44^{hi}CD62L⁻CXCR5⁺PD1⁺, T_{FR} as CD3⁺CD4⁺Foxp3⁺CXCR5⁺PD1⁺, Th2 cells as CD3⁺CD4⁺Foxp3⁻CD44^{hi}CD62L⁻GATA3⁺ and CD3⁺CD4⁺Foxp3⁻CD44^{hi}CD62L⁻RORYt⁺. Representative flow cytometry gating strategy.

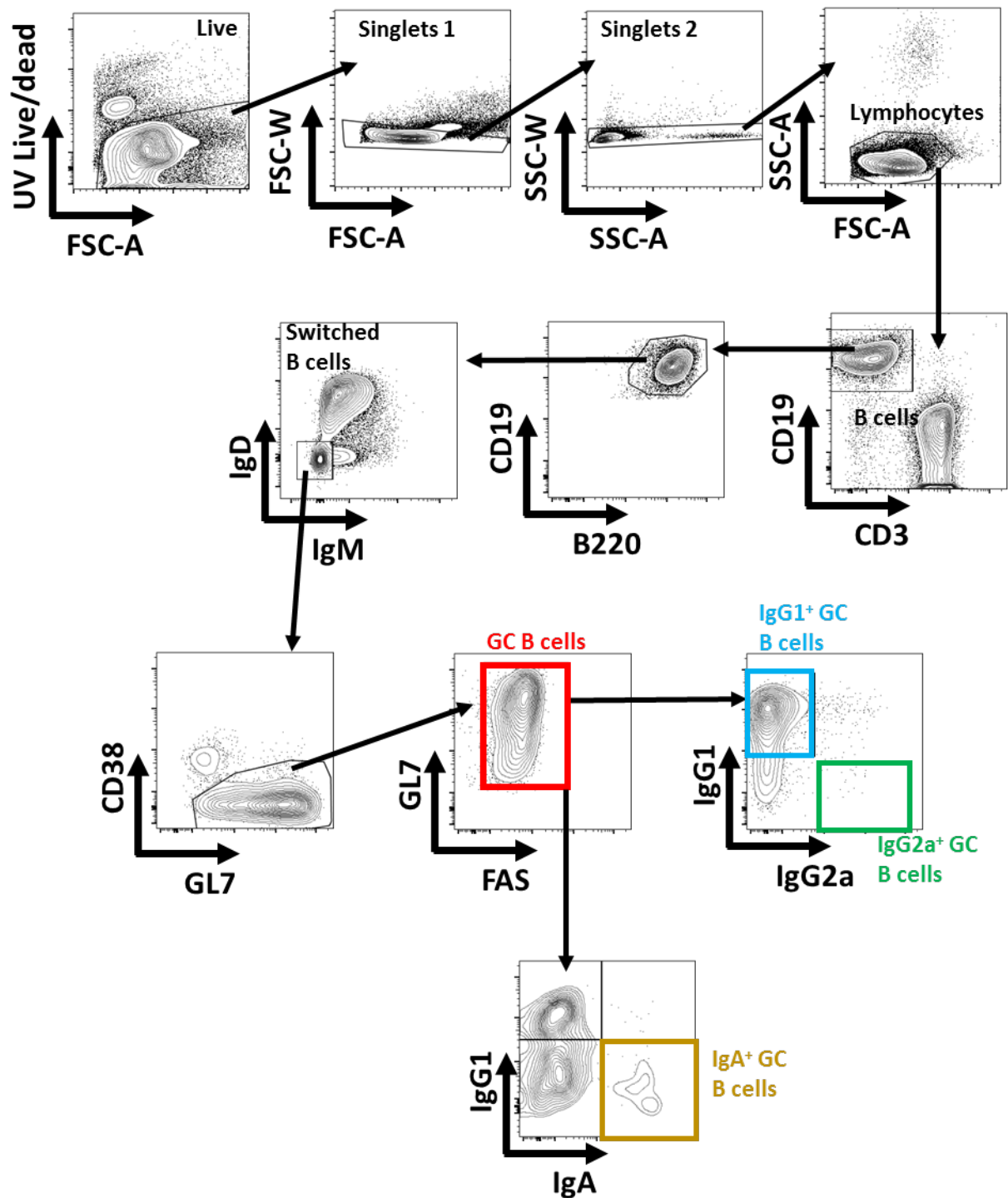


Figure 2.5: Gating strategy for germinal centre (GC) B cells.

Live cells were selected as UV live dead negative cells. Doublet cells were excluded and lymphocytes were selected. GC B cells were defined as CD19⁺B220⁺IgD⁻IgM⁻CD38⁻GL7⁺FAS⁺ lymphocytes. Representative flow cytometry gating strategy.

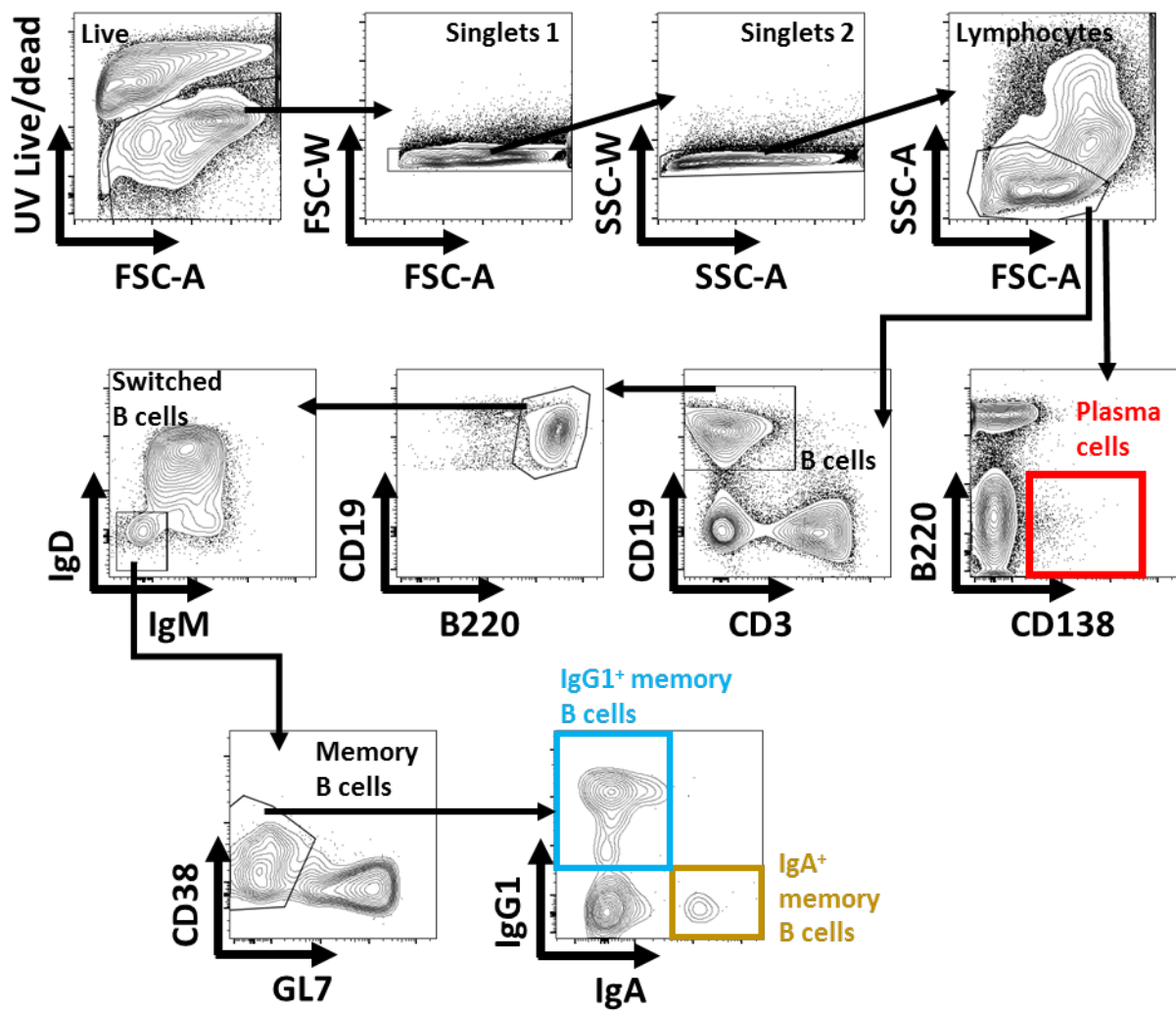


Figure 2.6: Gating strategy for plasma cells and memory B cells.

Live cells were selected as UV live dead negative cells. Doublet cells were excluded and lymphocytes were selected. Memory B cells were defined as CD19⁺B220⁺IgD⁻IgM⁻CD3⁺GL7⁻ lymphocytes. Plasma cells were defined as CD3⁻B220⁻CD138⁺ lymphocytes. Representative flow cytometry gating strategy.

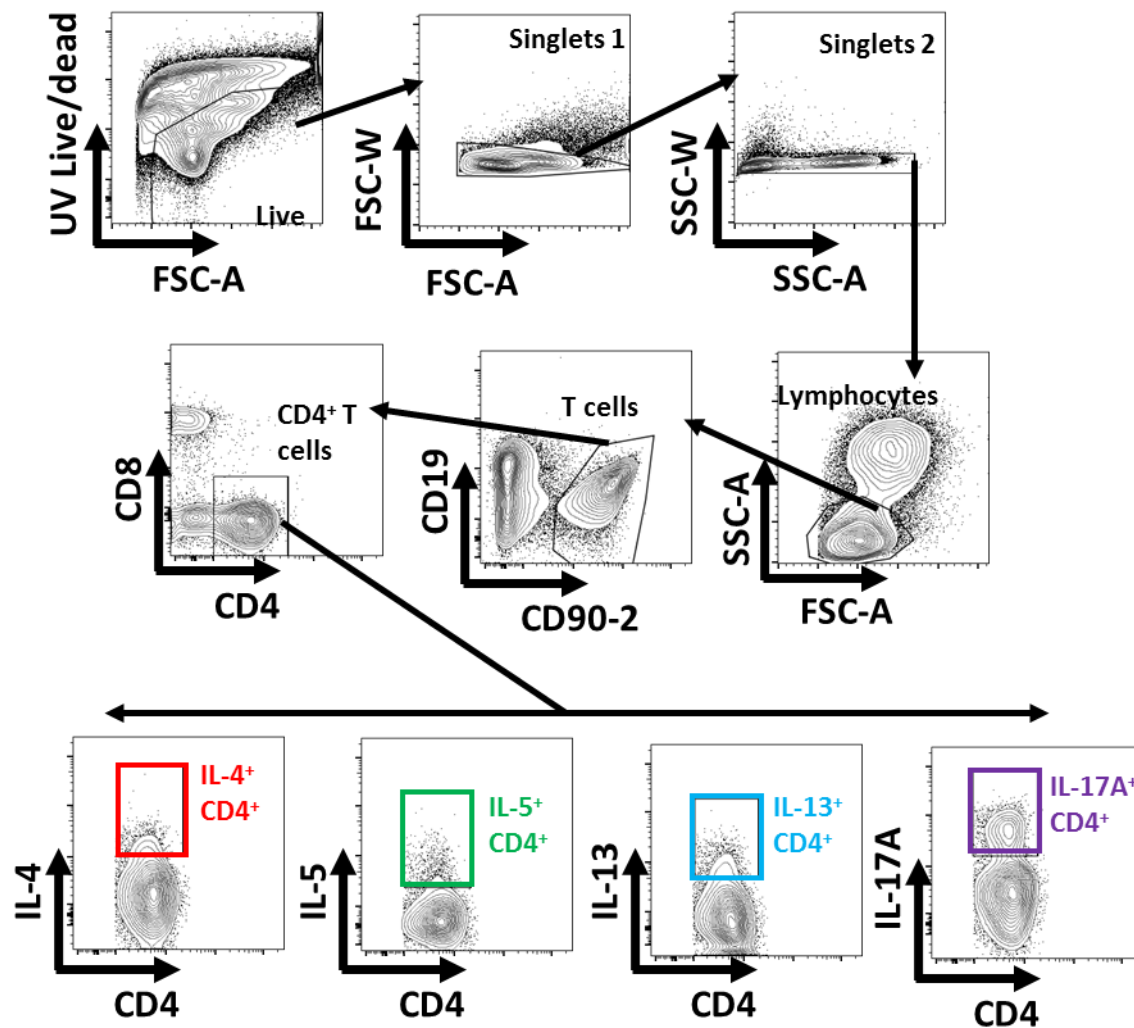


Figure 2.7: Gating strategy for cytokine positive CD4⁺ T cells.

Live cells were selected as UV live dead negative cells. Doublet cells were excluded and lymphocytes were selected. CD4⁺ T cells defined as CD90.2⁺CD4⁺ lymphocytes. Representative flow cytometry gating strategy.

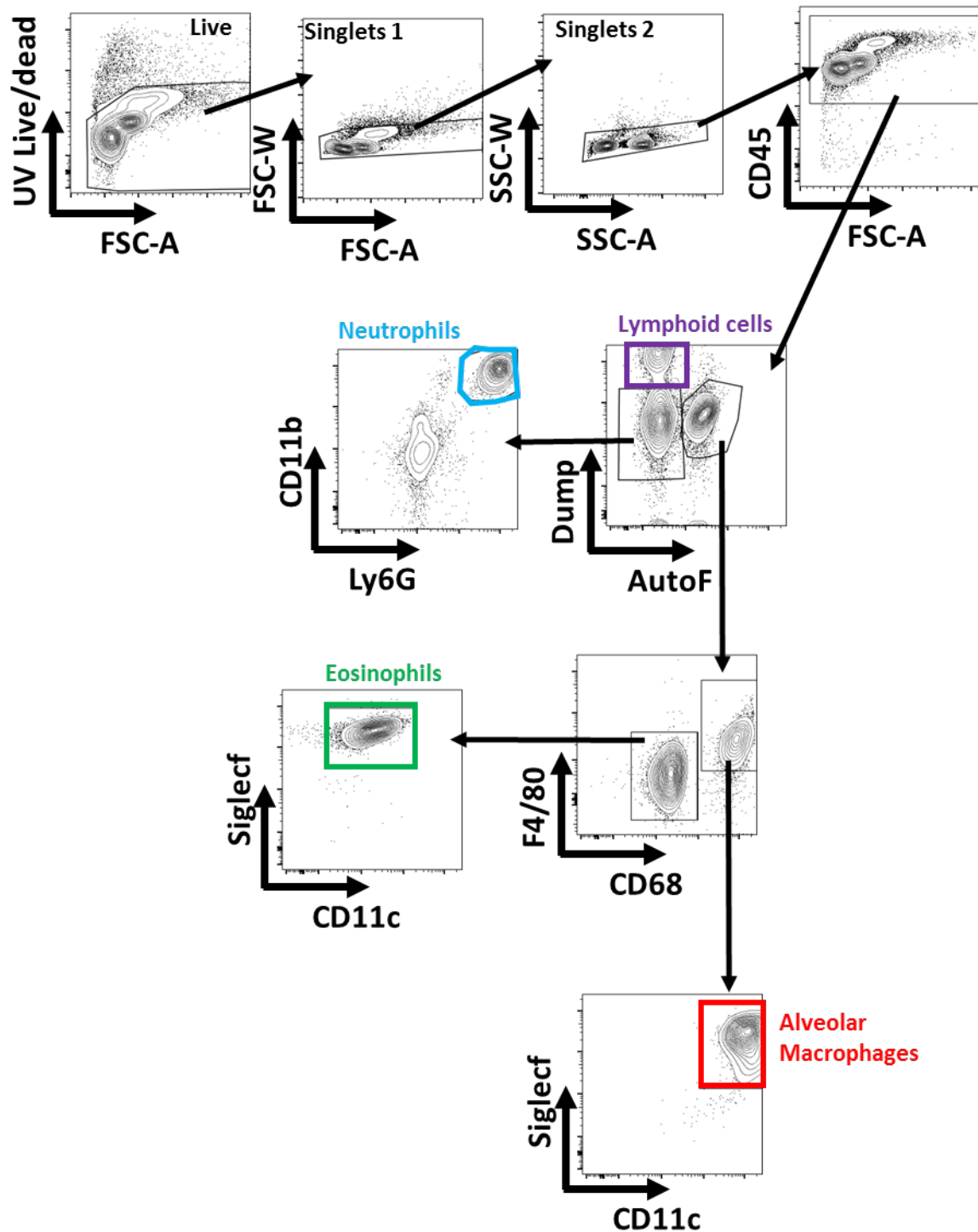


Figure 2.8: Gating strategy for myeloid and lymphoid cells.

Live cells were selected as UV live dead negative cells. Doublet cells were excluded and CD45 leukocytes were selected. Lymphoid cells were defined as CD45⁺Dump⁻AutoF⁻Ly6C⁻CD11b⁻ cells, neutrophils as CD45⁺Dump⁻AutoF⁻CD11b⁺Ly6G⁺ cells, eosinophils as CD45⁺Dump⁻AutoF⁺F4/80⁺CD68⁻Siglecf⁺CD11c⁻ cells and alveolar macrophages as CD45⁺Dump⁻AutoF⁺F4/80⁺CD68⁺Siglecf⁺CD11c⁺ cells. Representative flow cytometry gating strategy. Auto-fluorescence (AutoF) relates to an empty 488-530/30 filter channel. Dump includes CD90.2, CD19 and Nkp46.

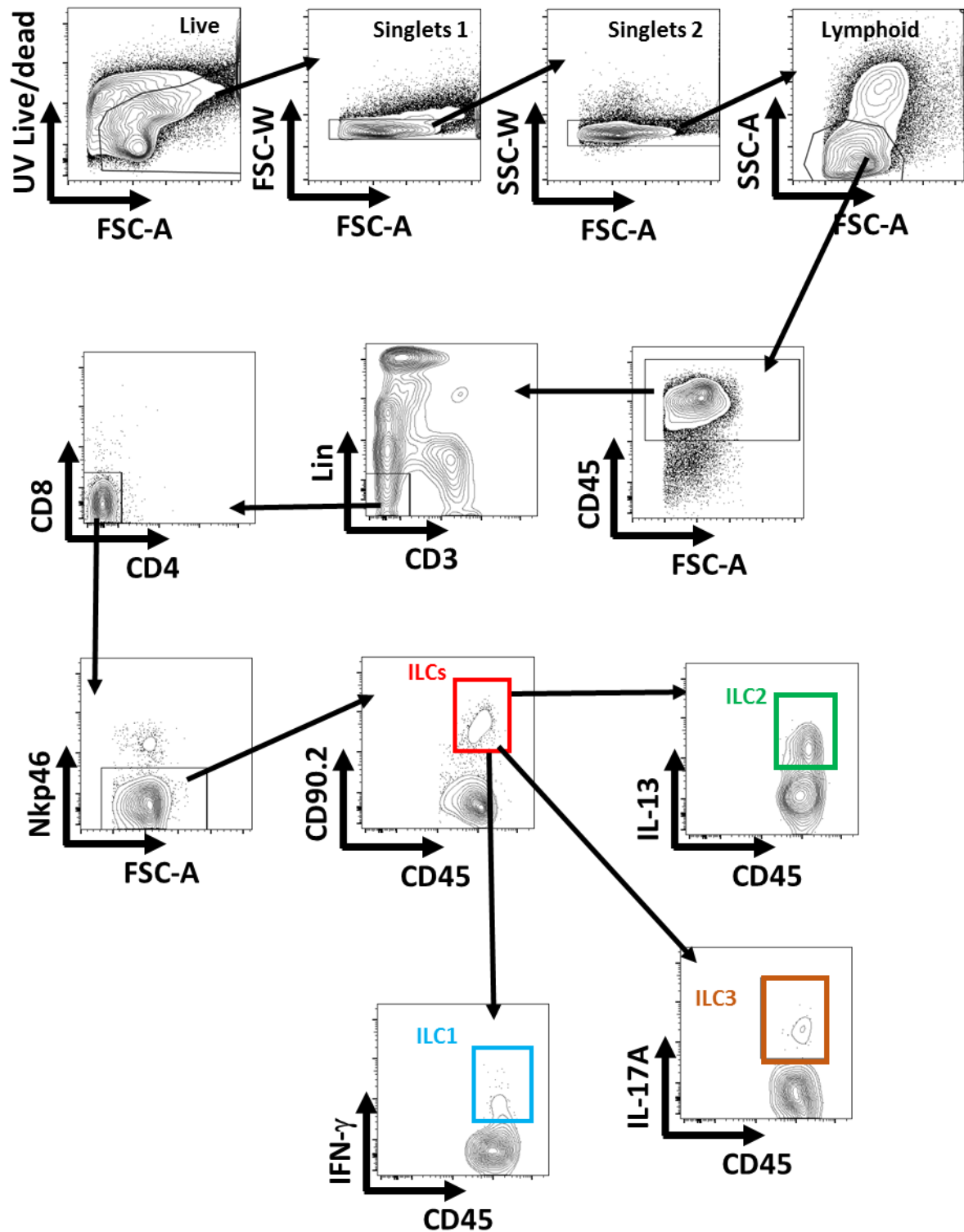


Figure 2.9: Gating strategy for innate lymphoid cells (ILCs).

Live cells were selected as UV live dead negative cells. Doublet cells were excluded and lymphoid cells were selected. ILCs were defined as Lin⁻ CD45⁺ CD90.2⁺ CD3⁺ CD4⁺ CD8⁺ Nkp46⁻ lymphoid cells. ILC1s were defined as IFN-γ⁺ ILCs, ILC2s as IL-13⁺ ILCs and ILC3s as IL-17A⁺ ILCs. Representative flow cytometry gating strategy. Lin cocktail contains CD19, CD11b, GR1, TER119, TCR-β and TCR-γδ.

2.9. Fluorescence activated cell sorting (FACS)

2.9.1. *in vivo* intravascular staining

Mice were heated at 38 °C for a maximum of 10 mins to make the tail vein more visible. Mice were then injected intravascularly via the tail vein with 3 µg FITC labelled rat anti-mouse CD45 antibody (Biolegend) in 300 µl PBS. Mice were culled after 3 mins, allowing enough time to stain vascular leukocytes while leaving tissue cells unstained. Blood was collected via the jugular artery. mLN, lung or spleen were dissected and when required BAL was acquired. Samples were processed as described previously in the cell recovery section (2.5) and tissue resident populations were defined as CD45⁻ (Figure 2.10).

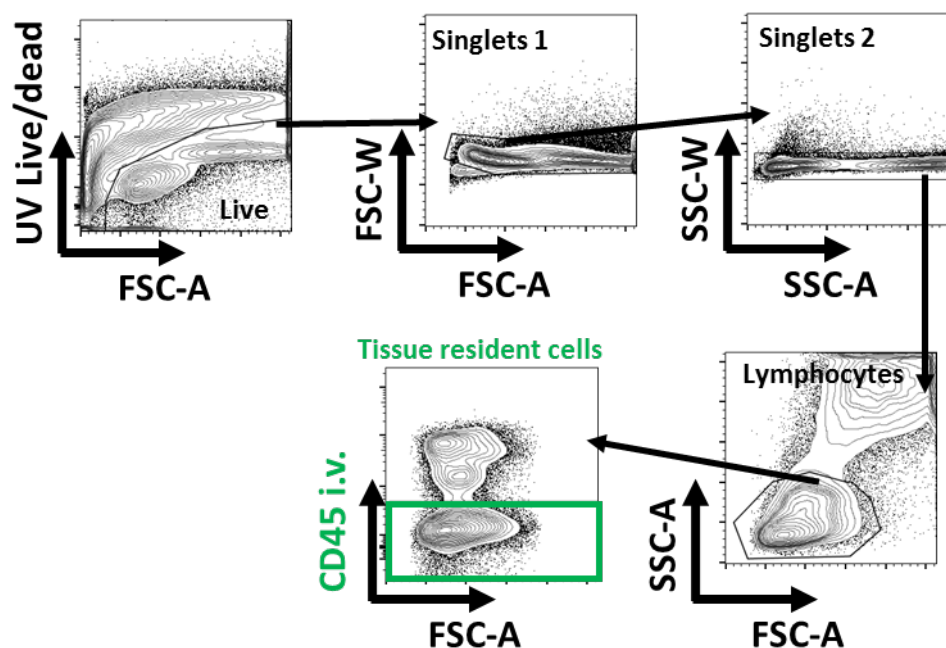


Figure 2.10: Gating strategy for tissue resident cells following *in vivo* fluorescent antibody injection.

Live cells were selected as UV live dead negative cells. Doublet cells were excluded and lymphocytes were selected. Tissue resident cells were defined as CD45⁻ cells. Representative flow cytometry gating strategy.

2.9.2. B cell sorting

CD19⁺ B cells were isolated from the lungs, mLN and spleen using the EasySep™ Mouse B cell isolation kit per manufactures' instructions (STEMCELL Technologies). Cell were stained in 5 ml FACS tubes (Falcon, UK) with extracellular antibodies against CD19, B220, IgD and IgM (**Table 1**). DAPI (Sigma-Aldrich) staining was used to determine cell viability. Tissue resident CD45⁻CD19⁺B220⁺ B cells were sorted to >95 % purity on BD Aria I and III (BD Bioscience). The gating strategy used is shown in **Figure 2.11**.

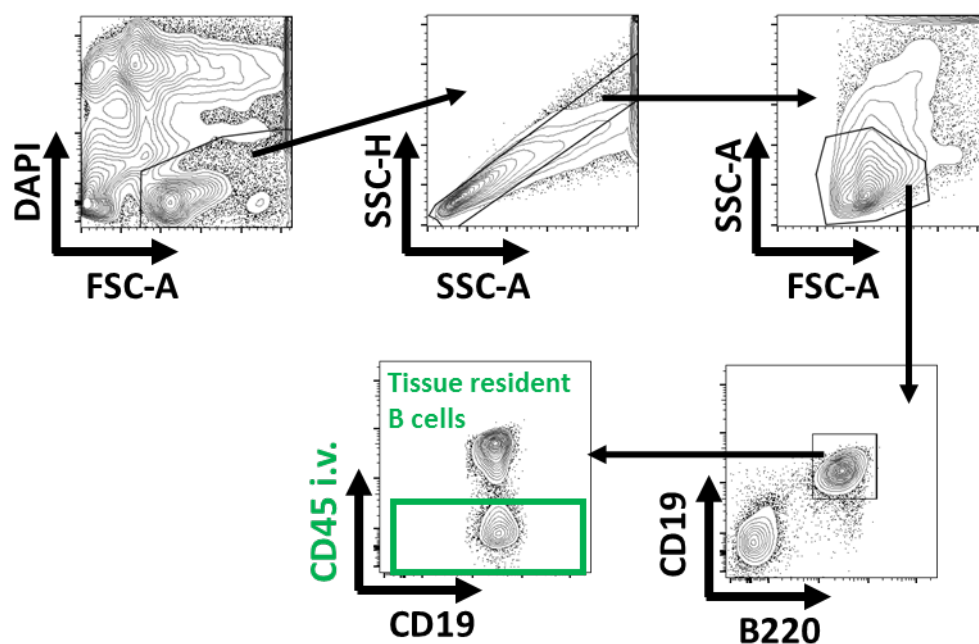


Figure 2.11: Gating strategy for sorting tissue resident B cells

Live cells were selected as DAPI negative cells. Doublet cells were excluded and lymphocytes were selected. Tissue resident B cells were defined as CD19⁺B220⁺CD45⁻ lymphocytes were sorted using the BD Aria III. Representative flow cytometry gating strategy.

2.9.3. T_{FH} sorting

CD4⁺ T cells were enriched from the lungs, mLN and spleen using the EasySep™ Mouse CD4⁺ T cell Isolation Kit per manufactures' instructions (STEMCELL Technologies, UK). CD4⁺ T cells were then stained in 5 ml FACS tubes (Falcon, UK) with extracellular antibodies against CXCR5, PD1, CD4, CD44, CD62L, CD19 and CD8 (**Table 1**). Cell viability was then determined by staining with TO-PRO™ Iodide-3 (Thermo Fisher Scientific) following manufacturers' instructions. T_{FH} (CD45⁻CXCR5⁺PD1⁺CD4⁺CD19⁻CD8⁻), non T_{FH} effector cells (T_{EFF}) (CD45⁻PD1⁺CXCR5⁻CD4⁺CD19⁻CD8⁻) and naïve T cells (CD45⁻CD44^{lo}CD62L⁺CD4⁺) were sorted to >95 % purity on BD Aria III (BD Bioscience). T_{FH} and T_{EFF} were sorted from the mLN and lungs, while naïve T cells were sorted from the spleen. The gating strategy used is shown in **Figure 2.11**.

2.10. Imaging

2.10.1. Sample preparation for imaging

Animals used for imaging were tracheotomised, cannulated and the lungs were inflated via the trachea with 1 ml 1 % low melting point agarose (Sigma-Aldrich) at 37 °C. Mice were then placed on ice for 5 minutes to allow the agarose to solidify. Lung lobes were excised from animals and fixed in 4 % PFA for between 45 and 90 mins. After fixation lung lobes were transferred into PBS supplemented with 0.05 % Sodium Azide (G-Biosciences, MO, USA) and stored at 4° C until required.

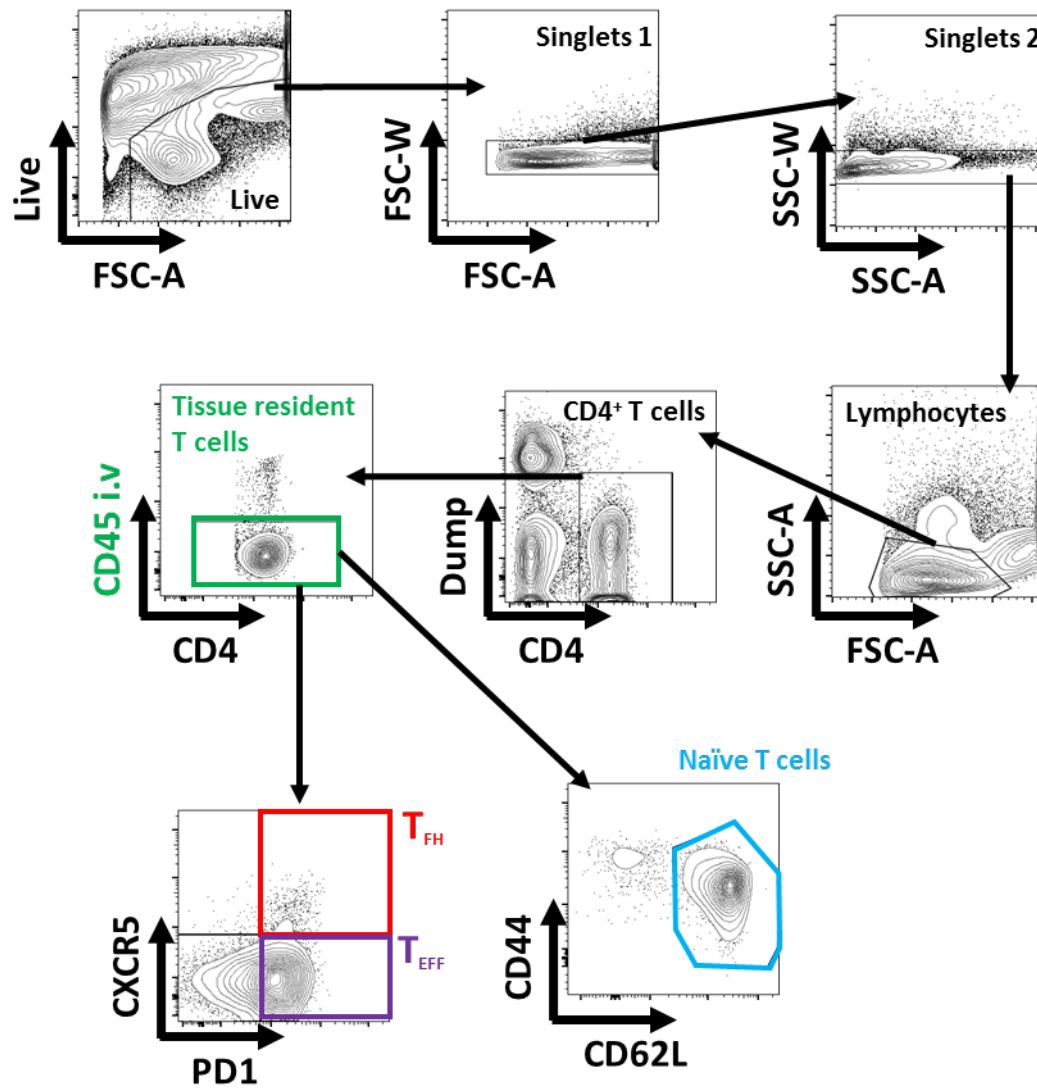


Figure 2.12: Gating strategy for sorting tissue resident T_{FH} , T_{EFF} cells and naïve T cells

Live cells were selected as TOPRO3 negative cells. Doublet cells were excluded and lymphocytes were selected. Tissue resident $CD4^+$ T cells were defined as $CD19^+CD8^-CD45^-CD4^+$ lymphocytes and T_{FH} ($CXCR5^+PD1^+$), T_{EFF} ($CXCR5^-PD1^+$) and naïve T cells ($CD44^loCD62L^+$) were sorted using the BD Aria III. T_{FH} and T_{EFF} cells were sorted from the mediastinal lymph node and lung, while naïve T cells were sorted from the spleen. Representative flow cytometry gating strategy.

2.10.2. Precision Cut Lung slices (PCLS) processing and staining

Agarose inflated lung lobes were sliced into 200 µm thick slices using the Compresstome R VF-300 (Precision Instruments, NC, USA). Lung slices were then transferred into a 24-well plate in blocking buffer (PBS supplemented with 3 % Bovine Serum Albumin (BSA), 0.1 % Sodium Azide and 0.3 % Tritonx-100) for 2 hours at RT. To reduce endogenous biotin, avidin or biotin-binding activity, samples were treated with the Avidin/Biotin blocking system (Biolegend) per manufacturer' instructions. Samples were then washed 3 times in PBS with gentle agitation. Biotinylated antibodies were diluted in staining buffer (PBS supplemented with 1 % BSA, 0.1 % Sodium Azide and 0.05 % TritonX-100) and incubated overnight at 4°C. Samples were then washed. Lung slices were later incubated for 2 hours at RT with the fluorescently bound streptavidin and directly conjugated antibodies. Antibodies used for PCLS staining are listed in **Table 2**. Stained slices were fixed in 4 % PFA for 5 mins then washed three times in PBS. Slices were then placed onto glass slides in Mowiol mounting medium supplemented with 2.5 % 1,4-diazabicyclo [2,2,2] octane (DABCO, Sigma-Aldrich).

2.10.3. Confocal microscopy

PCLS were imaged by confocal microscopy to allow in depth analysis of B cell aggregates. PCLS were imaged using a SP5 inverted confocal microscope (Leica) and analysed using the FIJI (FIJI is just ImageJ) or Imaris (Bitplane, Belfast, UK) imaging softwares.

2.10.4. Wide-field imaging and B cell aggregate quantification

B cells were identified in PCLS by staining with a fluorescent protein tagged anti-B220 antibody (**see table 2**) and lymphoid aggregates were quantified by wide-field imaging using the inverted wide-field microscope Zeiss Axio Observer (Carl Zeiss Microscopy, Jena, Germany). The lung slice was placed on the stage and the lung tissue was identified using 5 x magnification. To create an image of the whole lung slice a tile region was selected. The Zen acquisition software (Carl Zeiss Microscopy) then automatically split the tile region into

smaller squares which were imaged at 20 x magnification one at a time using the motorised stage. The individual square images were then stitched together to create a complete image of the whole lung (**Figure 2.13A**). The size and number of lymphoid aggregates in each image was then analysed using the FIJI software. Imaging analysis was performed by setting the threshold over 99 % (**Figure 2.13B**) and the wand (tracing) tool was utilised to select lymphoid aggregates (**Figure 2.13C**). The number of aggregates was assessed and the area of each aggregate was measured by the software in μm^2 .

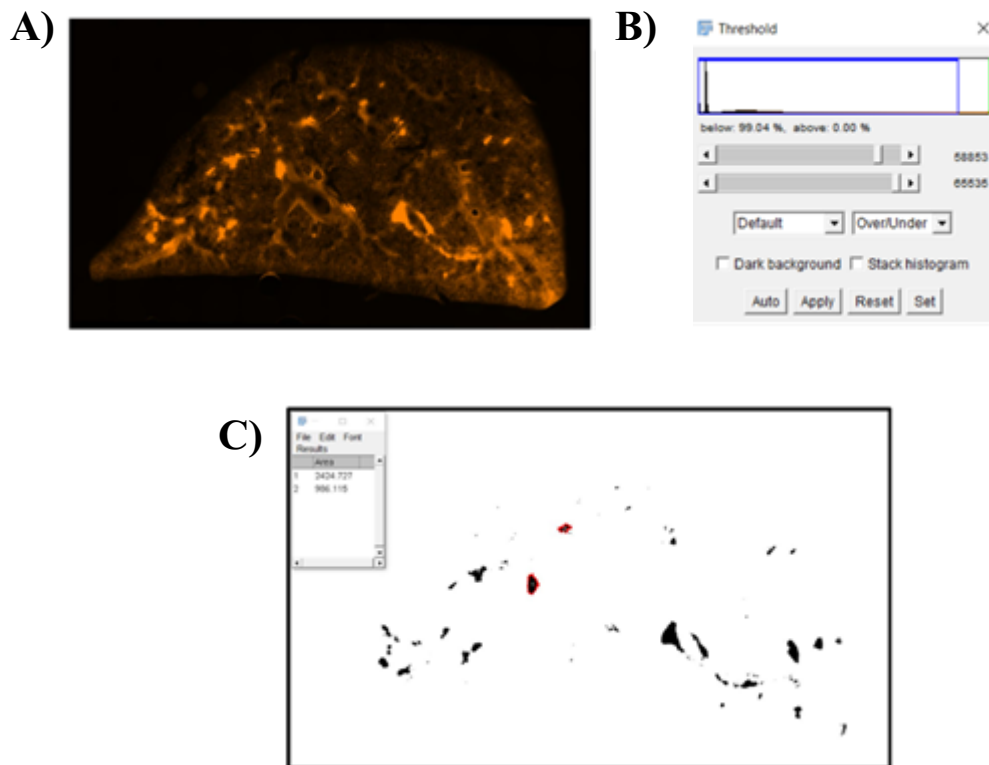


Figure 2.13: Quantification of B cell aggregates from wide-field microscopy images

A) Whole lung tissue image created from multiple individual square images stitched together. Staining represent B220⁺ cells within the lung tissue. **B)** Using the FIJI software the image exposure threshold was set to above 99 % to limit the amount of background staining. **C)** Image of the same lung tissue section displaying any staining above the 99 % set threshold. The wand feature of the FIJI software was used to select lymphocyte aggregates and determine the number and size of these structures.

2.11. Enzyme Linked ImmunoSorbent Assay (ELISA)

2.11.1. Lung homogenate preparation for ELISAs

Lung tissue from the inferior lung lobe was snap frozen in liquid nitrogen. Lungs were weighed and homogenised in 1 ml DMEM supplemented with 1 % Penicillin//Streptomycin using the FastPrep -24™ instrument (MP Biomedicals, CA, USA) at 6.5 m/s for 1 min. Lung homogenate was used for ELISAs.

2.11.2. General ELISA protocol:

96 well plate half area high binding plates (Corning™, NY, USA) were coated with the suitable antibody in coating buffer overnight at 4 °C. Plates were blocked with BSA in PBS at RT for 2 hours. Plates were washed using PBS supplemented with 0.05 % Tween-20). Samples are diluted in assay diluent (PBS supplemented with 1 % BSA and 0.01 % Tween-20) incubated overnight at 4 °C. Plates were washed 4 times. The appropriate biotinylated antibody was diluted in assay diluent at RT. Streptavidin-HRP (Vector Laboratories, Peterborough, UK) was used to detect bound antibody. Plates were washed 7 times and captured antibody, cytokine or chemokine were quantified using TMB Substrate Set (Biolegend) and the reaction was stopped using 0.18M H₂SO₄. Optical density was determined at 450 nm absorbance using the SpectraMax® i3x multi-mode MicroPlate reader (Molecular Devices, CA, USA).

2.11.3. Allergen specific antibody ELISAs:

Plates were coated with 50 µg/ml HDM or 10 µg/ml ALT in 0.1M NaHCO₃ (pH 9.6) overnight at 4 °C. Plates were blocked with 1 % BSA in PBS for 2 hours. Serum, BAL or lung samples were serially diluted to create an 8-point curve. Dilutions used in this study are outlined in **Table 3**. HDM specific IgG1 was detected using goat anti-mouse IgG1, Humans ads-HRP (SouthernBiotech, Birmingham, USA) while HDM specific IgA was detected using goat anti-mouse IgA, Humans ads-HRP (SouthernBiotech). HDM specific IgE was detected using biotin

rat anti-mouse IgE (BD Pharmingen), followed by the addition of streptavidin-HRP (Sigma-Aldrich).

2.11.4. Total antibody ELISAs:

Total IgE and IgG1 was measured in the lung and serum, while IgA was measured in the BAL and lung tissue using paired antibodies according to manufacturers' instructions (BD Pharmingen, UK). Paired antibodies used in this study are outline in **Table 4**.

2.11.5. Cytokine and chemokine ELISAs:

Lung and BAL IL-4, IL13, IL-17A IFN- γ , BAL IL-21 and serum MCP1 were determined using Invitrogen™ Ready-SET-GO! Kits (Thermo Fisher Scientific). Lung and serum Eotaxin-2/CCL21 and CXCL13/BLC/BCA-1 were measured using R&D DuoSet ELISA kits (R&D systems, MN, USA). Paired antibodies for murine IL-5 (R&D Systems, Abington, UK) were used in a standardised sandwich ELISA. All ELISAs were performed according to the manufacturers' instructions.

2.12. Real time quantification polymerase chain reaction

2.12.1. Whole lung tissue ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) conversion

Lung homogenate was made from the inferior lung lobe or post caval lung lobe as outlined in 2.11.1. 200 μ l of lung homogenate was stored in 500 μ l RLT lysis buffer (Qiagen, UK) supplemented with 1 % β -mercaptoethanol (Sigma-Aldrich) at -80 °C until required. Lung homogenate was further processed by centrifugation at 2000 rpm for 2 mins through a QiaShedder spin columns (Qiagen). RNA was extracted from the homogenised lung tissue using the RNeasy Mini Kit (Qiagen) following manufacturers' instructions. RNA was eluted in 32 μ l RNase free water and the concentration and quality of the RNA was assessed using the Nanodrop ND-1000 Spectrometer (Thermo Fisher Scientific). 1 μ g RNA was converted to

cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, MA, USA) following manufacturers' instructions.

2.12.2. Sorted cells RNA extraction and cDNA conversion

Sorted cells were lysed in 350 µl RLT lysis buffer supplemented with 1 % β-mercaptoethanol (Sigma-Aldrich) at -80° C until required. RNA was extracted from the homogenised lung tissue using the RNeasy Mini Kit (Qiagen) following manufacturers' instructions. RNA was eluted in 14-16 µl RNase free water and the concentration and quality of the RNA was assessed using the 2200 TapeStation system (Agilent Technologies, CA, USA). Up to 5 ng RNA was converted to cDNA using the GoScript Reverse Transcription Kit (Promega, WI, USA).

2.12.3. Real time quantitative polymerase chain reaction (RT-PCR)

Gene expression was quantified using TaqMan® gene expression assay sets (Thermo Fisher Scientific) along with TaqMan® fast master mix or using custom primer sequences with Fast SYBR® Green PCR master mix (Applied Biosystems, MA, USA). Both methods were used to quantify cDNA per manufacturers' instructions. Primers and gene expression sets used in this study are outlined in **Table 5**. RT-PCR was performed using the ViiA™ 7 Real time PCR system and expression relative *gapdh* was quantified.

2.13. Statistical analysis

Data was not assumed to be normally distributed and non-parametric analyses were used to determine statistical significance. Mann Whitney test was used to compare between two groups. Kruskal Wallis test with a multiple comparison test was used when comparing 3 or more groups. Paired analyses were performed using the Wilcoxon-signed rank test. Correlation was determined using the non-parametric Spearman's rank correlation coefficient.

Statistical significance was defined as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, All statistical analysis was performed using Prism Version 6.04 (GraphPad Software Inc, CA, USA).

Table 1: Antibodies used for flow cytometry

Target	Clone	Supplier	Fluorochrome	Concentration
Extracellular antibodies				
B220	RA3-6B2	Biolegend	BV510	1:200
CCR4	2G12	Biolegend	APC	1:100
CCR6	29-2L17	Biolegend	BV510	1:100
CCR7	4B12	Thermo	APC	1:100
CD3e	145-2C11	Thermo	PE/Dazzle	1:300
CD4	RM4-5	Biolegend	APC/CY7	1:100
CD8	53-6.7	Biolegend	BV711	1:200
CD8	53-6.7	Thermo	PERCP/Cy5.5	1:100
CD11b	M1/70	Thermo	PERCP/Cy5.5	1:100
CD11b	M1/70	Biolegend	BV711	1:200
CD11c	N418	Biolegend	PE/Dazzle	1:300
CD19	6D5	Biolegend	BV605	1:200
CD19	6D5	Biolegend	BV785	1:200
CD19	6D5	Thermo	PERCP/Cy5.5	1:100
CD38	90	Thermo	Alexa Fluro®700	1:200
CD38	90	Thermo	PE/CY7	1:200
CD44	IM7	Biolegend	PERCP/Cy5.5	1:200
CD45	30-F11	Biolegend	FITC	1:200
CD45.1	A20	Biolegend	BV711	1:100
CD45.2	104	Biolegend	BV510	1:100
CD45	30-F11	Biolegend	APC/CY7	1:200
CD62L	MEL-14	Biolegend	BV605	1:200
CD64	X54-5/7.1	Biolegend	PERCP/Cy5.5	1:100
CD69	H1.2F3	BD Pharmingen	PE	1:100
CD90-2	30-H12	Biolegend	BV605	1:200
CD90.2	30-H12	Biolegend	Alexa Fluor®700	1:100
CD103	2-E7	Biolegend	BV510	1:100
CD103	2-E7	Biolegend	APC	1:100
CD127	A7R34	Biolegend	BV785	1:200
CD138	281-2	Biolegend	BV711	1:100
CD138	281-2	Biolegend	PE	1:100
CXCR3	220803	R&D Systems	PE	1:100
CXCR4	L276F12	Biolegend	PE	1:100
CXCR5	L138D7	Biolegend	BV421	1:50
F4-80	BM8	Biolegend	PE/CY7	1:50
FAS	15A7	Thermo	PERCP/Cy5.5	1:100
GITR	DTA-1	BD Horizon	BV711	1:100
GL7	GL7	Biolegend	Alexa-Fluro®647	1:100
GL7	GL7	Biolegend	FITC	1:200
GR1	RB6-8C5	Thermo	PERCP/Cy5.5	1:100
Ia/Ie	M5/114.15.2	Biolegend	BV421	1:300
ICOS	C398.4A	Biolegend	BV510	1:200
ICOS-L	HK5.3	Biolegend	PE	1:100
IgD	11-26c.2a	Biolegend	Pacific Blue	1:100
IgE	R35-72	BD Biosciences	FITC	1:100
IgE	RME-1	BD Pharmingen	PE	1:100
IgG1	RMG1-1	Biolegend	PE/CY7	1:200
IgM	RMM-1	Biolegend	APC/CY7	1:50
Ki67	16A8	Biolegend	FITC	1:100

NK1.1	PK136	Biolegend	Alexa Fluor®647	1:100
PD1	RMP1-30	Biolegend	BV785	1:200
PD1	RMP1-30	Biolegend	PE/CY7	1:200
PSGL1	2PH1	BD Horizon	BV510	1:200
Ly6C	HK1.4	Biolegend	Alexa Fluor®700	1:100
Ly6C	HK1.4	Thermo	PERCP/Cy5.5	1:100
Ly6G	1A8	Biolegend	BV510	1:200
Nkp46	29A1.4	Biolegend	BV421	1:50
Nkp46		Biolegend	BV605	1:200
Siglec f	E50-2440	BD Pharmingen	PE	1:100
Siglec f	E50-2440	BD Pharmingen	Alexa Fluor®647	1:100
SLAM	TC15-12A2.2	Biolegend	FITC	1:100
TCR-β	H57-597	Biolegend	BV510	1:100
TCR-β	H57-597	Biolegend	PERCP/Cy5.5	1:100
TCR-γ/δ	GL3	Biolegend	PERCP/Cy5.5	1:100
TER119	TER119	Thermo	PERCP/Cy5.5	1:100
TruStain fcX™ (CD16/32)	93	Biolegend	N/A	1:100
Intracellular antibodies				
CD68	FA-11	Biolegend	PERCP/Cy5.5	1:200
IFN- γ	XMG1.2	Biolegend	APC	1:100
IFN- γ	XMG1.2	Biolegend	BV510	1:50
IL-4	11B11	Biolegend	BV421	1:100
IL-4	11B11	Biolegend	PE/Dazzle	1:100
IL-5	TRFK5	Biolegend	APC	1:100
IL-10	JESS-16E3	Thermo	PE	1:100
IL-13	eBio13A	Thermo	PE/CY7	1:100
IL-17A	TC11-18H10.1	Biolegend	APC	1:100
IL-17A	TC11-18H10.1	Biolegend	BV605	1:100
IL-22	Poly5164	Biolegend	PE	1:5-20
Human IgG	Polyclonal	Thermo	PE	1:200
Intranuclear antibodies				
BCL-6	K112-91	BD Pharmingen	PE	1:25
Foxp3	11-5773-82	Thermo	Alexa Fluor®700	1:50
Foxp3	11-5773-82	Thermo	FITC	1:100
GATA3	16E10A23	Thermo	Alexa Fluor®488	1:25
RORγt	AFKJS-9	Biolegend	APC	1:50
Purified antibodies				
rmIL-21 R subunit/Fc chimera	n/a	R&D Systems	Purified protein	1:25
IgA	RMA-1	Biolegend	Biotin	1:100
IgG2a	RMG2a-62	Biolegend	Biotin	1:100
Fluorescently conjugated streptavidin				
Biotin	N/A	Thermo	Streptavidin-APC	1:100

Table 1: Antibodies used for flow cytometry.

Antibodies were purchased from Biolegend, Thermo Fisher Scientific (Thermo), BD or R&D systems. Allophycocyanin (APC), Brilliant Violet (BV), Cyanine (Cy), Phycoerythrin (PE), Fluorescein isothiocyanate (FITC) and Peridinin Chlorophyll Protein Complex (PERCP).

Table 2: Antibodies used for staining precision cut lung slices

Target	Clone	Supplier	Fluorochrome	Concentration
Fluorophore conjugated antibodies				
B220	RA3-6B2	Biolegend	Alexa Fluro®594	1:200
B220	RA3-6B2	Biolegend	Alexa Fluro®647	1:200
CD90.2	30-H12	Biolegend	PE	1:100
GL7	GL7	Biolegend	Alexa-Fluro®647	1:200
Ki67	So1A15	Thermo	FITC	1:100
Biotinylated antibodies				
CD4	RM4-5	Biolegend	N/A	1:200
CD21/35 (CR2/CR1)	7E9	Biolegend	N/A	1:200
Fluorescently conjugated streptavidin				
Biotin	N/A	Biolegend	Streptavidin-BV421	1:200
Biotin	N/A	Biolegend	Streptavidin-APC	1:100
Fluorescently conjugated glycoprotein				
Isolectin GS-IB4	N/A	Thermo	Alexa-Fluro®488	1:200

Table 2: Antibodies used for staining precision cut lung slices

Antibodies were purchased from Biolegend or Thermo Fisher Scientific (Thermo). Allophycocyanin (APC), Brilliant Violet (BV), Cyanine (Cy) and Phycoerythrin (PE).

Table 3: Sample dilutions used to generate allergen specific antibody curves

Antibody	Sample type	Top dilution	Serial dilution
BALB/c – HDM specific ELISAs			
IgA	Lung	1:2	1:5
	BAL	Neat	1:4
IgE	Serum	1:5	1:4
	Lung	1:2	1:2
IgG1	Serum	1:100	1:5
	Lung	1:5	1:5
C57BL/6 – HDM Specific ELISAs			
IgA	Lung	Neat	1:2
	BAL	Neat	1:4
IgE	Serum	1:2	1:2
	Lung	Neat	1:2
IgG1	Serum	1:100	1:5
	Lung	1:5	1:5
BALB/c – ALT specific ELISAs			
IgA	Lung	Neat	1:4
	BAL	Neat	1:4
IgE	Serum	1:50	1:4
	Lung	1:2	1:2
IgG1	Serum	1:4000	1:3
	Lung	1:4	1:2
C57BL/6 – ALT specific ELISAs			
IgA	Lung	Neat	1:4
	BAL	Neat	1:4
IgE	Serum	1:50	1:4
	Lung	1:2	1:2
IgG1	Serum	1:4000	1:3
	Lung	1:4	1:2

Table 3: Sample dilutions used to generate allergen specific antibody curves

Table 4 Paired antibody sets

Target	Coating antibody	Standard antibody	Detection antibody
IgA	Purified rat anti-mouse IgA (C10-3)	Purified mouse IgA, k Isotype control (M18-254)	Biotin rat anti-mouse IgA (C10-1)
IgE	Purified rat anti-mouse IgE (R35-72)	Purified mouse IgE k Isotype control (C38-2)	Biotin rat anti-mouse IgE (R35-118)
IgG1	Purified rat anti-mouse IgG1 (A85-3)	Purified mouse IgG1, k isotype control (MOPC-31C)	Biotin rat anti-mouse IgG1 (A85-1)
IgG2a	Purified rat anti-mouse IgG2a (R11-89)	Purified mouse IgG2a, k isotype control (G155-178)	Biotin rat anti-mouse IgG2a (R19-15)

Table 4 Paired antibody sets

Antibody clones are displayed in brackets. All antibodies were purchased from BD Pharmingen™ UK.

Table 5: Real time qPCR primers

SYBR Green Primers	
Target	Sequence
<i>switch circle forward</i>	GACCTCTCCGAAACCAGGC
<i>Igg2a switch circle Reverse</i>	GGGCCAGTGGATAGACCGA
<i>Igg1 switch circle Reverse</i>	GGATCCAGAGTTCCAGGTCCT
<i>Gapdh Forward</i>	TCCCACTCTTCCACCTTCGA
<i>Gapdh Reverse</i>	AGTTGGGATAGGGCCTCTCTT
<i>Iε forward</i>	GAGATTCACAACGCCTGG
<i>Cε Reverse</i>	CTTTACAGGGCTTCAAGGG
<i>Iγ1 Forward</i>	TCGAGAAGCCTGAGGAATGT
<i>Cγ1 Reverse</i>	ATAGACAGATGGGGGTGTCTG
<i>Bcl6 Forward</i>	CCTGTGAAATCTGTGGCACTCG
<i>Bcl6 Reverse</i>	CGCAGTTGGCTTTTGTGACG
TaqMan® Gene Expression Assay Sets	
Target	Assay set number
<i>Bcl6</i>	Mm00477633_m1
<i>Cxcl13</i>	Mm04214185_s1
<i>Cxcr5</i>	Mm00432086_m1
<i>Foxp3</i>	Mm00475162_m1
<i>Gata3</i>	Mm00484683_m1
<i>Il4</i>	Mm00445259_m1
<i>Il5</i>	Mm00439646_m1
<i>Il9</i>	Mm00434305_m1
<i>Il13</i>	Mm00434205_g1
<i>Il17a</i>	Mm00439618_m1
<i>Il17f</i>	Mm00521423_m1
<i>Il21</i>	Mm00517640_m1
<i>Il22</i>	Mm01226722_g1
<i>Prdm1</i>	Mm00476128_m1
<i>Rorc</i>	Mm01261022_m1
<i>Tbx21</i>	Mm00450960_m1

Table 5: Real time qPCR primers

Primers used in this study are outlined in this table. Custom primers were purchased from Eurofins Scientific, UK and analysed using Fast SYBR® Green master mix. All primer sequences are listed in the 5' to 3' end. Taqman® gene expression assay sets utilised FAM as a fluorophore and were purchased from Thermo Fisher Scientific, MA, USA.

Chapter 3: The magnitude of the T_{FH} and GC response is determined by the length of allergen exposure

3.1. Introduction

T_{FH} are critical to humoral immunity, playing important roles in the regulation of the GC responses and antibody production. T_{FH} drive the generation of GC B cells and direct their proliferation and eventual differentiation into long lasting memory B cells and antibody secreting plasma cells (Vinuesa et al., 2016). Allergic asthma is characterised by the abnormal production of allergen specific antibodies, in particular IgE. Therefore, it was suggested that T_{FH} could be critical to the disorder. T_{FH} had been identified in the SLOs of mice exposed to Th2 inducing antigens and parasitic worms (King and Mohrs, 2009, Zaretsky et al., 2009). Furthermore, T_{FH} have now been identified in several sensitisation and challenge models of adjuvanted protein or allergen driven AAD (Coquet et al., 2015, Ballesteros-Tato et al., 2016, Noble and Zhao, 2016, Kobayashi et al., 2017). CXCR5⁺CD4⁺ T cells within the blood termed circulating T_{FH} (c T_{FH}) have also been found (Forster et al., 1994) and a few studies have now described c T_{FH} populations in the blood of patients with allergic rhinitis (Kamekura et al., 2015, Kim et al., 2016, Schulten et al., 2017). At the time of starting this PhD T_{FH} had not been evaluated in human studies with asthmatics or mouse models of AAD. Therefore, it was important to characterise where T_{FH} were found during allergic disease, using more clinically relevant models of AAD.

Allergic asthma is a disease driven by repeated low dose exposure to harmless agents, such as HDM, animal dander or fungal spores, resulting in the generation of allergen specific IgE (Lambrecht and Hammad, 2015). To date, studies of T_{FH} and AAD have utilised acute murine models of the disorder. However, T_{FH} responses can become dysregulated or exaggerated during chronic antigen exposure (Fahey et al., 2011, Harker et al., 2011). Firstly, the scale of the T_{FH} and GC response is dependent on antigen dose and therefore more T_{FH} and GC are generated with higher OVA doses (Baumjohann et al., 2013). Furthermore, chronic protein immunisations generates a greater proportion of T_{FH} and GC than a single acute dose (Baumjohann et al., 2013), while chronic LCMV infection generates a stronger T_{FH} response than the acute infection (Fahey et al., 2011, Harker et al., 2011). Lastly, excessive T_{FH}

responses are linked to abnormal auto-antibody production in several autoimmune disorders (Vinuesa et al., 2005, Linterman et al., 2009, Li et al., 2012, Zhu et al., 2012), indicating that the generation of too many T_{FH} can be problematic. Taken together, this suggests that during chronic AAD, the T_{FH} and humoral response should increase with allergen exposure time and this may contribute to abnormal antibody production.

During an asthma exacerbation, allergen challenge causes cross linking of IgE bound to FC ϵ RI receptors on mast cells and basophils leading to cellular degranulation and inflammation (Locksley, 2010). Asthmatic patients often undergo asthma exacerbations when they are re-exposed to their trigger allergen after a period of rest or exposed to high levels of an allergen (Singh and Busse, 2006). Although, multiple cellular populations, including eosinophils, Th2 cells and alveolar macrophages have been evaluated during the resolution and rechallenge stages of AAD (Johnson et al., 2004, Kearley et al., 2009, Mathie et al., 2015), the T_{FH} response has not. Th2 cells are rapidly increased in response to allergen re-exposure (Cates et al., 2004, Fattouh et al., 2005) and T_{FH} are dramatically elevated in other models during a secondary immune response and promote secondary antibody responses (Ise et al., 2014, Fairfax et al., 2015). Therefore, it is expected that T_{FH} should quickly response to allergen rechallenge, however this has not been evaluated.

Taken together it is clear that although a few groups have now started to evaluate the T_{FH} response in AAD, no extensive analysis has been performed using a clinically relevant mouse model. The purpose of this chapter was to examine the development of the T_{FH} and GC B cell response after 1, 3 and 5 weeks of allergen exposure. Furthermore, the T_{FH} and GC response was analysed following disease resolution and after allergen rechallenge. This was determined by analysing the T_{FH} response after a 4-week resolution period and after a 2-dose allergen challenge.

3.1.1 Hypothesis

The magnitude of the T_{FH} and GC response during allergic airway disease is determined by the length of allergen exposure.

3.1.2 Aims

- 1) To determine where T_{FH} and GC B cells are found during chronic allergic airway disease
- 2) To characterise the timing and the scale of the T_{FH} , GC B cell and antibody response during chronic allergic airway disease
- 3) To characterise the stability of the T_{FH} and GC response and their response to allergen re-challenge

3.2 Methods

3.2.1 ALT and HDM models of chronic allergic airway disease

Female adult BALB/c mice were exposed to intranasal (i.n) HDM (25µg), ALT (10µg) or a control of PBS (25µl) 3 times a week for 3 weeks. To examine allergen induced allergic airway disease mice were culled at the end of week 3 and the lungs were harvested. Lung function was assessed using the flexiVent system and cellular populations were determined by flow cytometry (**Figure 3.1A**).

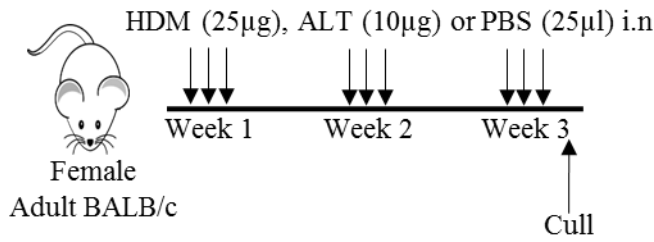
3.2.2 T_{FH} and humoral response time-course during chronic AAD

For the time-course studies female adult BALB/c mice were exposed to intranasal (i.n) HDM (25µg), ALT (10µg) or a control of PBS (25µl) 3 times a week for up to 5 weeks. To determine the timing and the location of the T_{FH} and GC B cell response allergen treated animals were culled at the end of week 1, 3 and 5. Blood was collected, and the lungs, mLN, ILN and spleen were harvested at each time-point. PBS treated animals were culled at the end of week 5. Mice were scarified 18 hours after the final dose for each time-point. Cellular populations were determined by flow cytometry and serum antibody levels were analysed by ELISA (**Figure 3.1B**).

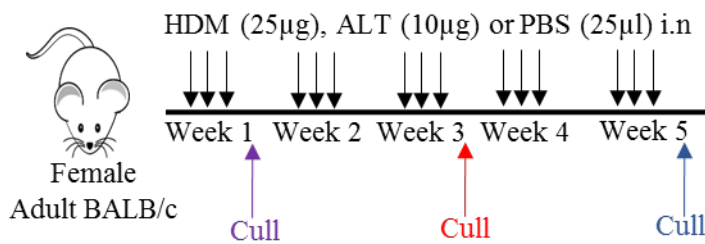
3.2.3 T_{FH} and humoral response rechallenge model of AAD

For re-challenge studies, female adult BALB/c mice were exposed to intranasal (i.n) HDM (25µg) or PBS (25µl) three times a week for 5 weeks to establish disease. Mice were then rested for 4 weeks to allow disease resolution before re-exposing mice to 2 alternate day doses of i.n. HDM (25µg) or PBS (25µl). 2 challenge doses were chosen as the re-challenge protocol as this has been previously been reported to exacerbate allergen driven allergic disease (Campbell et al., 2009). Mice were scarified 18 hours after the final dose and blood, BAL, lung tissue and mLN were collected from mice. Cellular populations were determined by flow cytometry and antibody levels in the serum and lungs were analysed by ELISA (**Figure 3.1C**).

A)



B)



C)

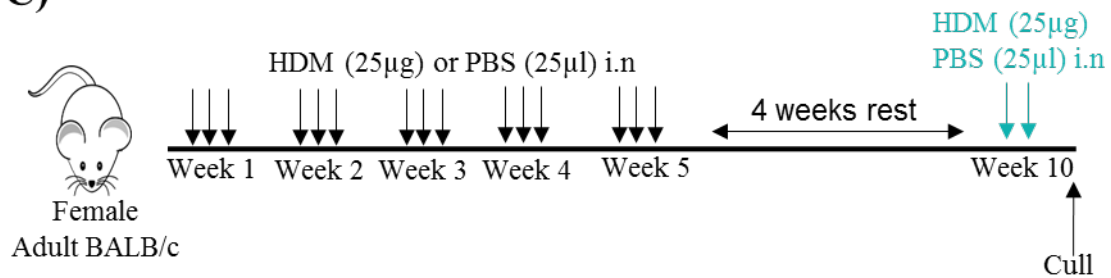


Figure 3.1: Methods

A) Adult female BALB/c mice were exposed to either 25 µg house dust mite (HDM), 10 µg alternaria alternata (ALT) or 25 µl phosphate buffered saline (PBS), 3 times a week for 3 weeks. Mice were culled at the end of week 3. **B)** Adult female BALB/c mice were exposed to either 25 µg HDM, 10 µg ALT or 25 µl PBS, 3 times a week for up to 5 weeks. Control mice were exposed to PBS for 5 weeks. To determine the timing and magnitude of the humoral response allergen exposed animals were culled at the end of week 1, 3 and 5. **C)** Adult female BALB/c mice were exposed to either 25 µg HDM or 25 µl PBS, 3 times a week for 5 weeks. Mice were rested for 4 weeks and re-exposed to 2 alternate day doses of 25 µg HDM or 25 µl PBS at the start of week 10. All mice were culled 18 hours after the final dose.

3.3 Results

3.3.1. Allergen exposed mice develop allergic airway disease

To determine if the allergen models were sufficient to drive allergic airway disease (Johnson et al., 2004, Gregory et al., 2009b), mice were exposed to ALT or HDM 3 times a week for 3 weeks (**Figure 3.1A**). Exposure to ALT or HDM increased the number of lung cells compared to PBS controls (**Figure 3.2A**). Characteristic of type 2 inflammation, there was an influx of eosinophils into the lung tissue (**Figure 3.2B**) and increased lung GATA3⁺ cells (Th2 cells) in both allergen models (**Figure 3.2C**). ALT induced a greater number of GATA3⁺ cells than HDM (**Figure 3.2C**). Both, ALT and HDM exposure induced AHR in allergen exposed animals characterised by increased airway resistance (**Figure 3.2D**) and elastance (**Figure 3.2E**) and decreased compliance (**Figure 3.2F**) compared to PBS controls (**Figure 3.2D-F**). Thus, these data suggest that exposure to either ALT or HDM was sufficient to drive AAD.

3.3.2. T_{FH} are found in secondary lymphoid organs and locally in the lungs during HDM exposure

T_{FH} have predominantly been identified in SLOs, while circulating T_{FH}-like cells have been found in the blood (Vinuesa et al., 2016). To determine where and when T_{FH} are found during chronic AAD we analysed multiple tissue sites over a 5-week period (**Figure 3.3A**). T_{FH} were identified in the mLN after 1 week of HDM exposure, increasing further and reaching significance relative to PBS treated mice at week 3, and remained stable after 5 weeks of HDM inhalation (**Figure 3.3A, B**). Splenic T_{FH} were increased from week 3 onwards, relative to PBS controls and remained elevated with further allergen exposure (**Figure 3.3A, C**). Circulating T_{FH} were not identified in the blood of any of the analysed animals (**Figure 3.3A, D**). Interestingly, T_{FH} were also found in the lungs tissue of HDM treated animals from week 3 onwards, remaining elevated compared to PBS treated controls at week 5 (**Figure 3.3A, D**).

No substantial T_{FH} populations were identified in the mLN, lung tissue, blood or spleen of PBS treated animals at any analysed time-points (**Figure 3.3A-E**).

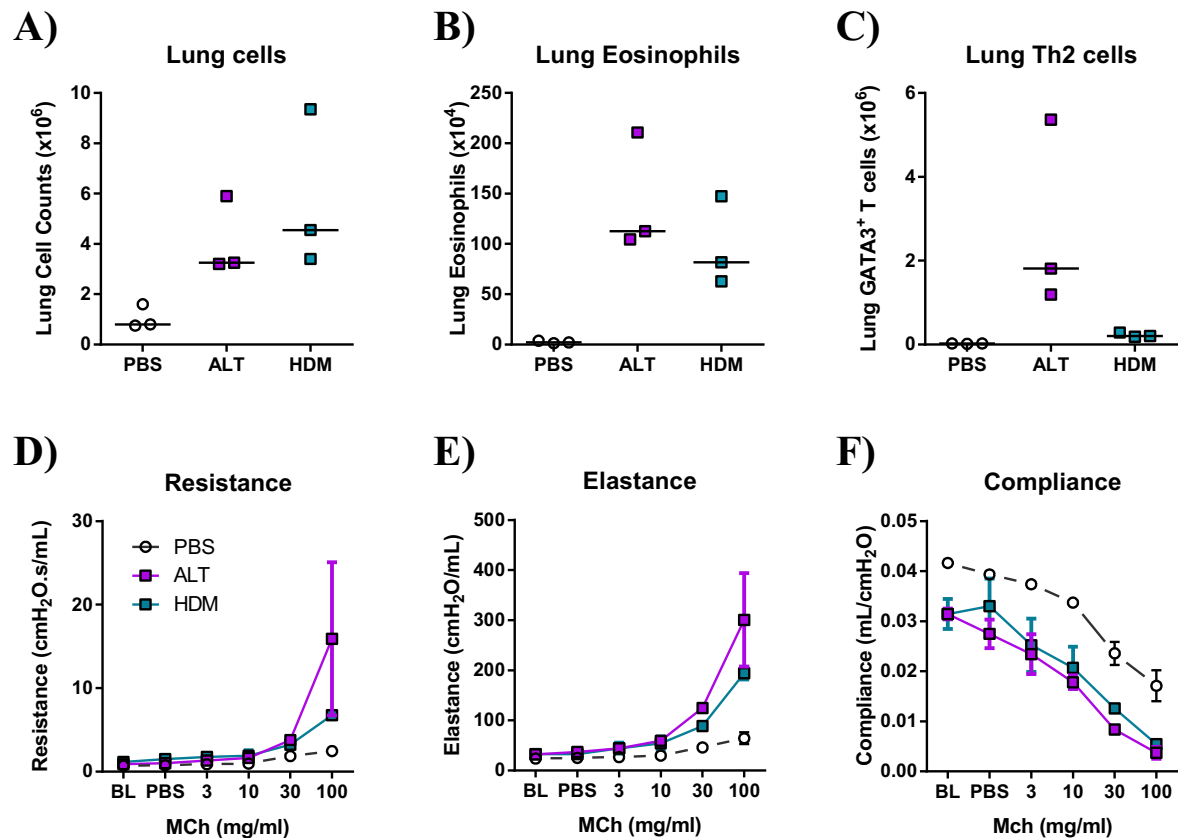


Figure 3.2: Chronic allergen exposure drives allergic airway disease.

A) Total lung cells. **B-C)** Flow cytometry was used to determine the number of cellular populations within the lungs. **B)** Eosinophils, **C)** GATA3⁺ Th2 cells. Median data is displayed. **D-F)** Airway hyperresponsiveness was measured by exposing mice to 0-100mg/ml methacholine (MCh) and the flexiVent system was used to measure **D)** Airway resistance, **E)** Airway elastance, **F)** Airway compliance. Curves display mean $\pm$ SEM. n=3 per group. Experiment, n=1.

A) Pre-gated on CD4⁺CD3⁺Foxp3⁻CD44^{hi}CD62L⁻ lymphocytes

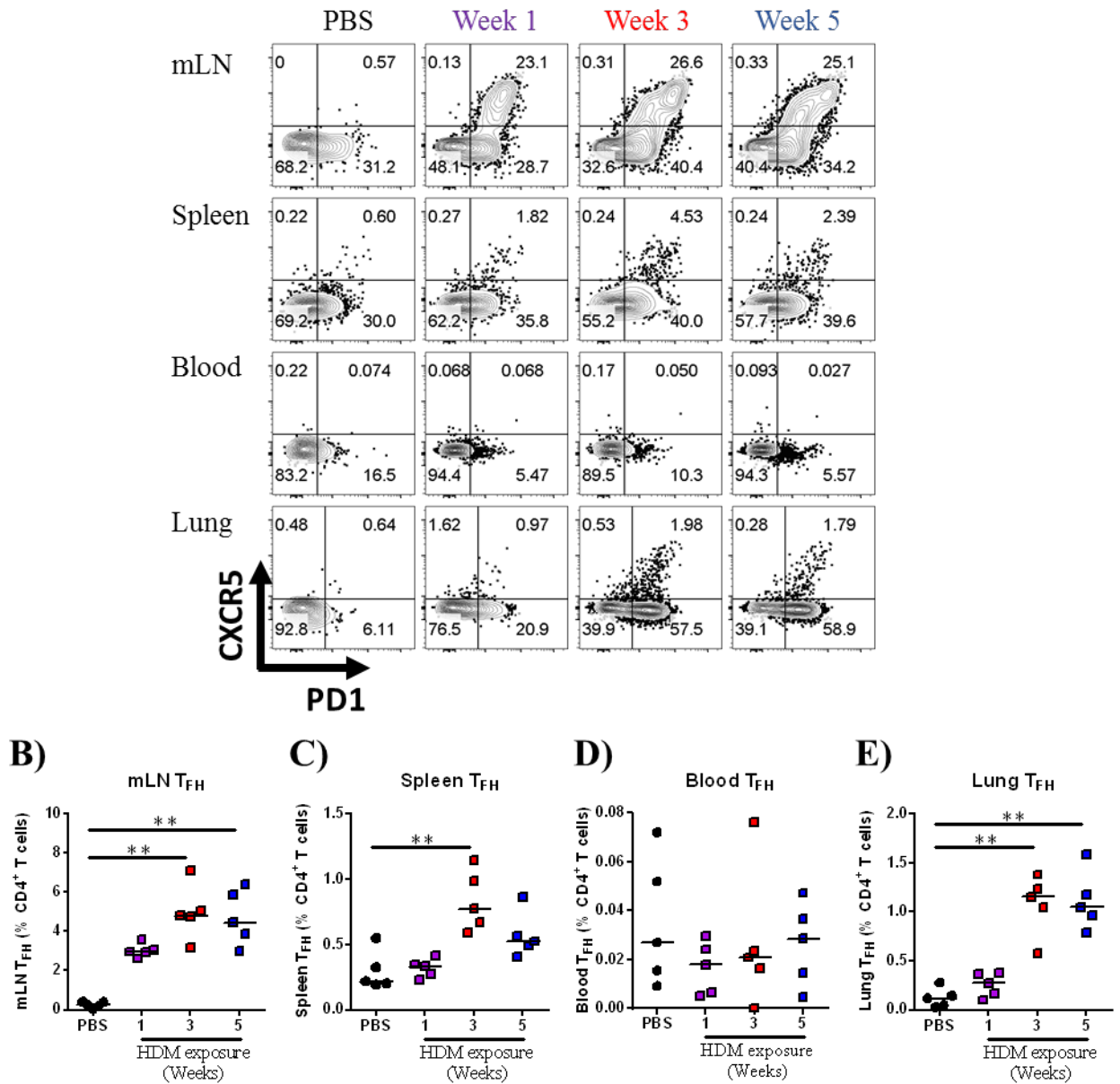


Figure 3.3: T_{FH} accumulate over time in the lungs and peripheral tissues following HDM exposure.

Flow cytometry was used to determine the frequency of T_{FH} within cellular compartments following HDM exposure. T_{FH} were defined as CXCR5⁺PD1⁺Foxp3⁻CD4⁺. **A)** Representative flow plots of T_{FH} in PBS and HDM treated animals, pre-gated on CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes. T_{FH} were quantified in the **B)** mLN, **C)** Spleen, **D)** Blood, **E)** Lung. Median data is displayed. n=5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

The timing of the T_{FH} response was similar when examining T_{FH} numbers instead of proportions with T_{FH} numbers found to increase from week 1 within the mLN (**Figure 3.4A**) and within the lung tissue and spleen from week 3 (**Figure 3.4B, C**). The greatest number of T_{FH} were found in the mLN at all analysed time-points (**Figure 3.4A**), followed by the spleen (**Figure 3.4B**), with the lowest numbers found in the lung (**Figure 3.4C**). Overall, T_{FH} were identified at greatest numbers at the week 5 time-point in HDM exposed animals (**Figure 3.4A-C**).

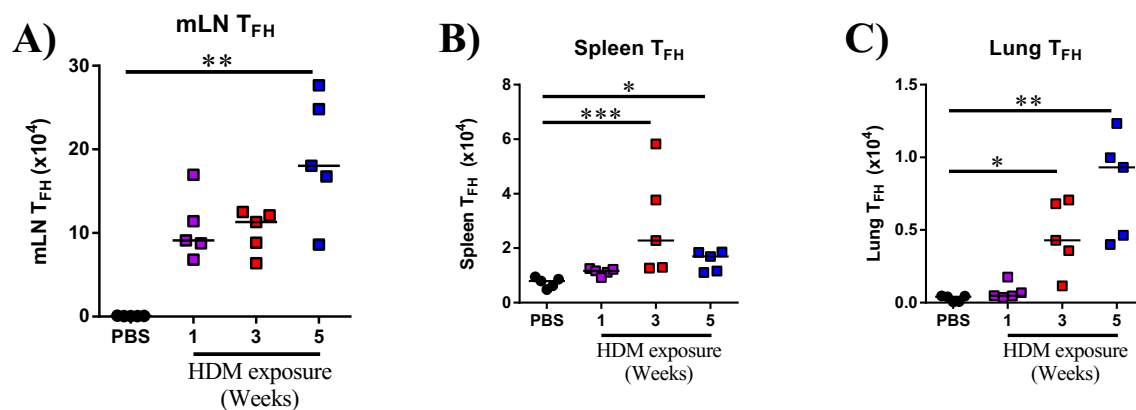


Figure 3.4: The majority of T_{FH} are found within the mLN of HDM treated animals.

Flow cytometry was used to determine the number of T_{FH} within cellular compartments following HDM exposure. T_{FH} were defined as CXCR5⁺PD1⁺Foxp3⁺CD4⁺. Numbers of T_{FH} were quantified in the **A**) mLN, **B**) Spleen, **C**) Lung. Median data is displayed. n=5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

3.3.3. T_{FH} are found in secondary lymphoid organs and locally in the lungs during ALT exposure

To determine if the type of allergen impacted the timing, magnitude and location of the T_{FH} response, animals were treated to the fungal allergen, ALT. T_{FH} appeared in the mLN after 1 week of ALT exposure (**Figure 3.5A, B**). T_{FH} proportions were maintained after 3 weeks of ALT exposure and were further increased after 5 weeks (**Figure 3.5A, B**). T_{FH} generation was

A) Pre-gated on CD4⁺CD3⁺Foxp3⁻CD44^{hi}CD62L⁻ lymphocytes

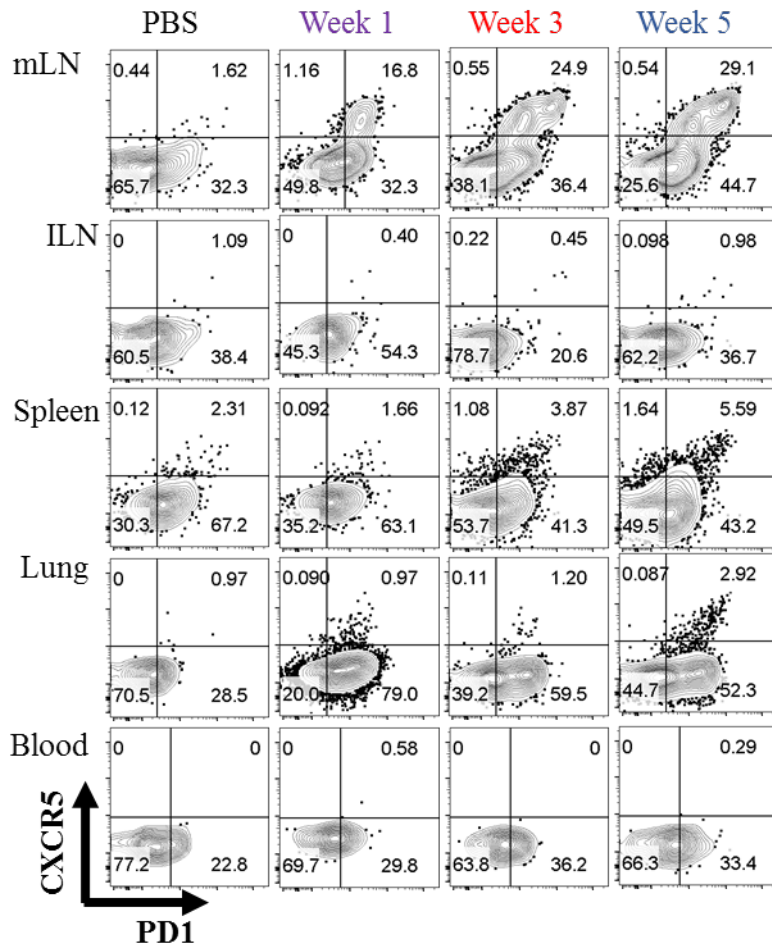
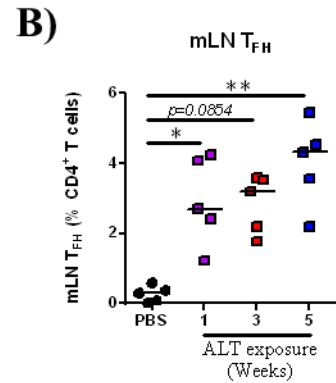
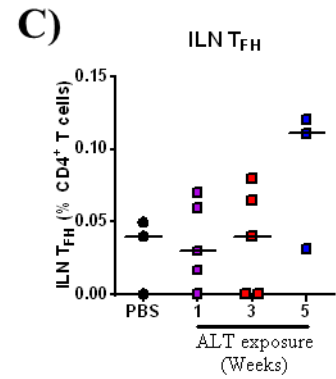
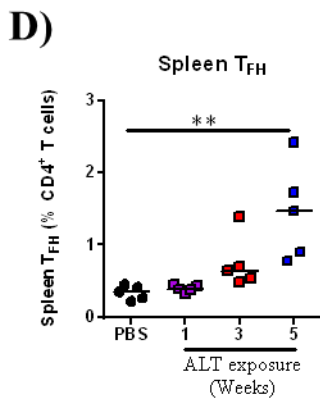
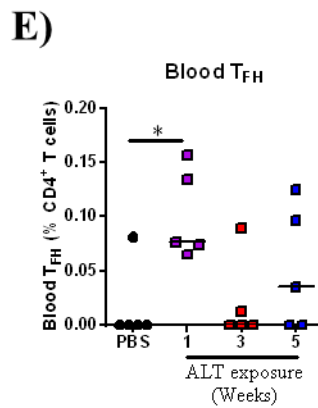
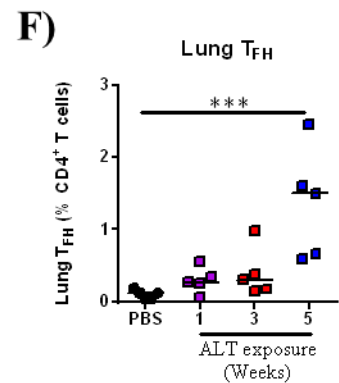


Figure 3.5: T_{FH} accumulate over time in the lungs and peripheral tissues with ALT exposure.

Flow cytometry was used to determine the frequency of T_{FH} within cellular compartments following HDM exposure. T_{FH} were defined as CXCR5⁺PD1⁺Foxp3⁻CD4⁺. **A)** Representative flow plots of T_{FH} in PBS and HDM treated animals, pre-gated on CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes. T_{FH} were quantified in the **B)** mLN, **C)** ILN, **D)** Spleen, **E)** Blood, **F)** Lung. Median data is displayed. n=3-5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

limited to lung draining lymph nodes as T_{FH} frequencies in the skin draining inguinal lymph nodes (ILN) following allergen exposure were comparable to PBS controls (**Figure 3.5A, C**). Splenic T_{FH} were elevated from 3 weeks of ALT exposure and were significantly increased compared to PBS treated mice from week 5 (**Figure 3.5A, D**). In HDM exposed animals, circulating T_{FH} were not readily identified in the blood at any analysed time-point (**Figure 3.5A**). Although, a very low frequency were significantly increased after 1 week of HDM exposure compared to PBS controls, however this represented very few cells falling into the CXCR5⁺PD1⁺ gate (**Figure 3.5A, E**). Reminiscent of HDM exposure T_{FH} were also found in the lung tissue of ALT treated animals but T_{FH} proportions were only significantly elevated at week 5 compared to PBS treated animals (**Figure 3.5A, F**).

T_{FH} numbers followed a similar pattern to proportions in ALT challenged animals (**Figure 3.6A-D**). The greatest number of T_{FH} were identified in the mLN, followed by the spleen and the lung tissue (**Figure 3.6A-C**). ILN T_{FH} numbers were very low (**Figure 3.6D**). The largest numbers of T_{FH} were identified after 5 weeks of ALT exposure (**Figure 3.6A-D**).

Collectively, the two aeroallergen exposure models show T_{FH} to exist in both the SLOs and locally within the lung tissue. T_{FH} are first present in the lung draining mLN and, with further allergen exposure, at other sites including the spleen and lungs.

3.3.4 T_{FR} are found during HDM driven chronic AAD and are not significantly elevated compared to PBS mice

T_{FR} are key regulators of humoral immunity and are known to directly suppress T_{FH} responses (Chung et al., 2011, Linterman et al., 2011). Whether T_{FR} are found during AAD allergic disease and what impact the length of allergen exposure has on the scale of the response was examined. T_{FR} were found in the mLN of HDM treated animals and appeared to increase with continued exposure. However, mLN T_{FR} were not significantly elevated compared to PBS controls at any time-point (**Figure 3.7A, B**). Splenic T_{FR} were increased from week 3 compared

to PBS treated mice, remaining elevated at week 5 (**Figure 3.7A, C**). Lung T_{FH} were found at very low frequency consistent with very few cells being observed within the representative gate, despite this they were significantly increased compared to PBS controls (**Figure 3.7A, D**). Few to no T_{FH} were found in the circulation of PBS or HDM exposed animals (**Figure 3.7A, E**).

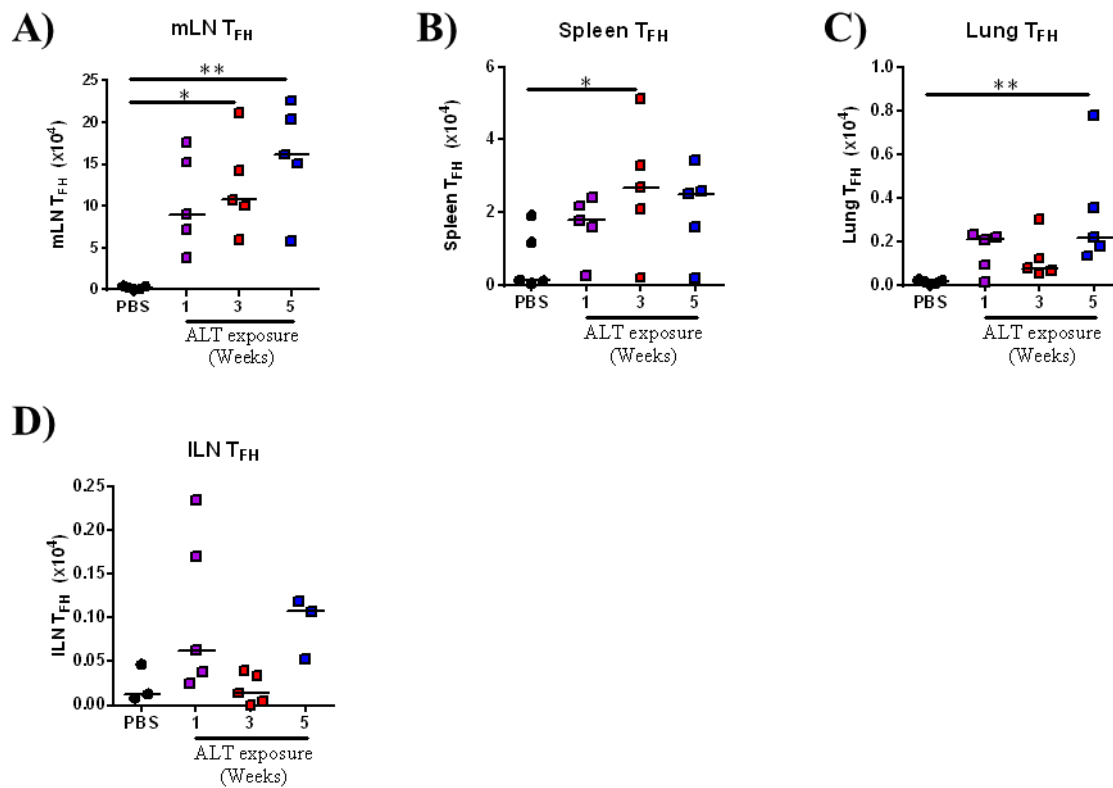


Figure 3.6: The majority of T_{FH} are found within the mLN of ALT treated animals.

Flow cytometry was used to determine the number of T_{FH} within cellular compartments following ALT exposure. T_{FH} were defined as $CXCR5^+PD1^+Foxp3^-CD4^+$. Numbers of T_{FH} were quantified in the **A**) mLN, **B**) Spleen, **C**) Lung, **D**) ILN. Median data is displayed. $n=3-5$ per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

A) Pre-gated on CD4⁺CD3⁺Foxp3⁺CD44^{hi}CD62L⁻ lymphocytes

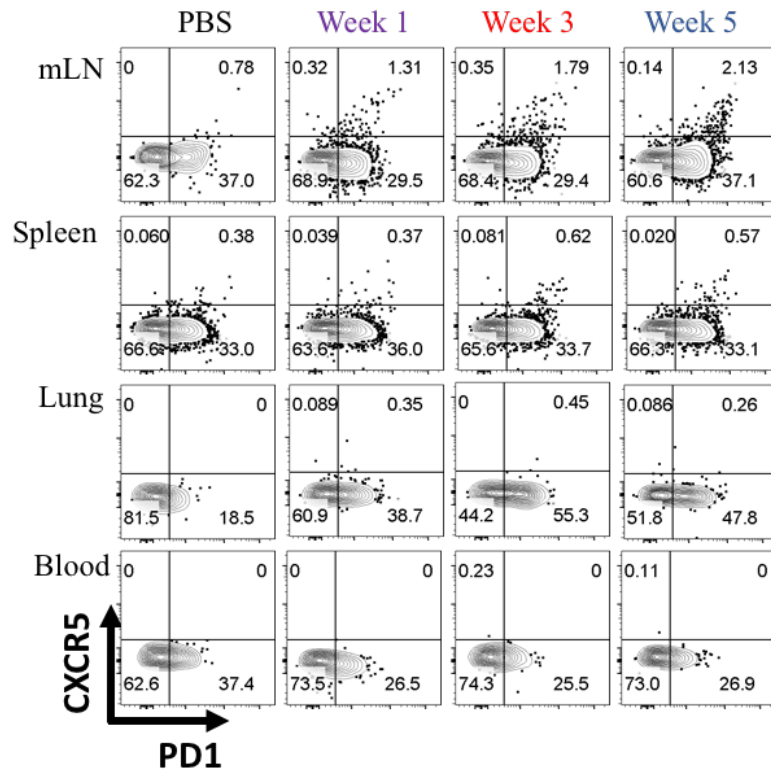
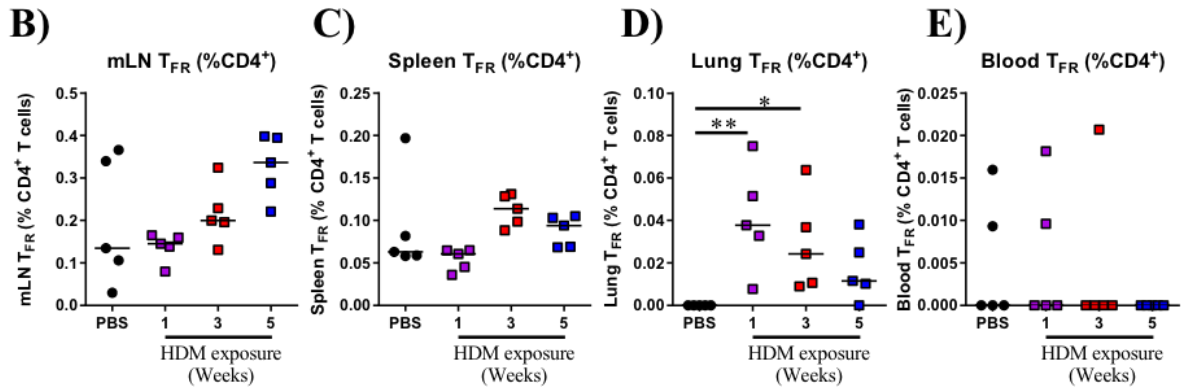


Figure 3.7: T_{FR} are found at multiple sites but are not further induced upon HDM exposure

Flow cytometry was used to determine the frequency of T_{FR} within cellular compartments following HDM exposure. T_{FR} were defined as CXCR5⁺PD1⁺Foxp3⁺CD4⁺ **A)** Representative flow plots of T_{FR} in PBS and HDM treated animals, pre-gated on CD4⁺CD3⁺Foxp3⁺ lymphocytes. T_{FR} were quantified in the **B)** mLN, **C)** Spleen, **D)** Lung, **E)** Blood. Median data is displayed. n=5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

Consistent with the T_{FH} data the greatest number of T_{FR} were identified after 5 weeks of HDM exposure within the mLN (**Figure 3.8A**), followed by the spleen (**Figure 3.8B**) and the lung tissue (**Figure 3.8C**). Examining the ratio of T_{FH} to T_{FR} in the mLN, spleen and lung, the T_{FH}/T_{FR} ratio was heavily skewed towards T_{FH} rather than T_{FR} in HDM treated animals (**Figure 3.8D-F**). In the lung tissue the T_{FH}/T_{FR} ratio was 2.5 and 4.2-fold higher than seen in the mLN at week 3 and 5, respectively (**Figure 3.8D, F**) and approximately 10-fold higher than the ratio in the spleen (**Figure 3.8E**). Thus, T_{FH} far outnumber T_{FR} during chronic AAD and the lung represents a particularly low site for T_{FR} .

3.3.5 T_{FR} are found during ALT driven chronic AAD

T_{FR} were found in the mLN of ALT treated animals (**Figure 3.9A, B**). T_{FR} proportions peaked after 1 week of exposure and appeared to decline with continued exposure (**Figure 3.9A, B**). T_{FH} were found at very low frequency in the ILN, peaking at 1 week of ALT exposure and decreasing at week 3 and 5 (**Figure 3.9A, C**). Splenic T_{FR} were increased in comparison of PBS treated controls after 5 weeks of ALT treatment (**Figure 3.9A, D**). Lung T_{FR} were found at low frequency and were found at the greatest frequency at week 1 and 3 (**Figure 3.9A, E**). Few to no circulating T_{FR} were found in the blood of PBS or ALT exposed animals (**Figure 3.9A, F**).

Consistent with HDM exposure, following ALT treatment T_{FR} were found at the highest frequency in the mLN (**Figure 3.10A**), followed by the spleen (**Figure 3.10B**) and lung tissue (**Figure 3.10C**). In general, T_{FR} numbers were found at highest levels at week 1 and declined with time (**Figure 3.10A-C**). Examining the ratio of T_{FH} to T_{FR} in the mLN, spleen and lung, the T_{FH}/T_{FR} ratio was strongly skewed towards T_{FH} rather than T_{FR} in ALT driven AAD **Figure 3.10D-F**). Thus, T_{FH} far outnumbered T_{FR} during chronic AAD.

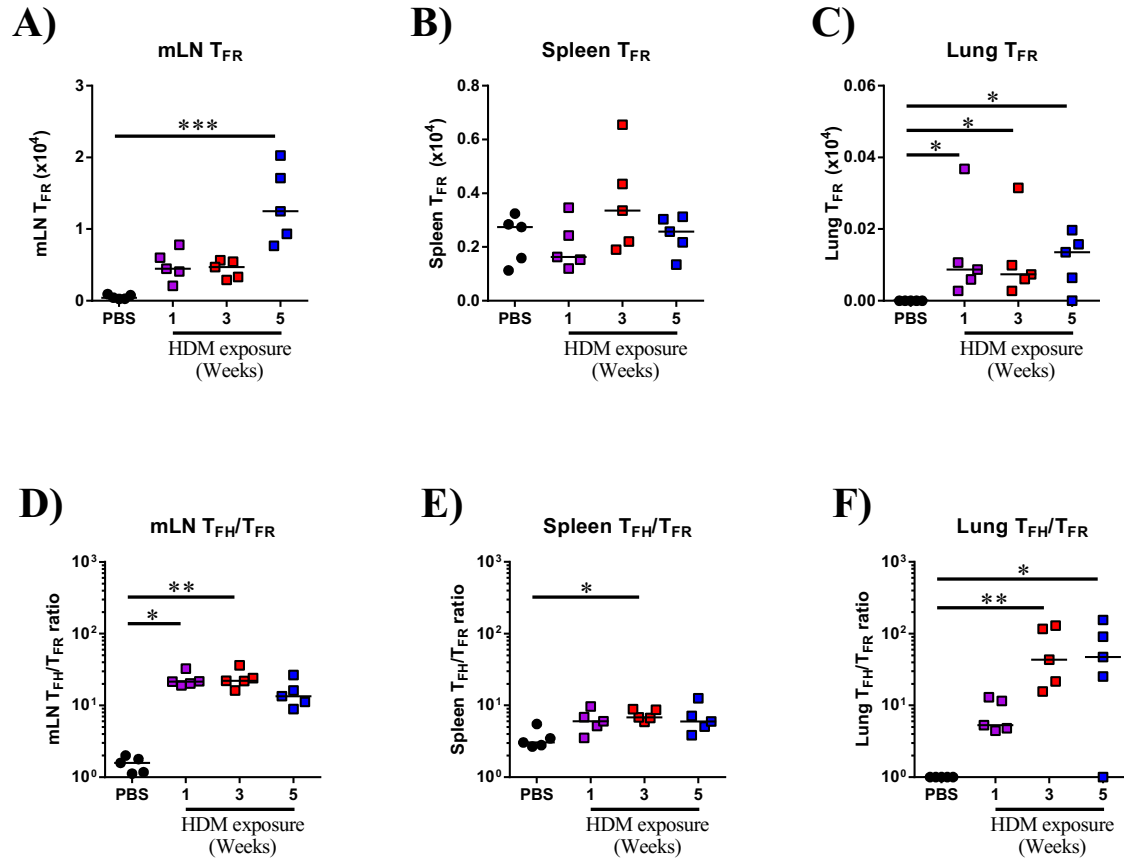


Figure 3.8: The majority of T_{FR} are found within the mLN of HDM treated animals. Flow cytometry was used to determine the total number of T_{FR} within cellular compartments following HDM exposure. T_{FR} were defined as CXCR5⁺PD1⁺Foxp3⁺CD4⁺. Numbers of T_{FR} were quantified in the **A)** mLN, **B)** Spleen, **C)** Lung. The ratio of T_{FH} to T_{FR} was determined during HDM driven disease in the **D)** mLN, **E)** Spleen, **F)** Lungs. Median data is displayed. n=5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

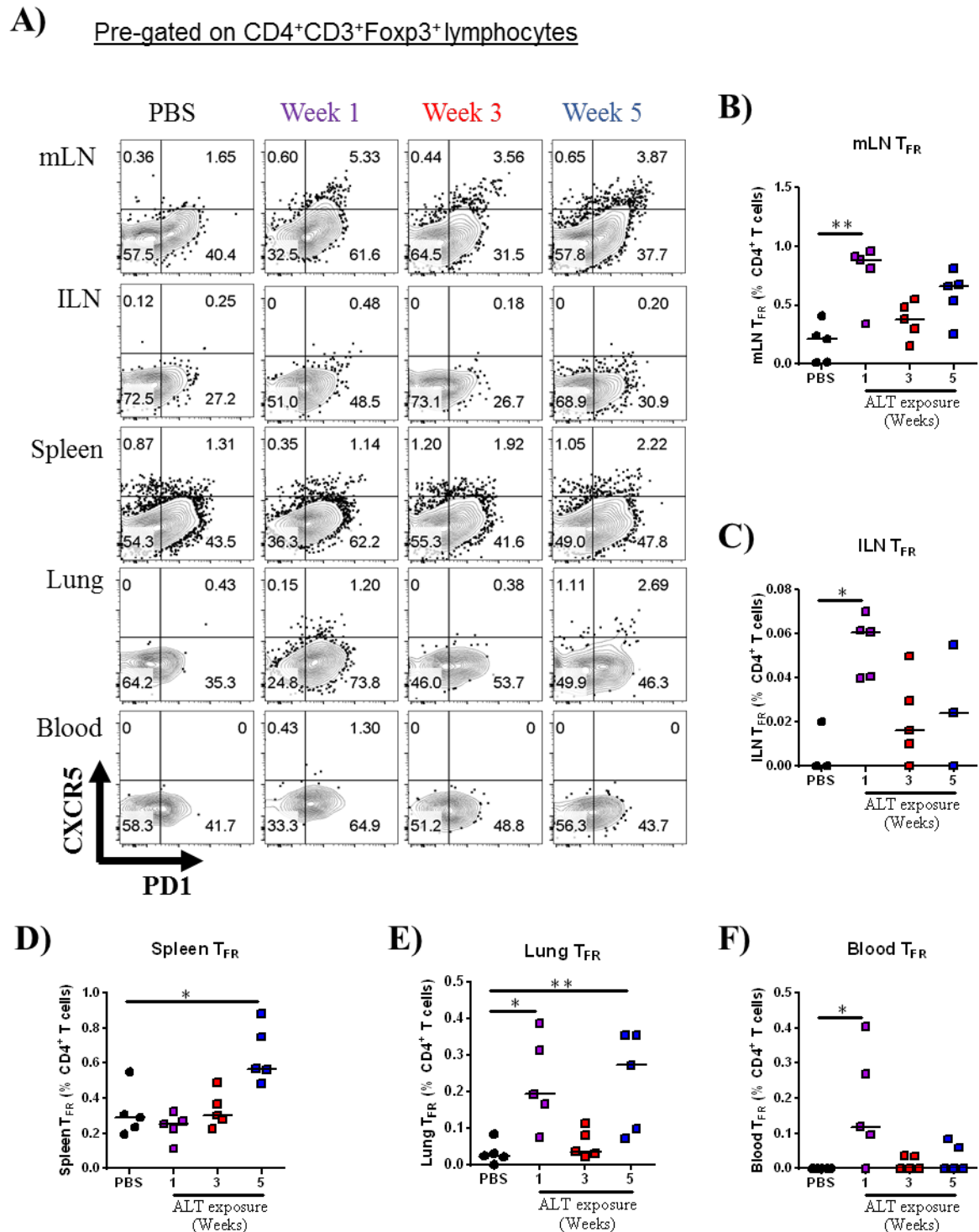


Figure 3.9: T_{FR} are found but not further induced upon ALT exposure.

Flow cytometry was used to determine the frequency of T_{FR} within cellular compartments following ALT exposure. T_{FR} were defined as CXCR5⁺PD1⁺Foxp3⁺CD4⁺ **A)** Representative flow plots of T_{FR} in PBS and ALT treated animals, pre-gated on CD4⁺CD3⁺Foxp3⁺ lymphocytes. T_{FR} were quantified in the **B)** mLN, **C)** ILN, **D)** Spleen, **E)** Lung, **F)** Blood. Median data is displayed. n=3-5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

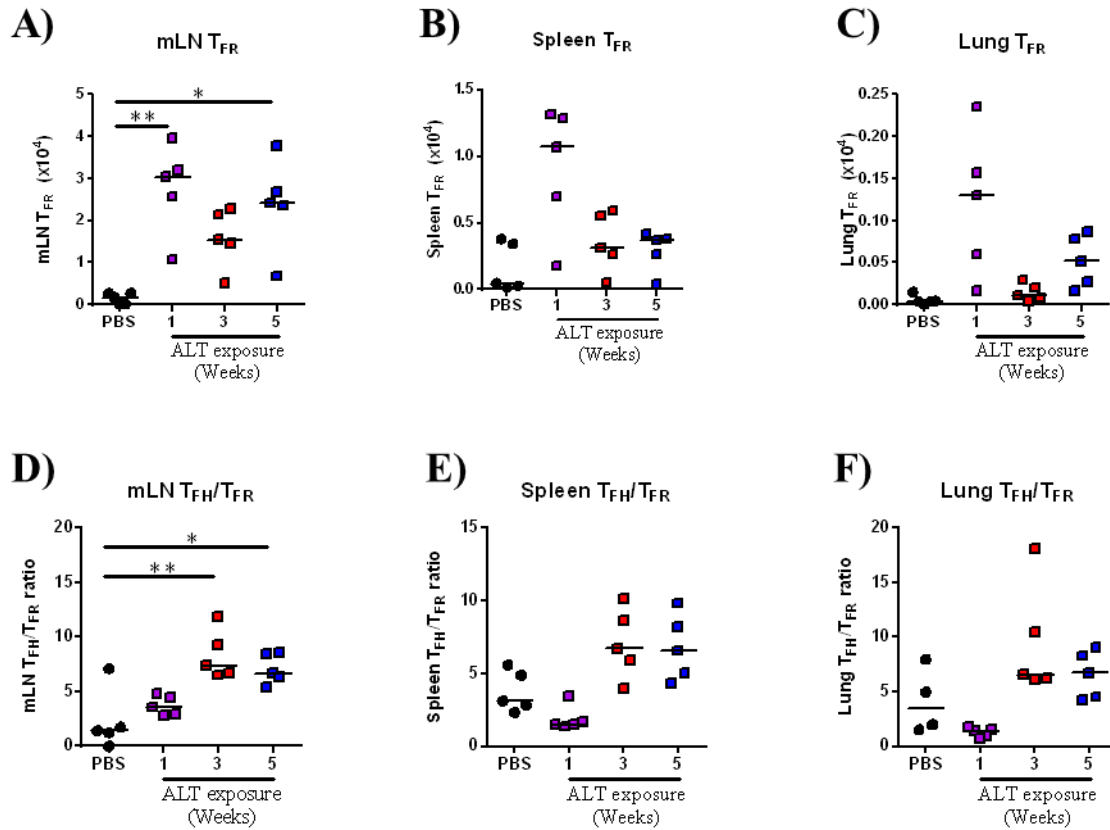


Figure 3.10: The majority of T_{FR} are found within the mLN of ALT treated animals. Flow cytometry was used to determine the number of T_{FR} within cellular compartments following ALT exposure. T_{FR} were defined as CXCR5⁺PD1⁺Foxp3⁺CD4⁺. Numbers of T_{FR} were quantified in the **A**) mLN, **B**) Spleen **C**) Lung. The ratio of T_{FH} to T_{FR} was determined during ALT driven disease in the **D**) mLN, **E**) Spleen, **F**) Lung. Median data is displayed. n=5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

3.3.6 GC B cells are found in secondary lymph nodes and locally within the lung tissue following allergen exposure

T_{FH} are critical regulators of humoral immunity, as they directly interact with activated B cells, driving their differentiation into GC B cells. Thus GC B cells are dependent on T_{FH} for their formation and survival (Vinuesa et al., 2016). GC B cells were not identified in the mLN, spleen or lung tissue of PBS control animals (**Figure 3.11A**) consistent with the lack of T_{FH} (**Figure 3.3A**). GC B cells were identified within the mLN after 1 week of HDM treatment, but were only significantly increased compared to PBS treated animals after 3 and 5 weeks of allergen

exposure (**Figure 3.11A, B**). Splenic GC B cells were increased after 3 weeks of allergen exposure (**Figure 3.11A, C**). Similarly, lung GC B cells were elevated after week 3 and were significantly increased after 5 weeks of HDM treatment (**Figure 3.11A, D**). Total numbers followed a similar pattern, and GC B cells found to be increased from week 3 at all analysed sites (**Figure 3.11E-G**) and further increased in the mLN and lung after 5 weeks of exposure (**Figure 3.11E, G**). The majority of GC B cells were found in the mLN, with lower numbers found in the lungs and spleen by week 5 (**Figure 3.11E-G**).

Following 3 weeks of ALT treatment, GC B cell frequencies were elevated in the mLN, remaining raised between weeks 3 and 5 (**Figure 3.12A, B**). Consistent with the lack of T_{FH} , ILN GC B cells were not increased relative to PBS controls at any analysed time-point (**Figure 3.12A, C**). Splenic GC B cells were slightly elevated at week 3 and further increased at week 5 (**Figure 3.12A, D**). Lung GC B cells were not significantly increased in comparison to PBS controls until 5 weeks of ALT treatment (**Figure 3.12A, E**). Consistent with the proportional data, total numbers of mLN GC B cells were significantly raised at week 3 and 5, compared to PBS controls (**Figure 3.13A**) and very low numbers of ILN GC B cells were found in all analysed groups (**Figure 3.13B**). Splenic GC B cells were found increased after 3 and 5 weeks of ALT treatment (**Figure 3.13C**) and significantly elevated in the lung tissue after 5 weeks (**Figure 3.13D**).

Total numbers and proportions of T_{FH} and GC B cells strongly positively correlated in the mLN, lung and splenic tissue of HDM (**Figure 3.14A-F**) and ALT (**Figure 3.14G-L**) exposed animals; thus confirming that T_{FH} and GC B cells induced during allergic airway disease have a highly dependent relationship.

Taken together the data from the two aeroallergen models show that GC B cells are predominantly found in the mLN following allergen exposure, but can also be identified within the lungs and spleen. Furthermore, these data show T_{FH} to precede GC B cell generation, with T_{FH} identified from week 1 of allergen exposure and GC B cells from 3 weeks onwards.

A) Pre-gated on CD19⁺B220⁺ lymphocytes

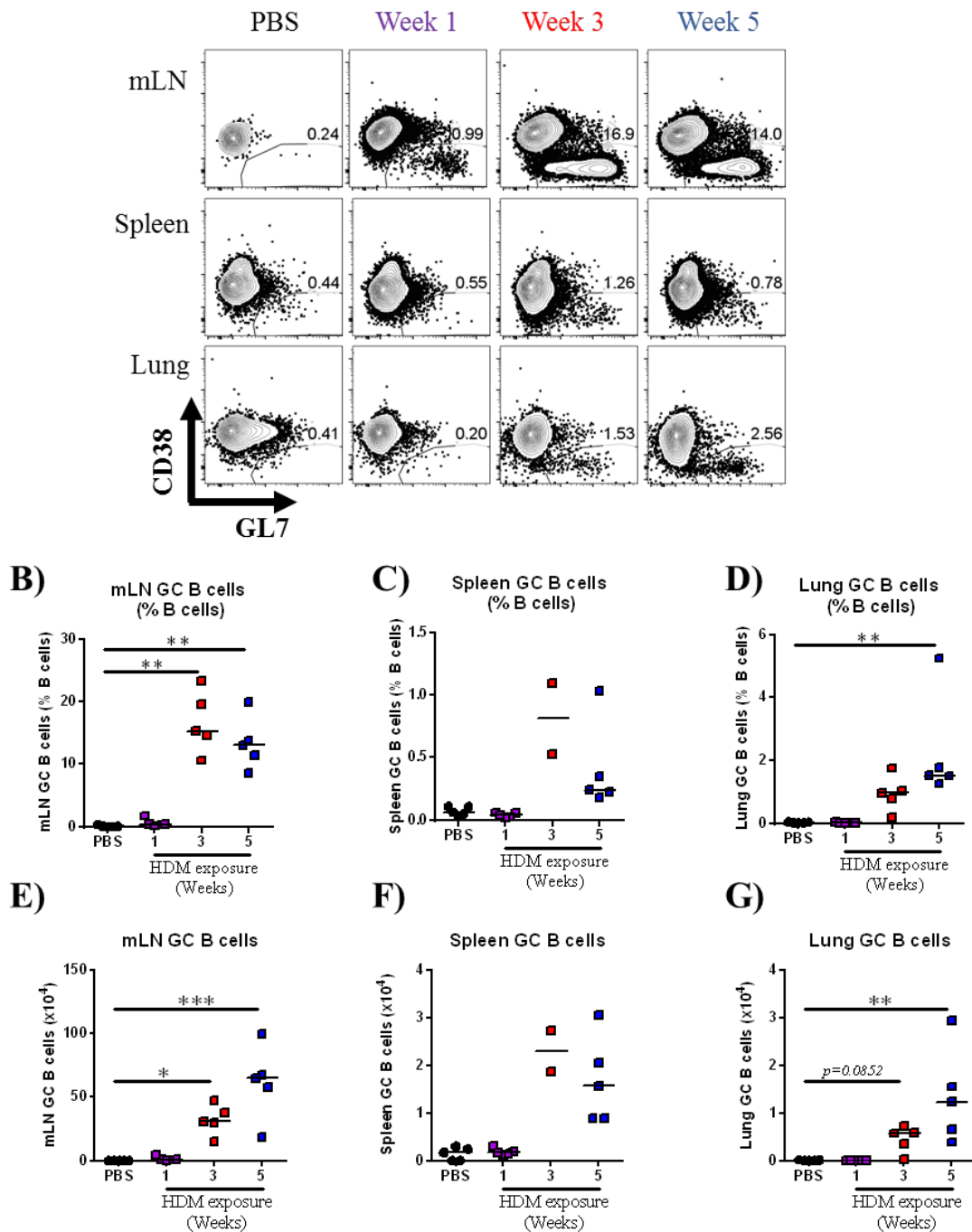


Figure 3.11: Germinal centres form in secondary lymphoid organs and the lung following HDM exposure.

Flow cytometry was used to determine the frequency of germinal centre (GC) B cells within cellular compartments following PBS and HDM exposure. GC B cell were defined as CD38⁺GL7⁺FAS⁺CD19⁺B220⁺ cells. **A)** Representative flow plots of GC B cells in PBS and HDM treated animals, pre-gated on CD19⁺B220⁺ lymphocytes, GC B cell frequencies were quantified in the **B)** mLN, **C)** Spleen, **D)** Lung. The numbers of GC B cells was quantified in the **E)** mLN, **F)** Spleen, **G)** Lung. Median data is displayed. n=2-5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

A) Pre-gated on CD19⁺B220⁺ lymphocytes

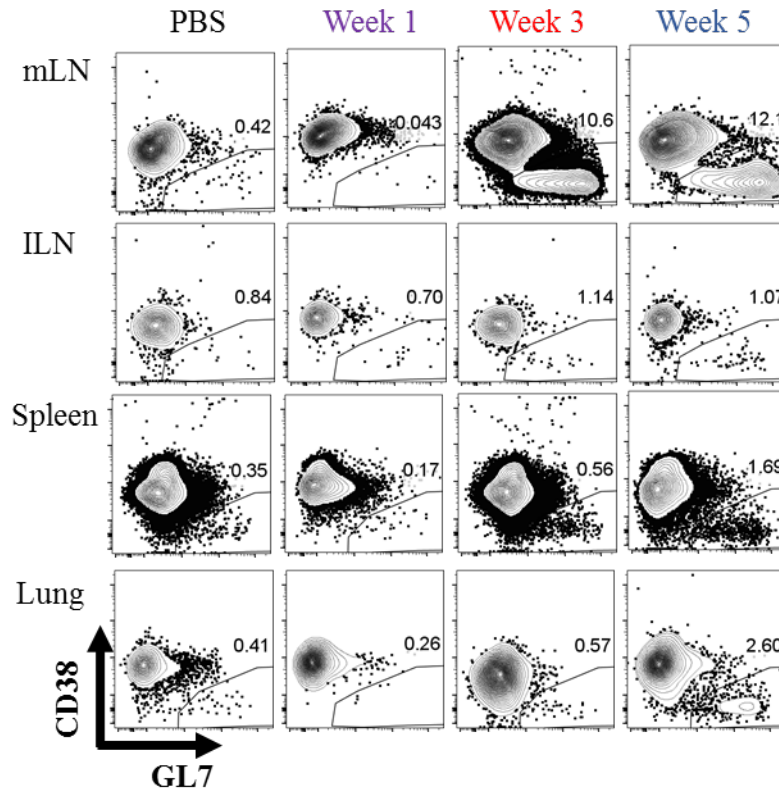
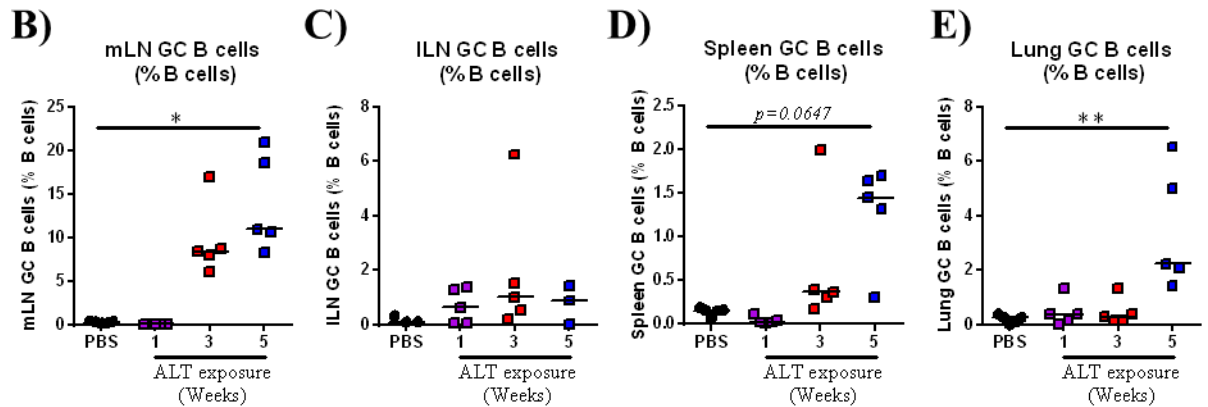


Figure 3.12: Germinal centres form in secondary lymphoid organs and the lung following ALT exposure.

Flow cytometry was used to determine the frequency of germinal centre (GC) B cells within cellular compartments following ALT exposure. GC B cells were defined as CD38⁺GL7⁺FAS⁺CD19⁺B220⁺ cells. **A)** Representative flow plots of GC B cells in PBS and ALT treated animals, pre-gated on CD19⁺B220⁺ lymphocytes. GC B cell frequencies were quantified in the **B)** mLN, **C)** ILN, **D)** Spleen, **E)** Lung. Median data is displayed. n=3-5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

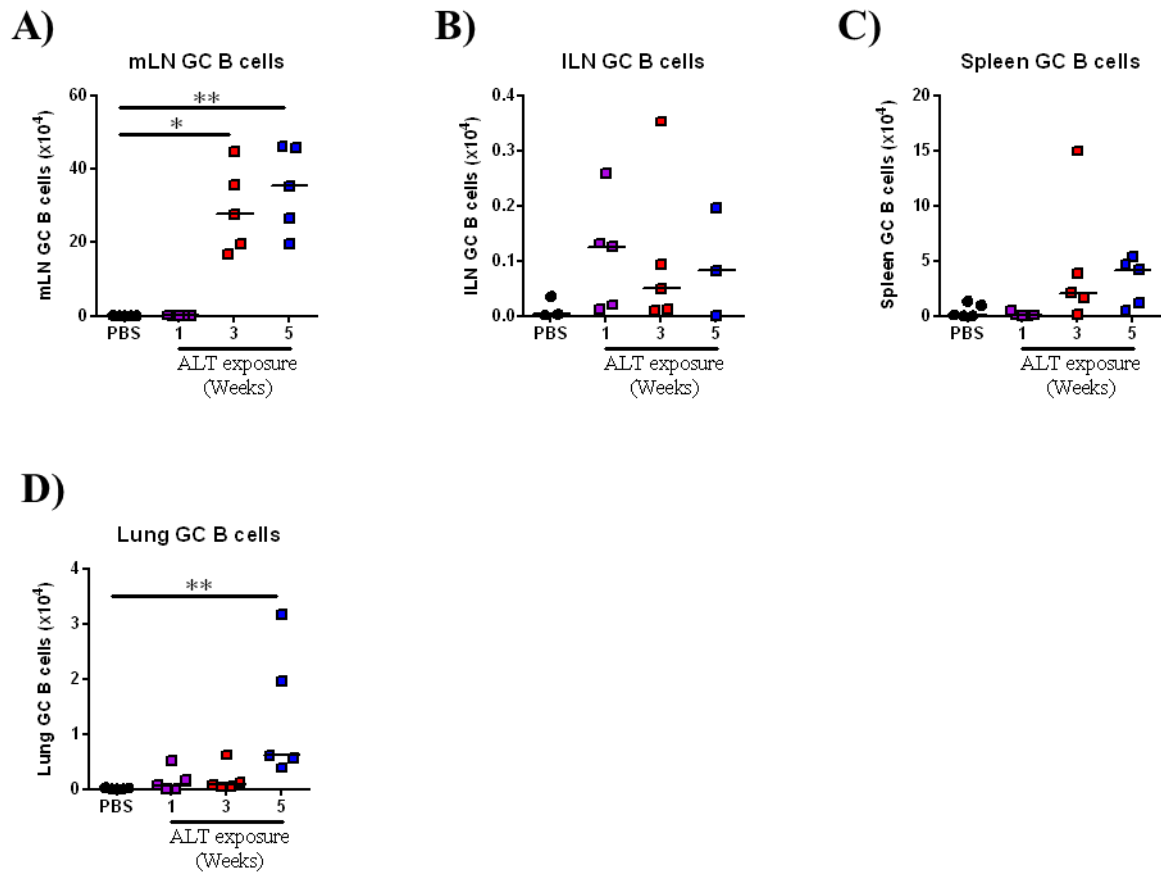


Figure 3.13: The majority of GC B cells are found within the mLN of ALT treated animals.

Flow cytometry was used to determine the number of GC B cells within cellular compartments following HDM exposure. GC B cells were defined as CD38⁺GL7⁺FAS⁺CD19⁺B220⁺ cells. Numbers of GC B cells were quantified in the **A)** mLN, **B)** ILN, **C)** Spleen, **D)** Lung. Median data is displayed. n=3-5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

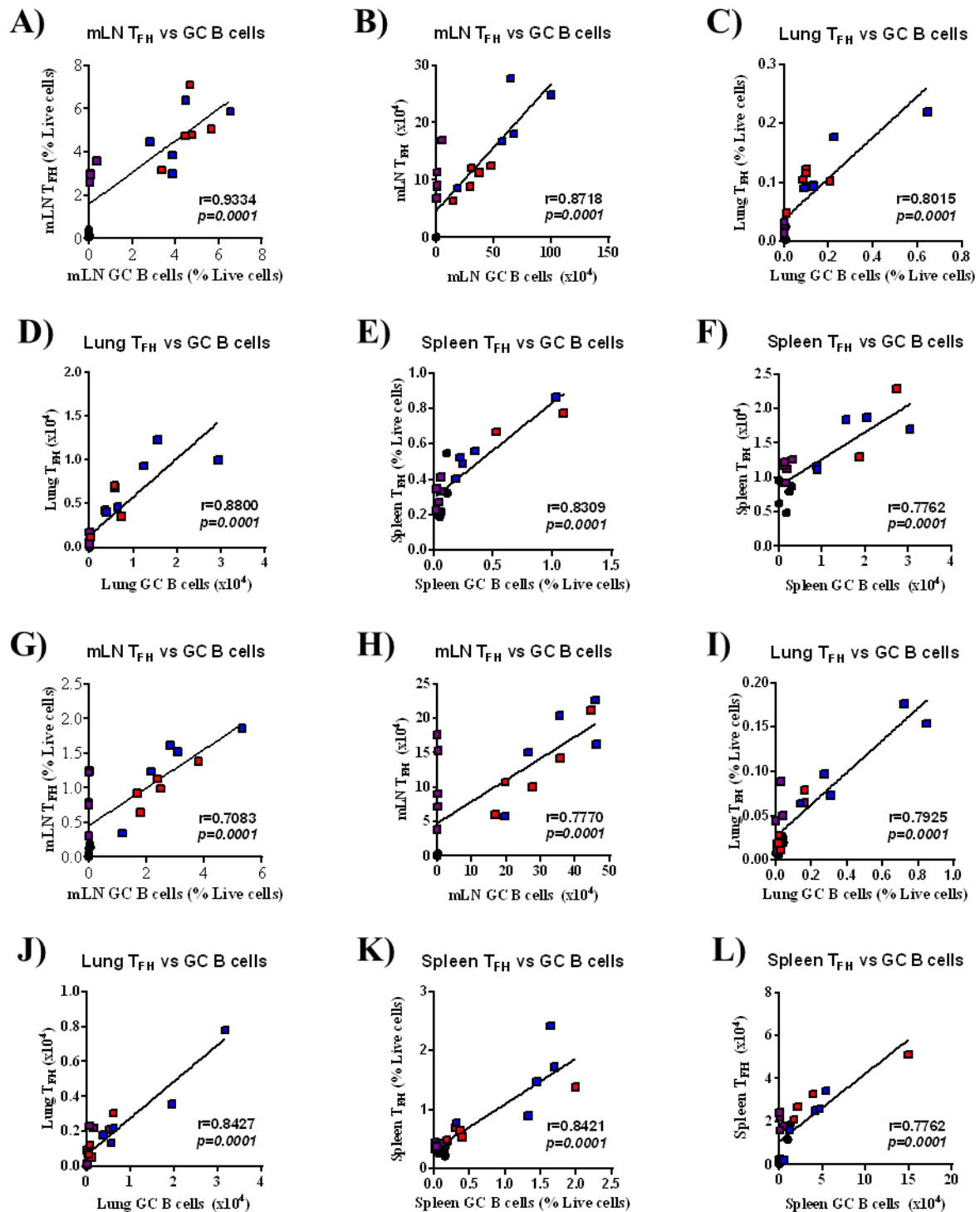


Figure 3.14: T_{FH} and GC B cells positively correlate

Flow cytometry was used to determine the frequency and number of T_{FH} and GC B cells following HDM or ALT exposure. T_{FH} and GC frequencies and total numbers were correlated. **A-F) HDM**, **A)** mLN frequencies, **B)** mLN Totals, **C)** Lung frequencies, **D)** Lung totals, **E)** Spleen frequencies, **F)** Spleen totals. **G-L) ALT**, **G)** mLN frequencies, **H)** mLN totals, **I)** Lung frequencies, **J)** Lung totals, **K)** Spleen frequencies, **L)** Spleen totals. Spearman rank correlation was calculated. $n=2-5$ per time-point. Representative data from 2 independent experiments.

3.3.7 IgG1⁺ GC B cells are the predominant GC B cells during chronic AAD

To determine the phenotype of GC B cells that were produced during chronic AAD, flow cytometry was used to identify IgG1⁺ and IgG2a⁺ GC B cells at week 3 and 5 (**Figure 3.15 and 3.16**). Following 3 and 5 weeks of HDM treatment, more than 80% of mLN GC B cells were IgG1⁺ (**Figure 3.15A, B**). However, in the lung tissue IgG1⁺ GC B cells were less dominant accounting for approximately 55.7% and 66.7% of GC B cells at week 3 and 5, respectively (**Figure 3.15A, C**). While, in the spleen 48.7% and 62.3% of the GC B cells were IgG1⁺ at week 3 and 5 (**Figure 3.15A, D**). IgG2a⁺ GC B cells accounted for less than 5% of GC B cells during HDM exposure (**Figure 3.15A**).

Similarly, in the mLN of ALT exposed animals IgG1⁺ GC B cells also represented more than 80% of the GC B cells at week 3 and 5 (**Figure 3.15E, F**). Following 3 weeks of ALT exposure, significant GC B cells were not identified in the lung tissue (**Figure 3.12E**), but those found approximately 20% were IgG1⁺, but at 5 weeks where a substantial GC B cell population was identified, approximately 58.3% of the GC B cells were IgG1⁺ (**Figure 3.15E, G**). After 3 weeks of ALT treatment, splenic IgG1⁺ GC B cells accounted for 45-49% of GC B cells (**Figure 3.15E, H**). IgG2a⁺ GC B cells defined less than 2% of GC B cells generated during ALT driven AAD (**Figure 3.15E**).

Collectively, the predominant GC B cell induced during chronic AAD was IgG1⁺. However, the dominance of IgG1 was altered depending on the tissue site, with IgG1⁺ GC B cells accounting for the majority of GC B cells in the mLN, but were less dominant in the lung tissue or spleen, where other isotype positive GC B cells may be more predominant.

3.3.8 Memory B cells were increased following allergen exposure

Memory B cells are vital in long term immunity, enabling long lasting protection, governed by the production of antigen specific antibodies (De Silva and Klein, 2015). Memory B cells were defined in this study as IgG1⁺CD38⁺GL7⁺IgD⁻IgM⁻ B cells. Memory B cells were increased after

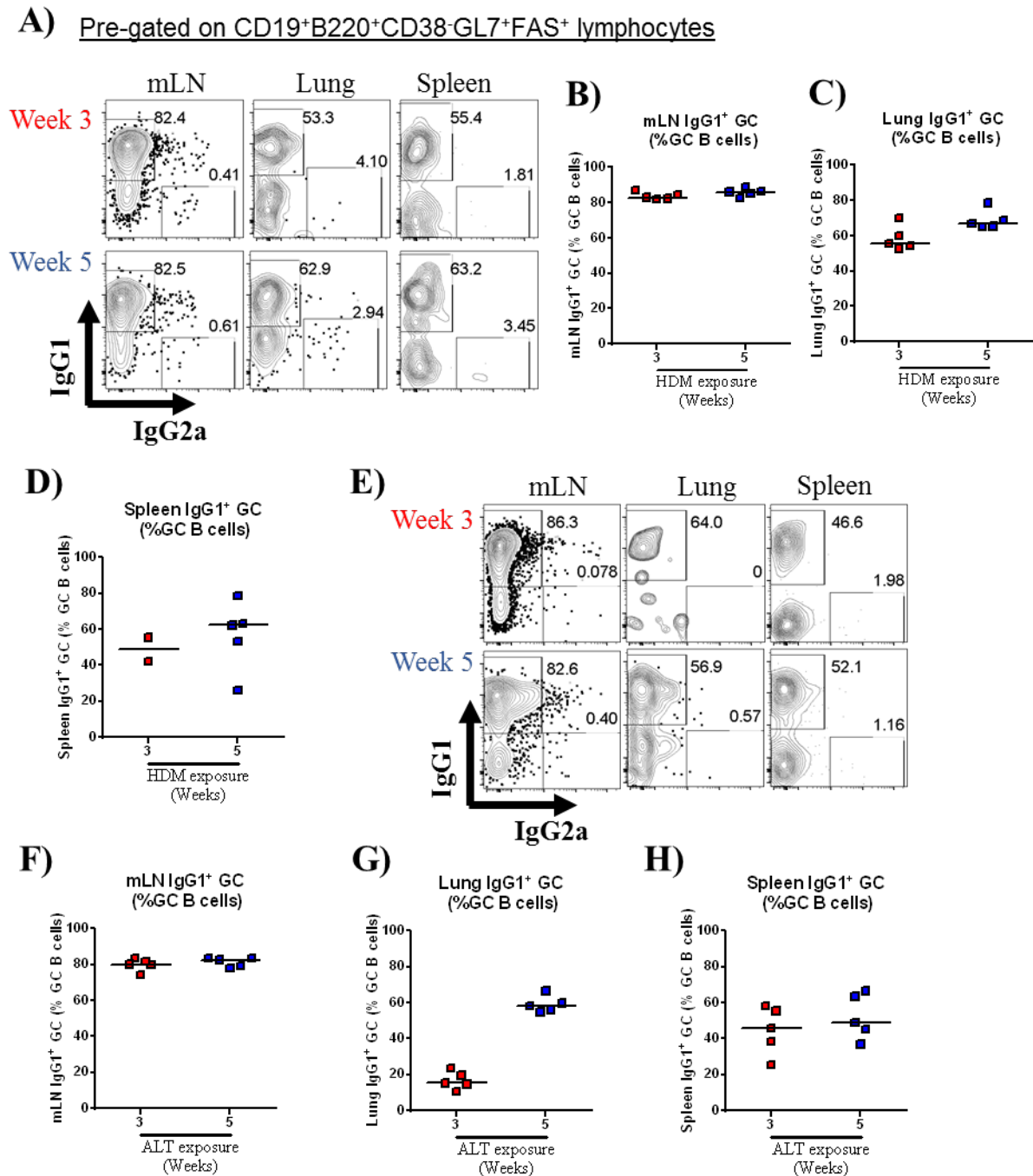


Figure 3.15: IgG1⁺ cells represent the predominant germinal centre B cell after allergen exposure

Flow cytometry was used to determine the type of germinal centre (GC) B cells in different tissue compartments following house dust mite (HDM) and alternaria alternata (ALT). **A-D)** HDM, **A)** Representative flow plots of IgG1⁺ and IgG2a1⁺ GC B cells, pre-gated on CD19⁺B2201⁺CD38⁻GL7⁺FAS⁺ lymphocytes. IgG1⁺ GC B cell frequencies were quantified at week 3 and 5. **B)** mLN, **C)** Lung, **D)** Spleen. Median data is displayed. n=2-5 per time-point. **E-H)** ALT, **E)** Representative flow plots. IgG1⁺ GC B cell frequencies were quantified at week 3 and 5. **F)** mLN, **G)** Lung, **H)** Spleen. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

week 3 by proportion (**Figure 3.16A**) and total number (**Figure 3.16B**) in the mLN, and further elevated after 5 weeks (**Figure 3.16A, B**). Splenic memory B cells were unaltered proportionally throughout the study, but total numbers were elevated after 5 weeks compared to PBS treated animals (**Figure 3.16C, D**). Lung memory B cells were increased at week 3 and further increased after 5 weeks (**Figure 3.16E, F**).

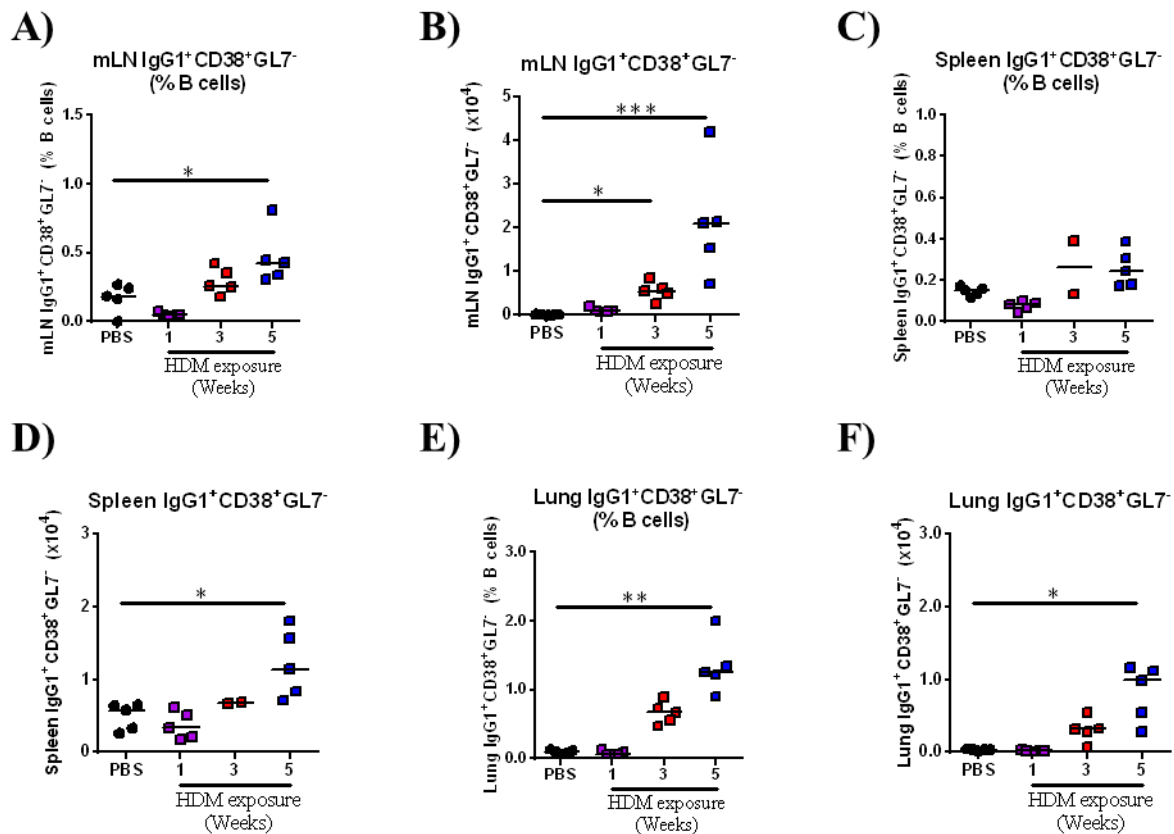


Figure 3.16: Memory B cell compartment is increased following HDM exposure

Flow cytometry was used to determine the frequency and total number of IgG1⁺ memory B cells within cellular compartments following HDM exposure. IgG1⁺ memory cells were defined as IgG1⁺CD38⁺GL7⁺IgD⁻IgM⁻CD19⁺B220⁺ lymphocytes and quantified. **A)** mLN frequency, **B)** mLN total, **C)** Spleen frequency, **D)** Spleen total, **E)** Lung frequency, **F)** Lung total. Median data is displayed. n=2-5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

Following ALT aeroallergen exposure mLN memory B cells were increased by proportion at week 5 (**Figure 3.17A**) and at total number at week 3 and 5 (**Figure 3.17B**) compared to PBS treated animals. Splenic memory B cells increased with continued ALT exposure (**Figure 3.17C, D**). While, lung memory B cells were significantly elevated after 5 weeks of ALT exposure (**Figure 3.17E, F**).

Overall, memory B cells were found at multiple sites during chronic AAD and increased with prolonged allergen exposure.

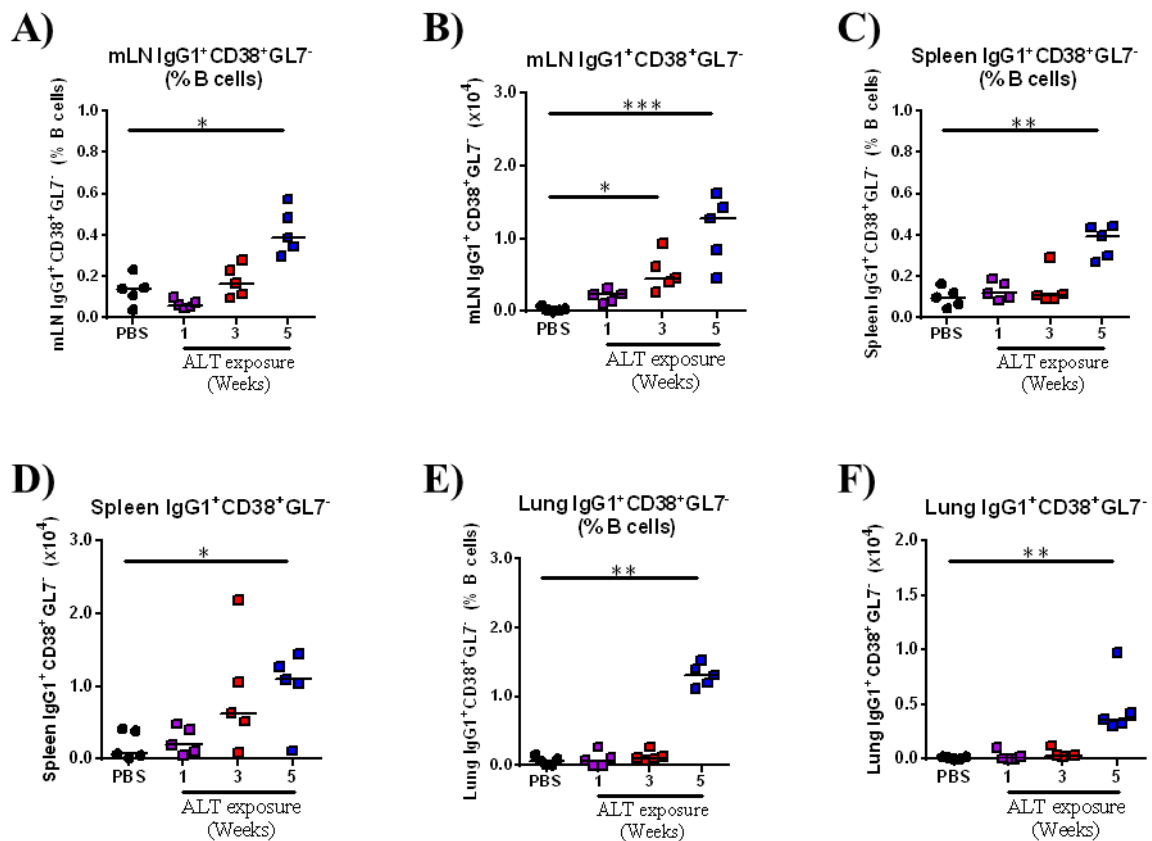


Figure 3.17: Memory B cell compartment is increased following ALT exposure

Flow cytometry was used to determine the frequency and total of IgG1⁺ memory B cells within cellular compartments following ALT exposure. IgG1⁺ memory cells were defined as IgG1⁺CD38⁺GL7⁺IgD⁺IgM⁺CD19⁺B220⁺ lymphocytes and quantified. **A)** mLN frequency, **B)** mLN total, **C)** Spleen frequency, **D)** Spleen total, **E)** Lung frequency, **F)** Lung total. Median data is displayed. n=5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

3.3.9. Plasma cells are induced after allergen driven allergic disease

The production of allergen specific antibodies is one of the hallmark symptoms of AAD (Lambrecht and Hammad, 2012) and plasma cells are the major antibody producing cell of the immune system, therefore the kinetics of their response was also examined. The frequency of plasma cells was increased in the mLN after 3 weeks and significantly increased compared to PBS controls after 5 weeks (**Figure 3.18A, B**). By total number mLN plasma cells were increased at both week 3 and 5 (**Figure 3.18C**). Lung plasma cells were increased by frequency and total number after 3 weeks and significantly increased compared to PBS controls after 5 weeks (**Figure 3.18A, D, E**). Few plasma cells were found in the spleen and were reduced by proportion after 1 week and did not change significantly with further allergen exposure (**Figure 3.18F**). Overall, the number of splenic plasma cells did not change regardless of continued HDM exposure (**Figure 3.18G**).

During ALT driven AAD plasma cells were induced in the mLN and lungs (**Figure 3.19A-E**). mLN plasma cells were induced significantly after 5 weeks compared to PBS treated control mice (**Figure 3.19A-C**). Lung plasma cells were significantly increased after 1 week and remained constant over the rest of the time-course (**Figure 3.19A, D, E**). Splenic plasma cells were not increased by neither proportion nor total number over the time-course (**Figure 3.19A, F, G**). Thus, plasma cells were induced during chronic AAD and the timing aligned with the presence of GC B cells and the emergence of allergic specific antibody responses.

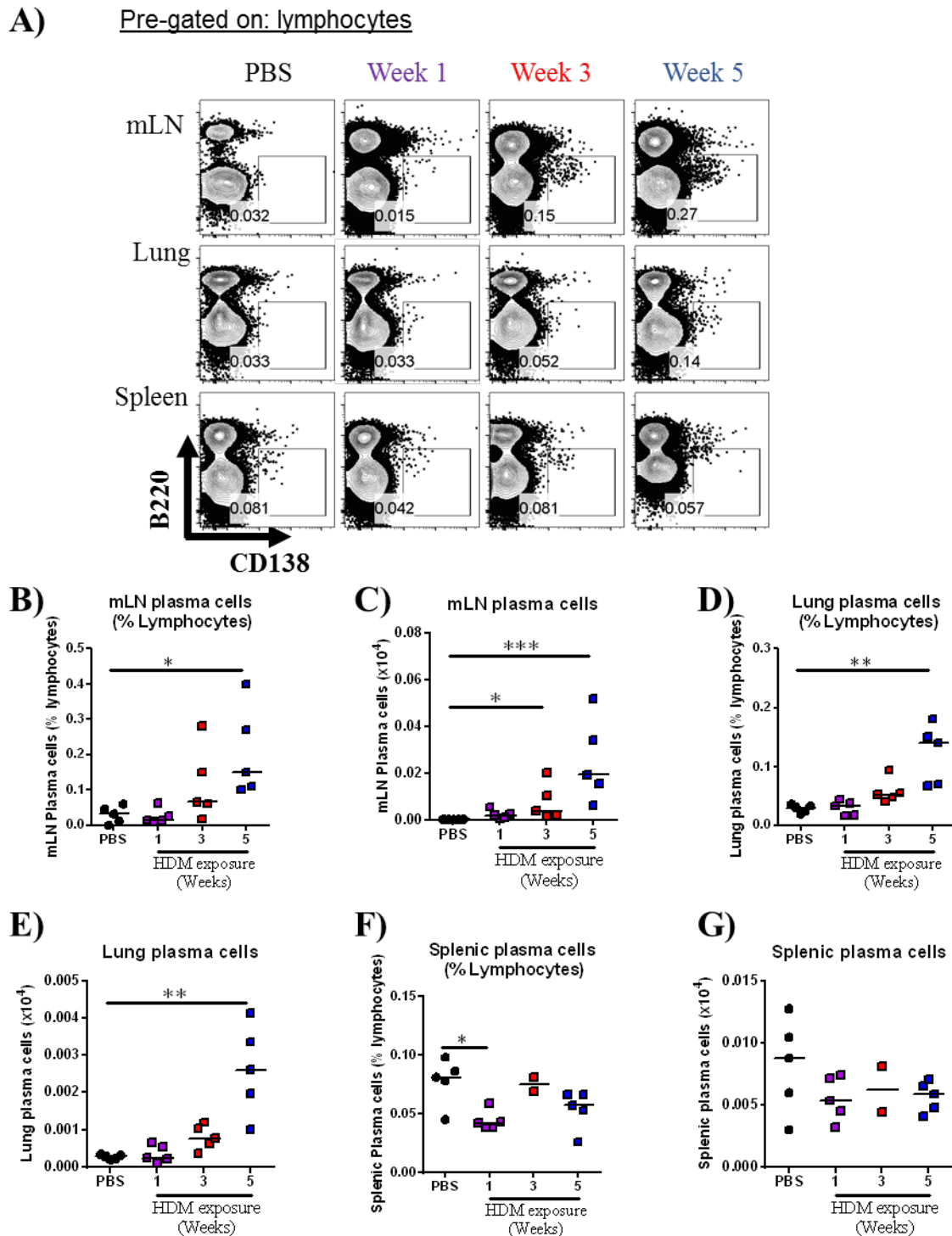


Figure 3.18: Plasma cells are generated found in secondary lymphoid organs and the lung following HDM exposure.

Flow cytometry was used to determine the frequency and number of plasma cells within cellular compartments following HDM exposure. Plasma cells were defined as B220^{lo}CD138⁺ lymphocytes. **A)** Representative flow plots of plasma cells in PBS and HDM treated animals, pre-gated on lymphocytes, plasma cell frequency and number were quantified **B)** mLN frequency, **C)** mLN total, **D)** Lung frequency, **E)** Lung total, **F)** Splenic frequency, **G)** Splenic total. Median data is displayed. n=2-5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

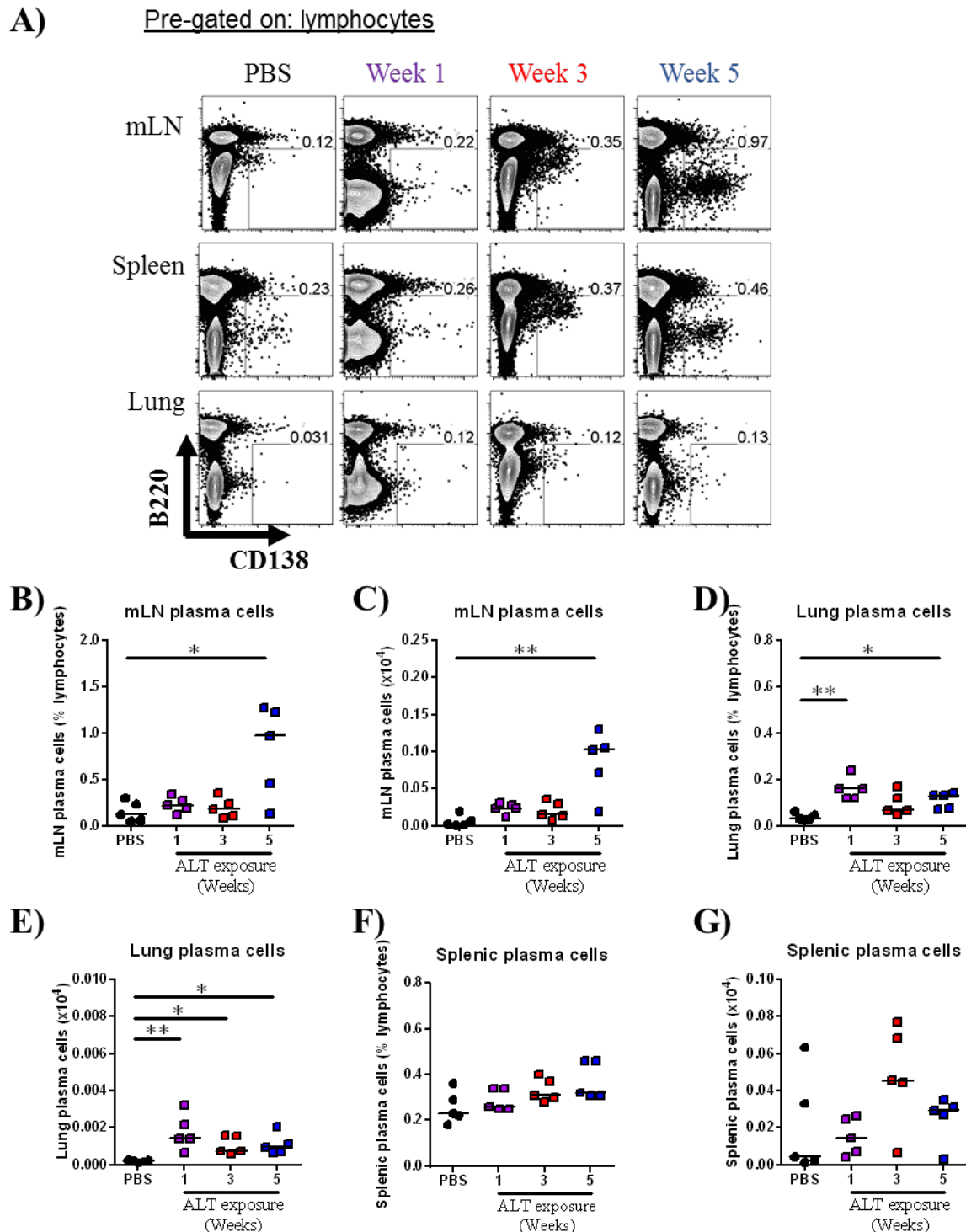


Figure 3.19: Plasma cells are generated found in secondary lymphoid organs and the lung following ALT exposure.

Flow cytometry was used to determine the frequency and number of plasma cells within cellular compartments following ALT exposure. Plasma cells were defined as B220^{lo}CD138⁺ lymphocytes. **A)** Representative flow plots of plasma cells in PBS and HDM treated animals, pre-gated on lymphocytes, plasma cell frequency and number were quantified **B)** mLN frequency, **C)** mLN total, **D)** Lung frequency, **E)** Lung total, **F)** Splenic frequency, **G)** Splenic total. Median data is displayed. n=5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

3.3.10 Allergen specific and total antibody increases in the serum with prolonged allergen exposure

Allergen specific antibody, in particular IgE, is a hallmark feature of AAD (Lambrecht and Hammad, 2015). HDM specific and total IgE were only detected in the serum of allergen exposed animals after 3 weeks (**Figure 3.20A, B**). Continued allergen exposure for an additional 2 weeks did not alter the level of serum allergen specific or total IgE (**Figure 3.20A, B**). Similarly, allergen specific and total IgG1 was only observed in the serum of allergen treated mice after 3 weeks of exposure (**Figure 3.20C, D**) and was increased at week 5 relative to week 3 (**Figure 3.20C, D**).

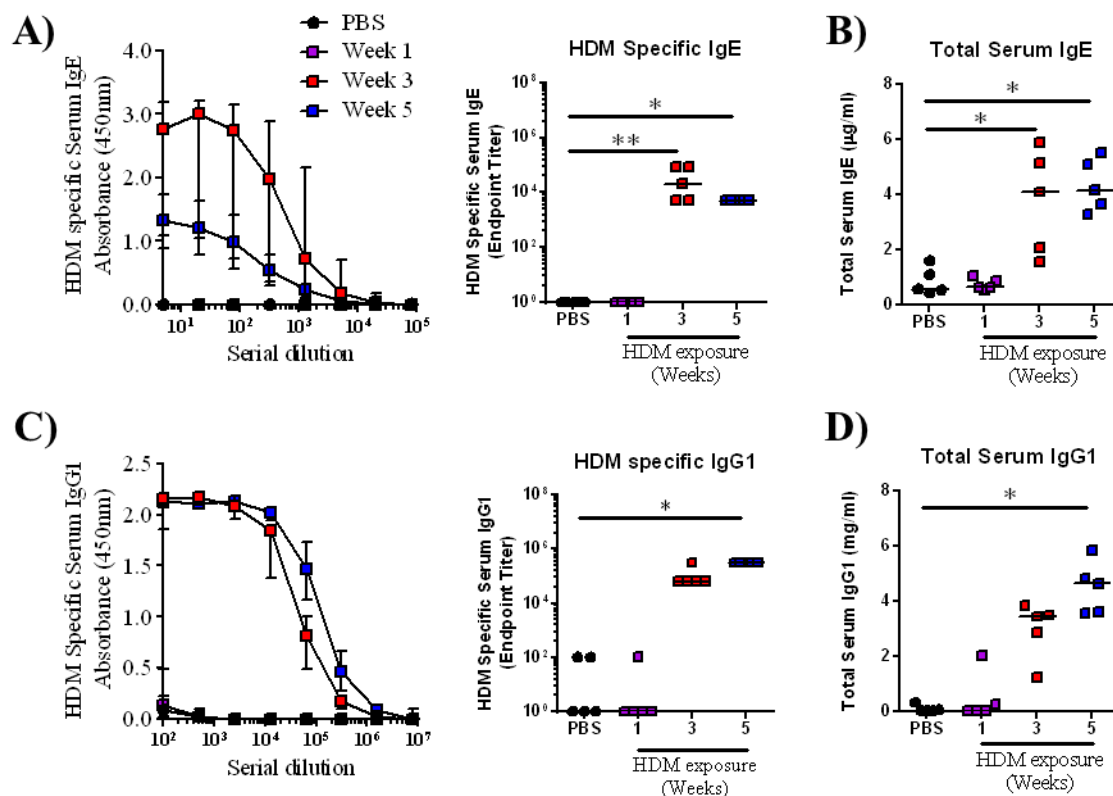


Figure 3.20: Serum HDM specific antibody is detectable after 3 weeks.

HDM specific and total antibody levels in the serum was assessed by ELISA. **A)** Serum was titrated, allergen specific IgE was measured and endpoint titers calculated, **B)** Total IgE, **C)** Serum was titrated, allergen specific IgG1 was measured and endpoint titers calculated, **D)** Total IgG1. Median data is shown and allergen-specific curves display median $\pm$ interquartile range. $n=5$ per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

ALT specific and total IgE and IgG1 was detected from week 3 and were further increased after 5 weeks of ALT exposure (**Figure 3.21A-D**). Allergen specific antibodies were undetectable in the serum of PBS exposed animals (**Figure 3.20, 21**). These data show the ability of chronic allergen exposure to generate systemic allergen specific antibody which lines up with the timing of GC B cell generation.

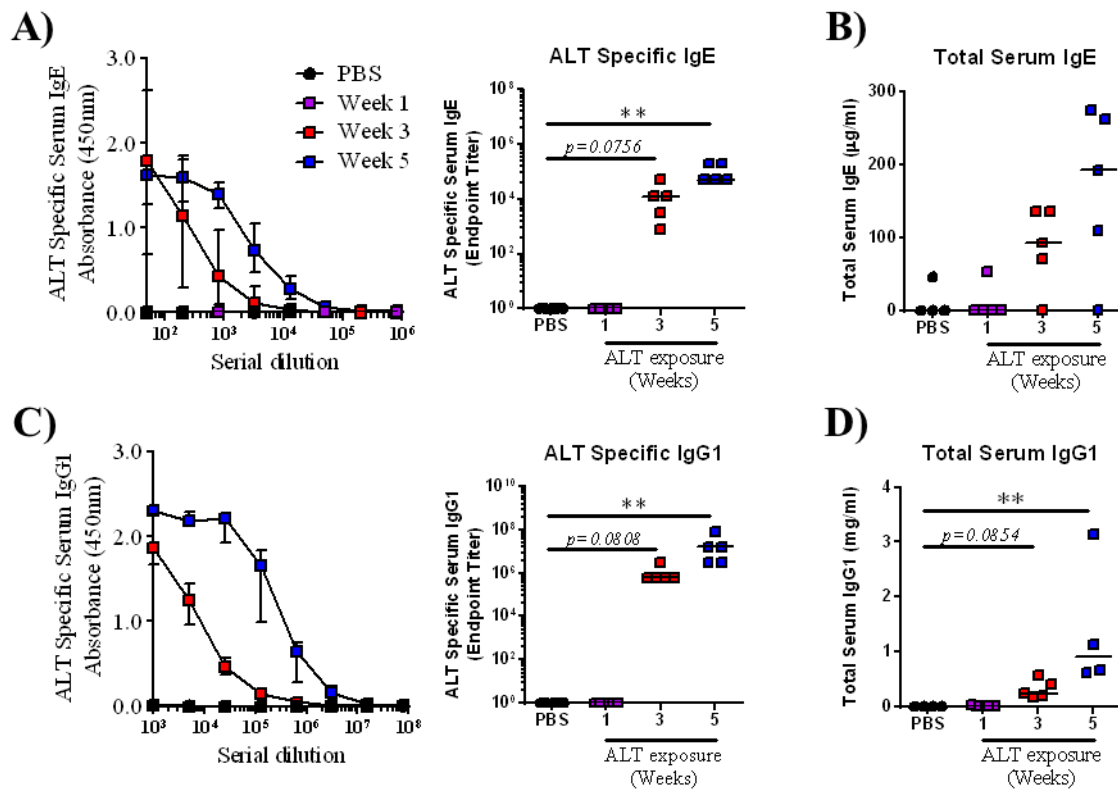


Figure 3.21: Serum ALT specific antibody is detectable after 3 weeks.

ALT specific and total antibody was assessed in the serum by ELISA. **A)** Serum was titrated, allergen specific IgE was measured and endpoint titers calculated, **B)** Total IgE, **C)** Serum was titrated, allergen specific IgG1 was measured and endpoint titers calculated, **D)** Total IgG1. Median data is shown and allergen-specific curves display median $\pm$ interquartile range. $n=4-5$ per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

3.3.11 Allergen specific and total antibody is detected within the lung tissue

HDM specific and total IgE was detected in the lung tissue after 3 weeks of allergen exposure and was not altered with further allergen exposure (**Figure 3.22A, B**). HDM specific and total IgG1 was identified after 3 weeks and remained stable at 5 weeks (**Figure 3.22C, D**). HDM specific and total IgA was detected in the lung tissue after 3 week (**Figure 3.22E, F**). Specific IgA was increased with further exposure (**Figure 3.22E**), while total IgA was unchanged (**Figure 3.22F**).

Similarly, when mice were treated with ALT, specific and total IgE was increased after 3 weeks of allergen exposure and appeared to trend upwards when mice were treated for 5 weeks (**Figure 3.23A, B**). ALT specific and total IgG1 and IgA were detected after 3 weeks and was further elevated after 5 weeks of ALT exposure (**Figure 3.23C-F**). Analysing levels of IgA within the BAL showed that total IgA and allergen specific IgA in both models were elevated after 3 weeks of allergen exposure (**Figure 3.24A-D**). Continued allergen exposure further elevated allergen specific IgA within the airways (**Figure 3.24A, C**). Interestingly, total BAL IgA was not altered by further aeroallergen exposure in either AAD model (**Figure 3.24B, D**). Taken together, antibody can be detected in the lung and airways after 3 weeks of allergen exposure, fitting with the presence of GC B cells at this time-point.

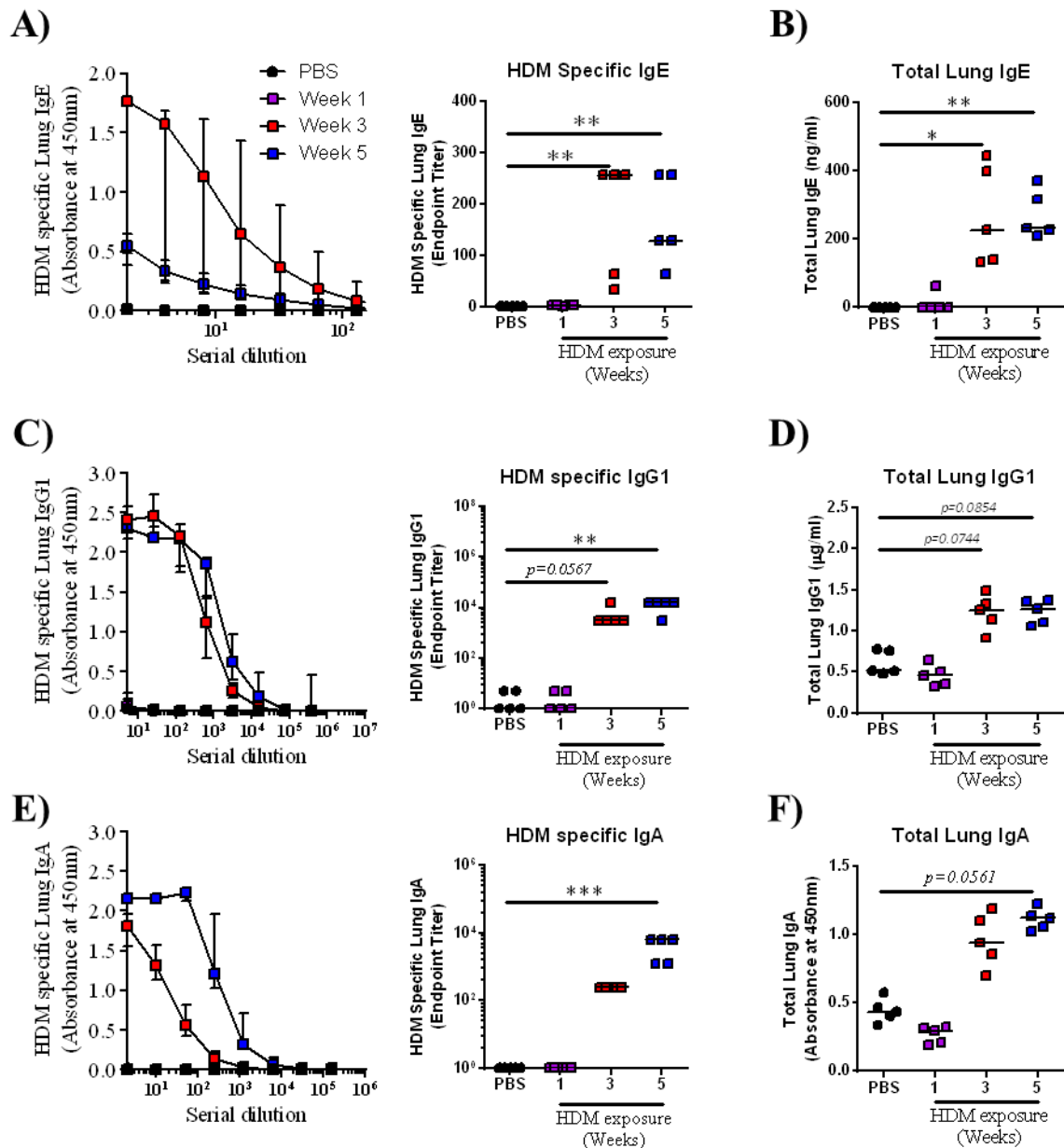


Figure 3.22: HDM specific antibody is detectable after 3 weeks in the lung tissue. HDM specific and total antibody was assessed in the lung tissue by ELISA. For HDM specific ELISAs, lung homogenate was titrated, allergen specific antibody was measured and endpoint titers calculated. **A)** HDM specific IgE **B)** Total IgE, **C).** HDM specific IgG1, **D)** Total IgG1. **E)** HDM specific IgA, **F)** Total IgA. Median data is shown and allergen-specific curves display median $\pm$ interquartile range. n=5 per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

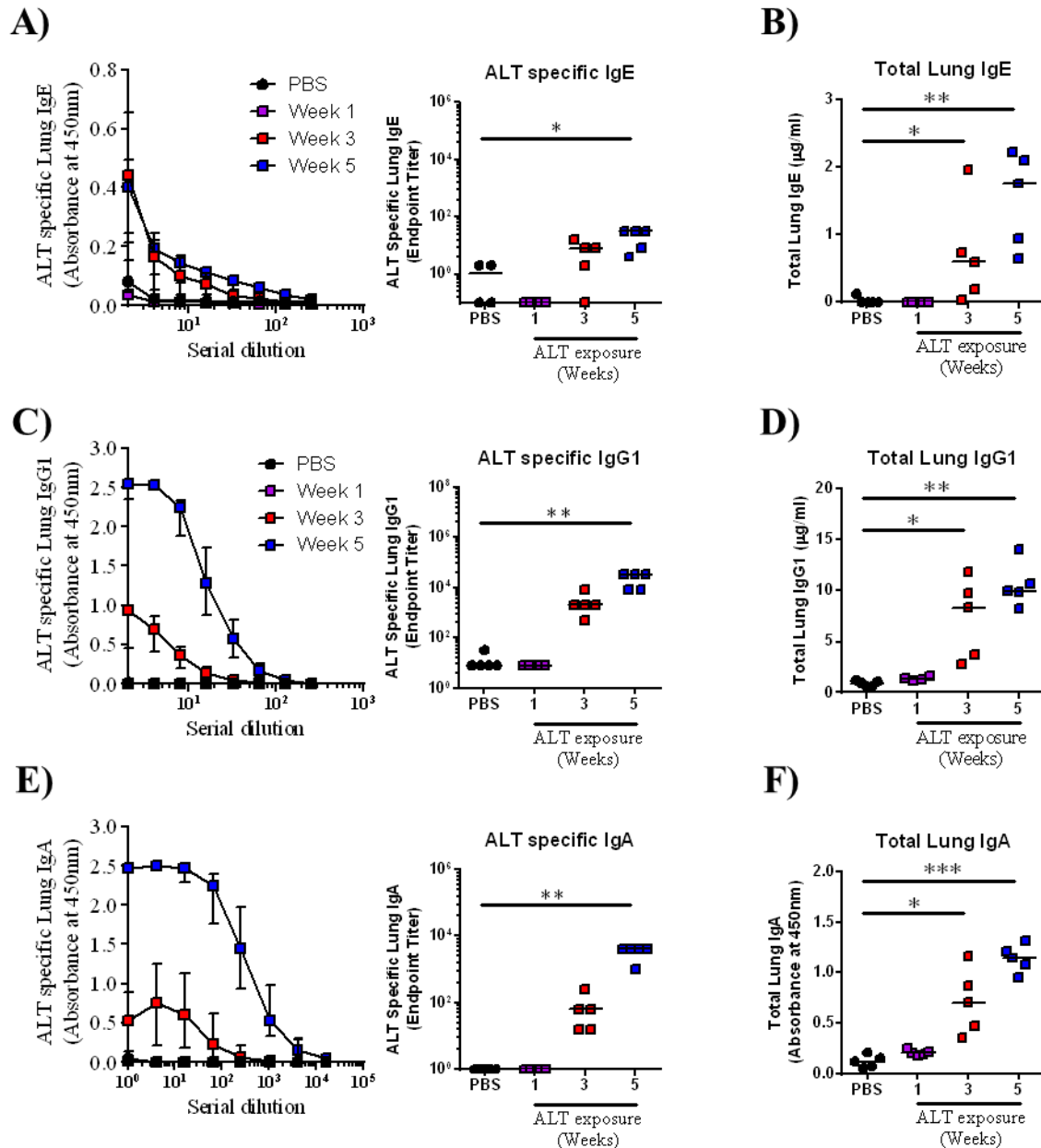
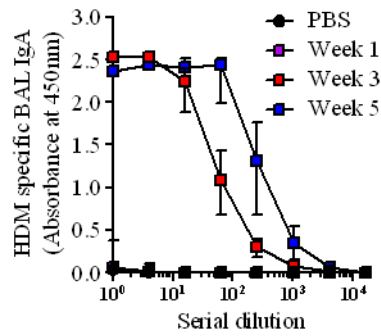
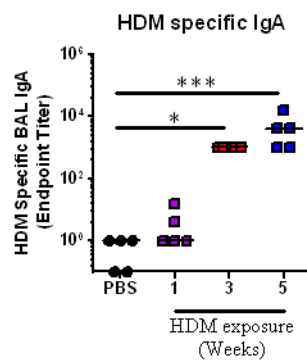


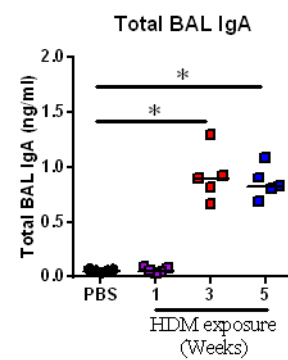
Figure 3.23: Lung ALT specific antibody is detectable after 3 weeks.

HDM specific and total antibody was assessed in the lung tissue by ELISA. For specific ELISAs, lung homogenate was titrated, allergen specific antibody was measured and endpoint titers were calculated. **A)** HDM specific IgE, **B)** Total IgE, **C)** HDM specific IgG1, **D)** Total IgG1, **E)** HDM specific IgA, **F)** Total IgA. Median data is shown and allergen-specific curves display median $\pm$ interquartile range. $n=4-5$ per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

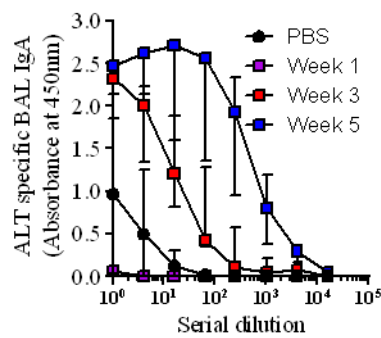
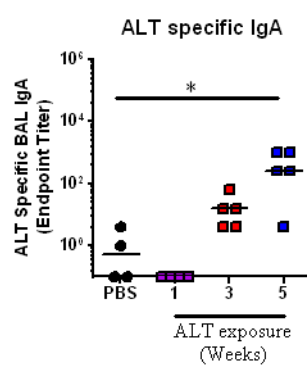
A)



B)



C)



D)

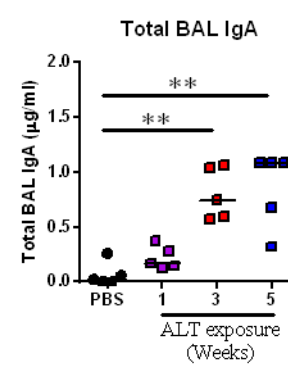


Figure 3.24: IgA is detectable in the BAL after 3 weeks of allergen exposure.

Specific and total IgA in the BAL was assessed by ELISA. For specific antibody ELISAs, BAL was titrated, allergen specific IgA was measured and endpoint titers calculated, **A)** HDM specific IgA, **B)** Total IgA – HDM study, **C)** ALT specific IgA **D)** Total IgA – ALT study. Median data is shown and allergen-specific curves display median $\pm$ interquartile range. $n=4-5$ per time-point. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Representative data from 2 independent experiments.

3.3.12. HDM rechallenge after disease resolution exacerbates HDM driven allergic disease

To examine the secondary humoral response during HDM driven allergic airway disease, mice were exposed to PBS or HDM for 5 weeks, rested for 4 weeks and re-challenged to either two doses of PBS or HDM and culled 18 hours after the final dose as described in **Figure 3.1C**. HDM rechallenge (HDM+HDM) increased the number of cells in the lung tissue (**Figure 3.25A**), BAL (**Figure 3.25B**) and mLN (**Figure 3.25C**) compared to HDM primed and PBS rechallenge animals (HDM+PBS). The level of cellular influx was greater in HDM+HDM mice than mice exposed to HDM for the first time (PBS+HDM) (**Figure 3.25A-C**). After 4 weeks of rest the number of eosinophils in the BAL and lungs of mice previously exposed to HDM and re-challenged with PBS (HDM+PBS) was similar to the level observed in PBS+PBS controls (**Figure 3.25D, E**). However, eosinophils were dramatically increased following HDM rechallenge in the BAL and lungs (**Figure 3.25D, E**). This HDM re-exposure was further characterised by increased IL-13 secretion into the airways (**Figure 3.25F**) and an increase in lung IL-4⁺ CD4⁺ T cells (**Figure 3.25G**), IL-13⁺ CD4⁺ T cells (**Figure 3.25H**) and IL-17A⁺ CD4⁺ T cells (**Figure 3.25I**). Concomitant with this increase in lung cytokine producing cells, the lungs retained a small population of non-T_{FH} effector cells, PD1⁺CXCR5⁻ (T_{EFF}) after the rest period and the lung T_{EFF} cell population were rapidly increased in the lungs upon allergen rechallenge (**Figure 3.26A, B**). However, this rapid response in T_{EFF} cells was not observed in the mLN or the spleen (**Figure 3.26C-F**) consistent with a local response upon allergen rechallenge. Taken together, these data show that re-exposure to HDM after a period of allergen withdrawal elicits a strong allergic disease recall response.

3.3.13. HDM re-exposure increases the lung T_{FH} response

To determine the secondary T_{FH} response, T_{FH} were examined after allergen rechallenge. Even after 5 weeks of rest, HDM+PBS mice had increased proportions and total numbers of T_{FH} in the mLN compared to PBS+PBS exposed animals or those exposed to HDM for the

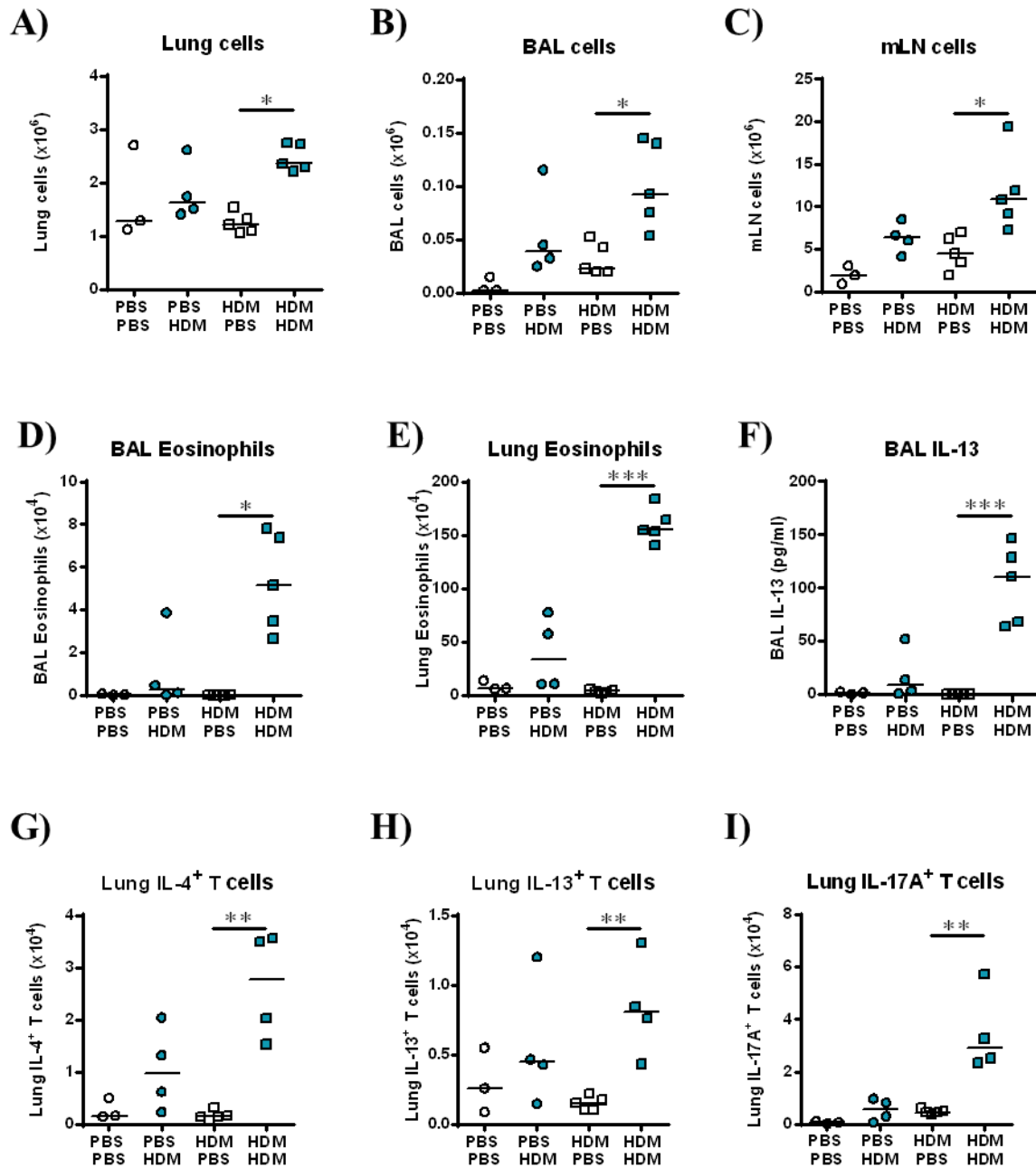


Figure 3.25: Mice re-exposed to HDM display an allergic disease recall response.

Following a 5 weeks primary exposure of HDM or PBS and a 4 week rest period, recall responses were assessed by rechallenge with 2 doses of either PBS or HDM. Cell counts were determined by trypan blue exclusion. **A)** Lung, **B)** BAL, **C)** mLN. Flow cytometry was used to determine cellular populations. **D)** BAL Eosinophils, **E)** Lung Eosinophils, **F)** IL-13 was measured in the BAL by ELISA, **G)** Lung IL-4⁺ CD4⁺ T cells, **H)** IL-13⁺ CD4⁺ T cells, **I)** IL-17A⁺ CD4⁺ T cells. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Experiment, n=1.

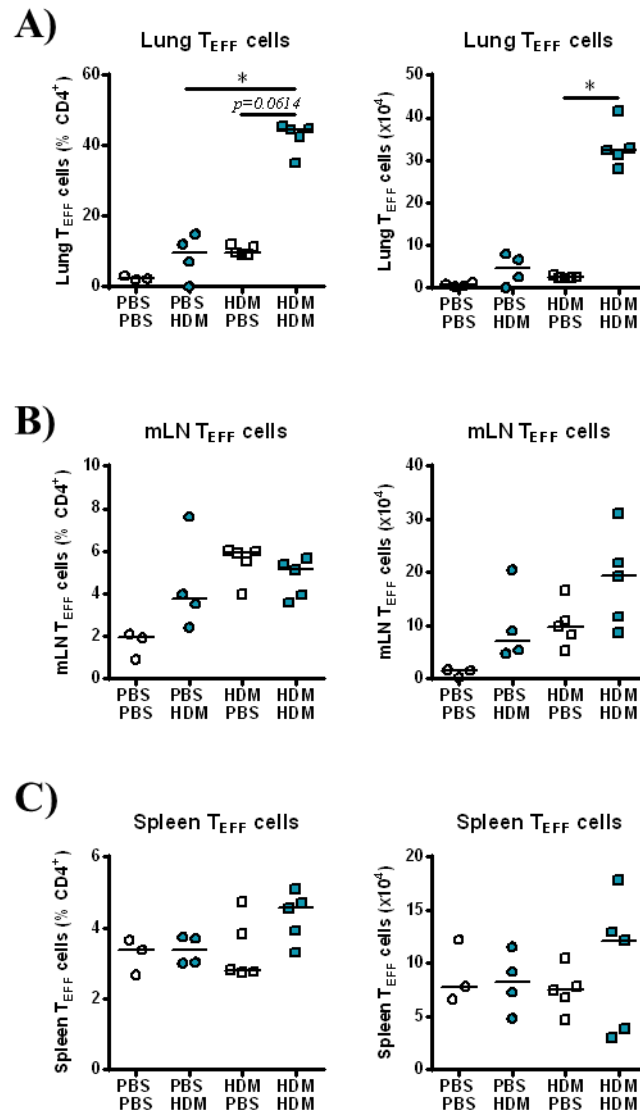


Figure 3.26: Lung non- T_{FH} effector cells are increased after HDM re-challenge

Following a 5 weeks primary exposure of HDM or PBS and a 4 week rest period, recall responses were assessed by rechallenge with 2 doses of either PBS or HDM. **A-C)** Flow cytometry was used to determine the frequency and number of non T_{FH} effector cells, termed T_{EFF} cells (PD1⁺CXCR5⁺CD44^{hi}CD62L⁺CD4⁺Foxp3⁻). The frequency and total number of T_{EFF} cells was quantified for **A)** Lung, **B)** mLN, **C)** Spleen. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Experiment, n=1.

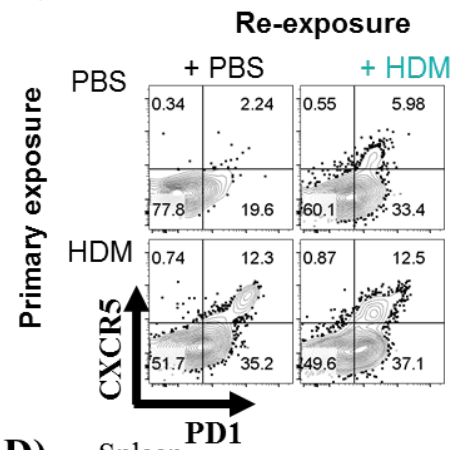
first time (PBS+HDM) (**Figure 3.27A-C**). Re-exposing HDM primed mice to HDM (HDM+HDM) did not further increase T_{FH} proportion or total numbers in the mLN (**Figure 3.27A-C**). After the rest period, Splenic T_{FH} were not found elevated in HDM exposed and PBS challenged (HDM+PBS) mice and were found at similar proportion and total as PBS+PBS mice (**Figure 3.27D-F**). Re-exposure of mice to HDM appeared to increase splenic T_{FH} proportions (**Figure 3.27D, E**) but not total numbers (**Figure 3.27F**). T_{FH} populations within the lungs did not persist after the rest period and HDM+PBS treated mice had similar proportions and numbers of T_{FH} compared to PBS+PBS mice and PBS+HDM animals (**Figure 3.27G-I**). Interestingly, after HDM re-exposure, the HDM+HDM had a dramatically elevated proportions and total number of T_{FH} within the lung tissue (**Figure 3.27G-I**). Therefore, T_{FH} persisted after rest within the mLN but not the lungs, but only the lung T_{FH} populations increased after allergen rechallenge.

3.3.14. HDM re-exposure reduced mLN T_{FR} populations

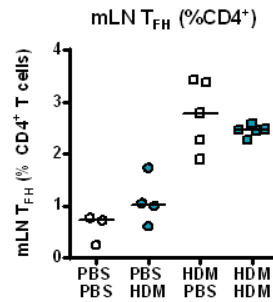
T_{FR} populations were identified within the mLN of PBS+PBS and HDM+PBS exposed animals (**Figure 3.28A-C**). Upon initial HDM treatment (PBS+HDM) or re-exposure (HDM+HDM) T_{FR} were reduced by proportion and total (**Figure 3.28A-C**). Splenic T_{FR} were not altered between the groups (**Figure 3.28D-F**) and similar results were observed for T_{FR} within the lungs (**Figure 3.28G-I**). Therefore, allergen re-exposure reduced regulatory T_{FR} populations within the mLN, but did not alter lung or spleen T_{FR} .

Pre-gated on CD4⁺CD3⁺Foxp3^{hi}CD44^{hi}CD62L⁻ lymphocytes

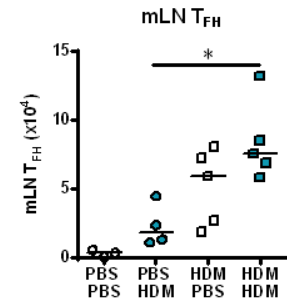
A) Mediastinal lymph node



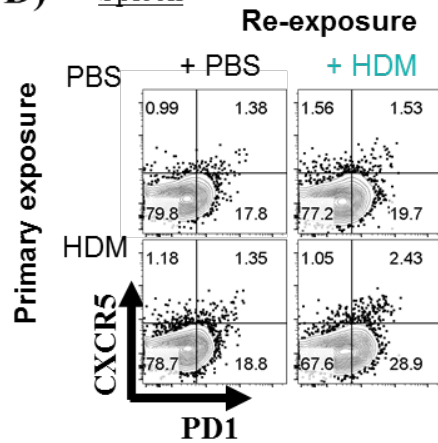
B)



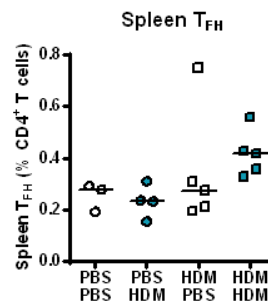
C)



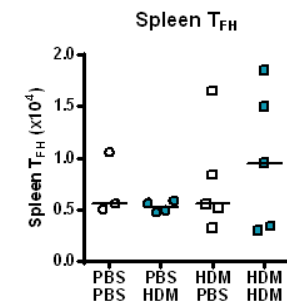
D) Spleen



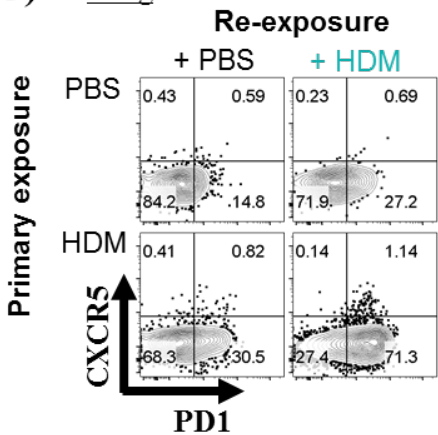
E)



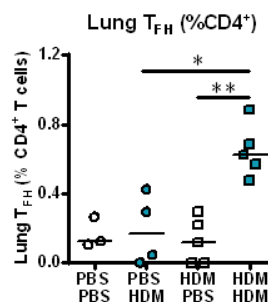
F)



G) Lung



H)



I)

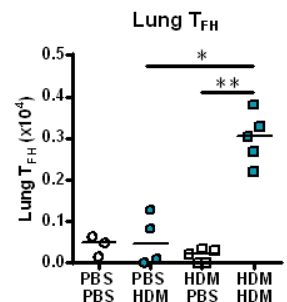


Figure 3.27: Lung T_{FH} are increased after HDM re-challenge

Following a 5 weeks primary exposure of HDM or PBS and a 4 week rest period, recall responses were assessed by rechallenge with 2 doses of either PBS or HDM. Flow cytometry was used to determine the frequency and number of T_{FH}. **A)** Representative flow plots of T_{FH} in the mLN, pre-gated on CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes. mLN T_{FH} quantified by **B)** frequency, **C)** total, **D)** Representative flow plots of T_{FH} in the spleen. Splenic T_{FH} quantified by **E)** frequency, **F)** total. **G)** Representative flow plots of T_{FH} in the lung. Lung T_{FH} quantified by **H)** frequency, **I)** total. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Experiment, n=1.

Pre-gated on CD4⁺CD3⁺Foxp3⁺CD44^{hi}CD62L⁻ lymphocytes

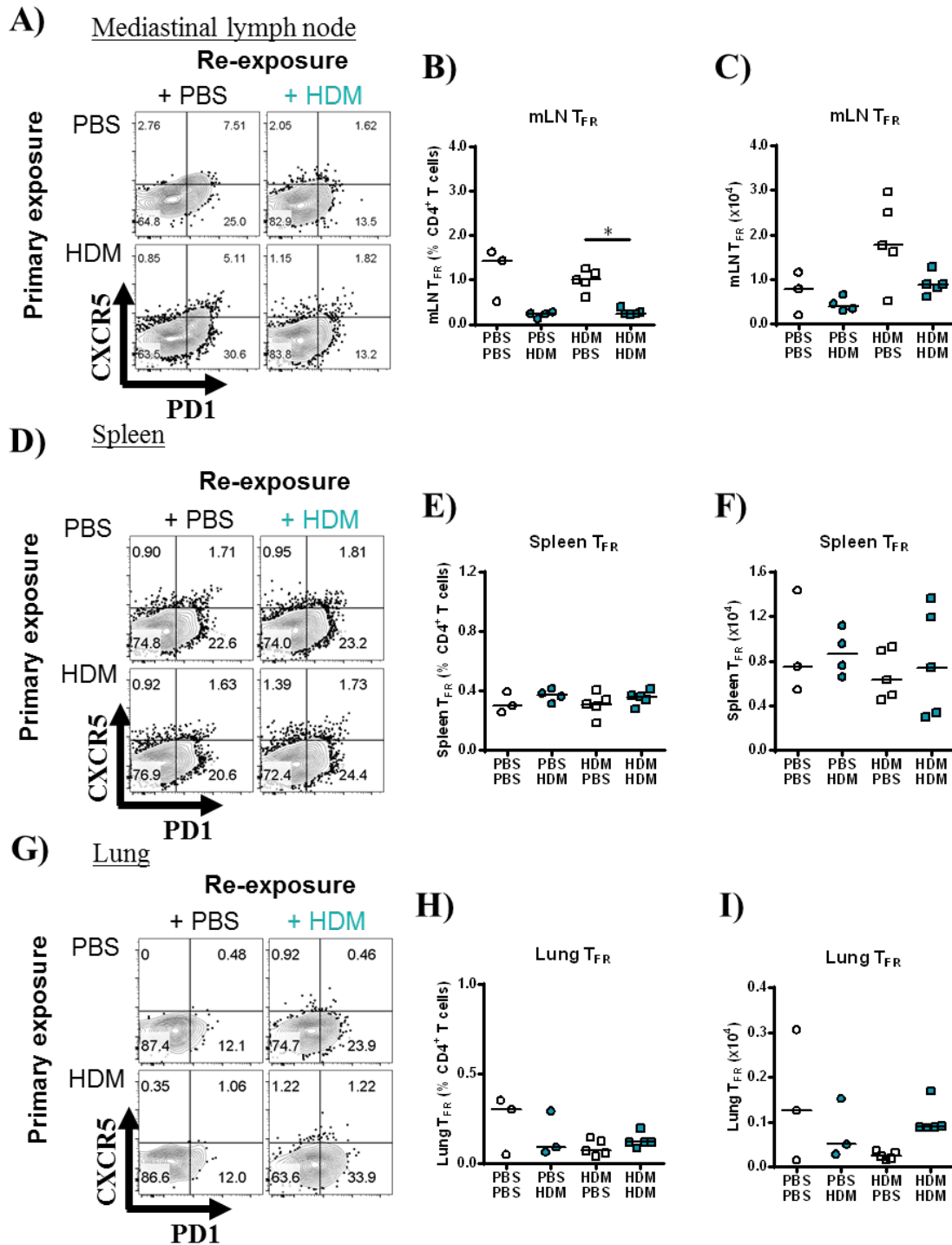


Figure 3.28: mLN T_{FR} are reduced upon allergen rechallenge.

Following a 5 weeks primary exposure of HDM or PBS and a 4 week rest period, recall responses were assessed by rechallenge with 2 doses of either PBS or HDM. Flow cytometry was used to determine the frequency and number of T_{FR}. **A)** Representative flow plots of T_{FR} in the mLN, pre-gated on CD4⁺ CD3⁺Foxp3⁺ lymphocytes. mLN T_{FR} quantified by **B)** frequency, **C)** total. **D)** Representative flow plots of T_{FR} in the spleen. Splenic T_{FR} quantified by **E)** frequency, **F)** total. **G)** Representative flow plots of T_{FR} in the lung. Lung T_{FR} quantified by **H)** frequency, **I)** total. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Experiment, n=1.

3.3.15. HDM re-exposure does not drive a strong secondary GC B cell response

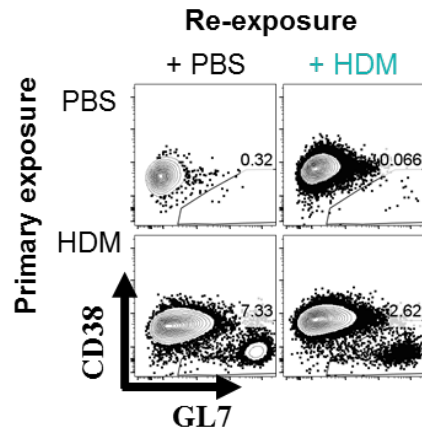
The secondary GC B cell response was also examined. Exposure to only 2 doses of HDM (PBS+HDM) did not induce GC B cell development (**Figure 3.29A, B**), consistent with data showing GC B cells were identified from 3 weeks of HDM exposure (**Figure 3.11**). Following the rest period, GC B cells were still identified in the mLN of the HDM+PBS group (**Figure 3.29A-C**). Interestingly, following HDM re-exposure GC B cell proportions were reduced but the total number of GC B cells was unchanged (**Figure 3.29A-C**). Similar data was observed within the spleen but the proportion and total numbers of GC B cells was considerably lower than observed in the mLN (**Figure 3.29D-F**). Furthermore, locally within the lungs, GC B cells were still identified after rest and showed a trend to decrease by proportion but not in total number (**Figure 3.29G-I**). Overall, this data suggests GC B cells persist during the disease resolution period and were not strongly induced upon allergen rechallenge.

3.3.16. Memory B cell population do not respond to HDM re-exposure

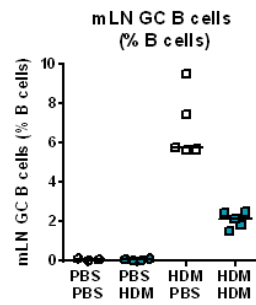
IgG1⁺ memory B cells were elevated in HDM+PBS animals compared to PBS+PBS and PBS+HDM groups in the mLN, spleen and lung tissue (**Figure 3.30A-F**). Proportionally IgG1⁺ memory B cells were reduced in the mLN and spleen following HDM rechallenge (HDM+HDM) compared to the HDM+PBS groups (**Figure 3.30A, C**). This reduction was not observed in the lung tissue (**Figure 3.30E**). Total numbers of IgG1⁺ memory cells were not altered following HDM rechallenge (**Figure 3.30B, D, F**). Thus, IgG1⁺ memory cells could be identified after 5 weeks of rest but did not rapidly increase in number following allergen exposure.

Pre-gated on CD19⁺B220⁺ lymphocytes

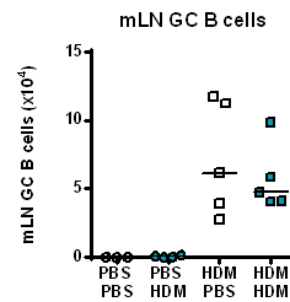
A) Mediastinal lymph node



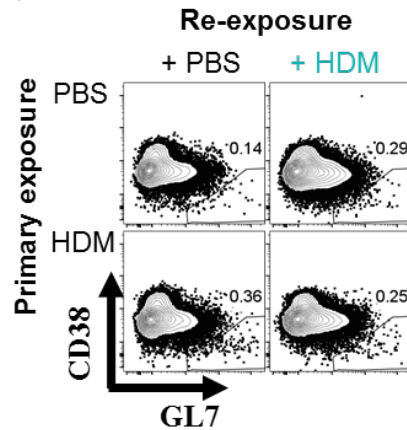
B)



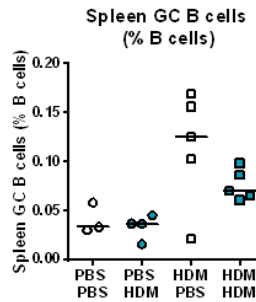
C)



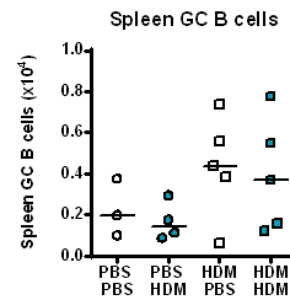
D) Spleen



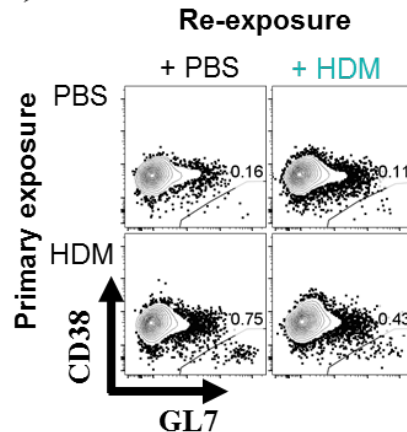
E)



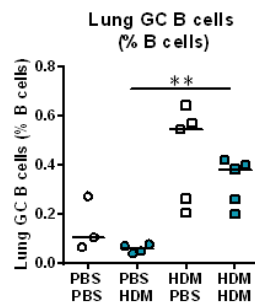
F)



G) Lung



H)



I)

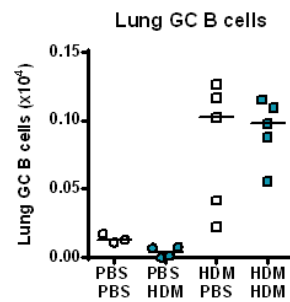


Figure 3.29: Rechallenge with HDM after a period of rest does not alter GC B cell numbers in secondary lymph organs.

Flow cytometry was used to determine the frequency and number of GC B cells within cellular compartments, **A)** Representative flow plots of GC B cells in the mLN, pre-gated on CD19⁺B220⁺ lymphocytes. mLN GC B cells were quantified by **B)** frequency, **C)** total. **D)** Representative flow plots of GC B cells in the spleen, Splenic GC B cells quantified by **E)** frequency, **F)** total. **G)** Representative flow plots of GC B cells in the lungs. Lung GC B cells quantified by **H)** frequency, **I)** total. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Experiment, n=1.

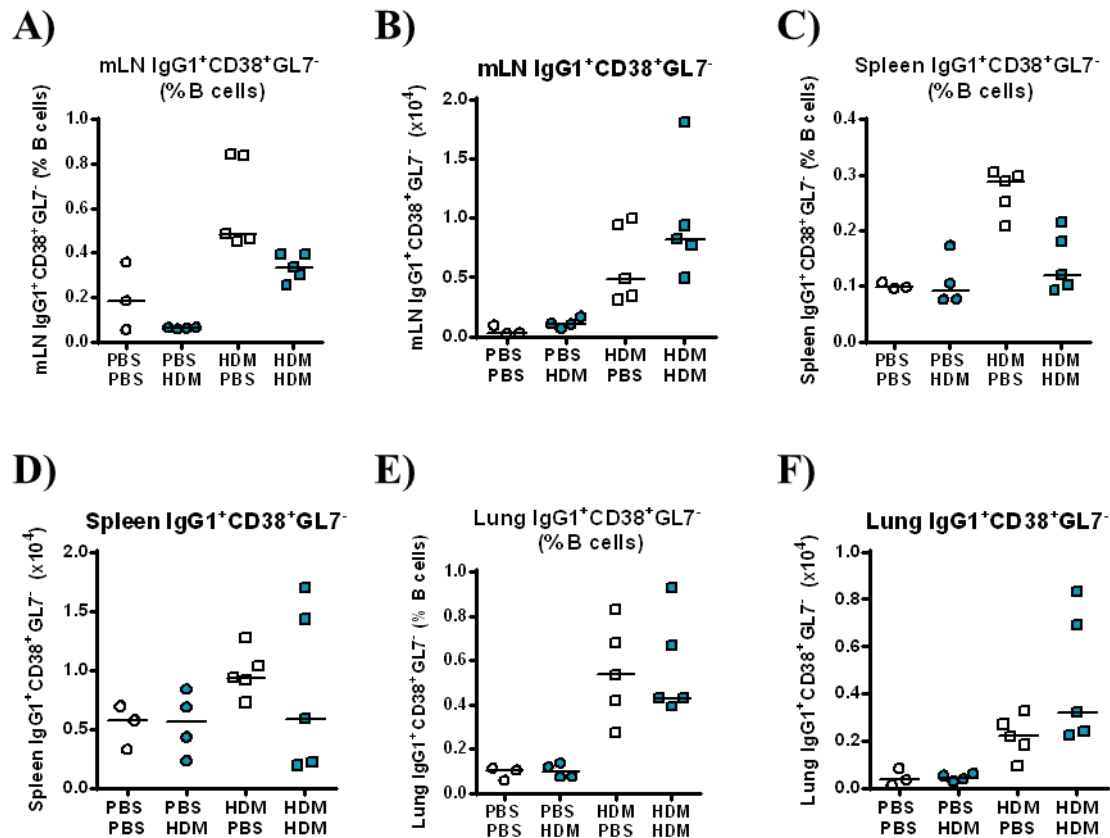


Figure 3.30: Memory B cells are long lasting and fail to respond to HDM re-exposure

Flow cytometry was used to determine the frequency and total of IgG1⁺ memory B cells within cellular compartments IgG1⁺ memory cells were defined as IgG1⁺CD38⁺GL7⁻IgD⁻IgM⁻CD19⁺B220⁺ lymphocytes and quantified. **A)** mLN frequency, **B)** mLN total, **C)** Spleen frequency, **D)** Spleen total, **E)** Lung frequency, **F)** Lung total. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Experiment, n=1.

3.3.17. mLN plasma cells are increased after HDM re-exposure

After a 4 week rest period, the proportion and number of mLN plasma cells had returned to levels seen in PBS+PBS controls (**Figure 3.31A-C**). However, after HDM re-exposure mLN plasma cell proportions and total numbers were significantly increased (**Figure 3.31A-C**). Splenic plasma cells were similar in all groups and numbers were unchanged by HDM re-exposure (**Figure 3.31D-F**). Finally, lung plasma cell proportions were similar between HDM+PBS and HDM+HDM groups (**Figure 3.31G-I**), but the total number of lung plasma cells

were increased after HDM re-exposure (**Figure 3.31G-I**). Therefore, HDM re-exposure generated a strong plasma cell recall response, in particular in the mLN.

3.3.18. HDM rechallenge increased allergen specific IgE

Given the change in plasma cell populations the impact that HDM re-exposure had on HDM-specific antibody responses was measured in the rechallenge study. Following 5 weeks of rest, IgG1 was still detected in the serum and lung of HDM+PBS group (**Figure 3.32A, B**). No difference was observed in antibody titer between the HDM+PBS and HDM+HDM group (**Figure 3.32A, B**). Similar results were observed with IgA in the airways and lungs, where following rest IgA was readily detected and this was unchanged by HDM re-exposure (**Figure 3.32C, D**). Interestingly, IgE was considerably less stable and was no longer detected in serum and lung tissue of the HDM+PBS mice (**Figure 3.32E, F**). However, after HDM rechallenge, IgE was dramatically increased in the serum and lungs of mice previously exposed animals (**Figure 3.32E, F**). Therefore, this data suggested allergen specific IgE to be less stable than other immunoglobulin isotypes but to be rapidly produced following allergen re-exposure.

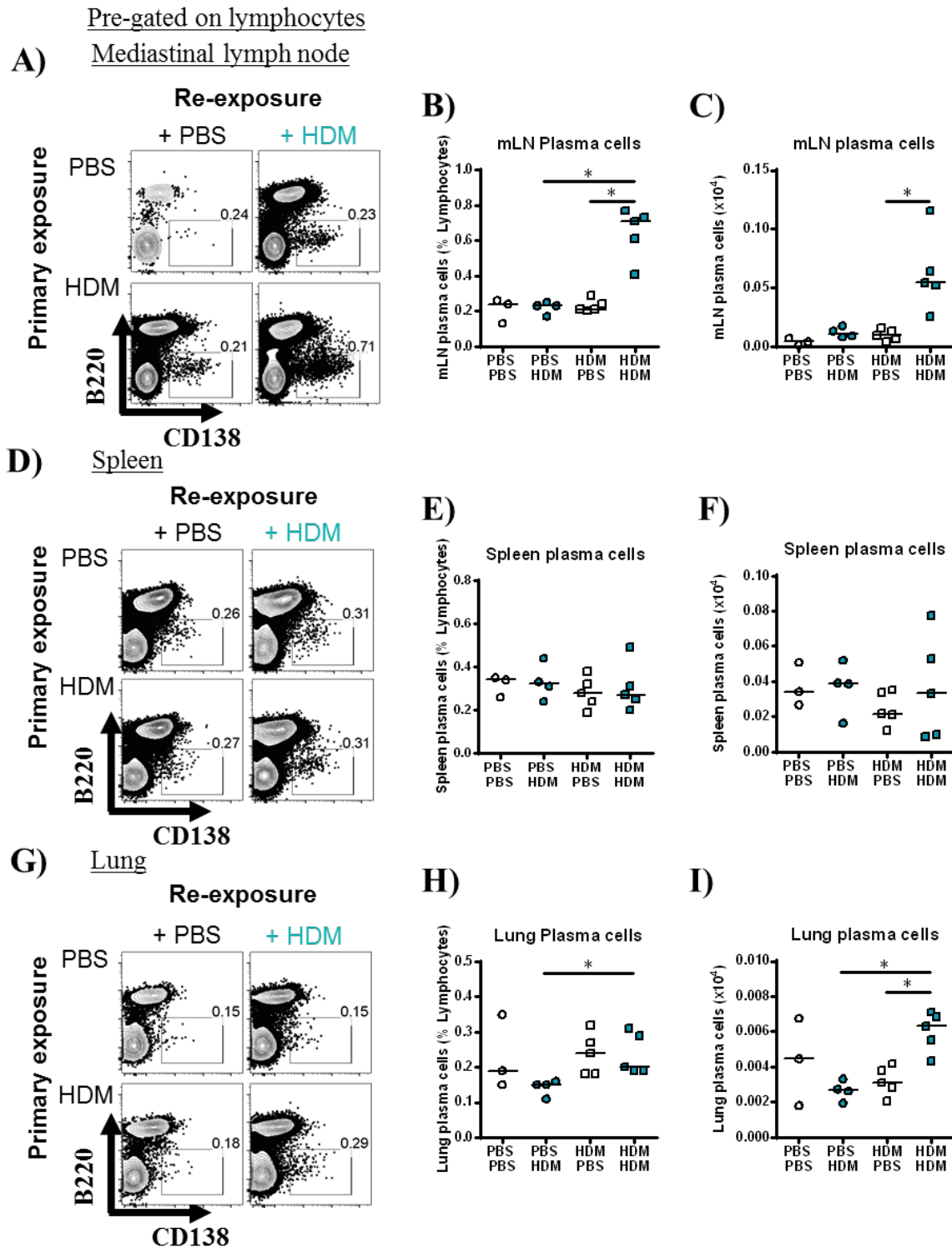


Figure 3.31: HDM rechallenge increases lymph node and lung plasma cells

Flow cytometry was used to determine the frequency and number of plasma cells within cellular compartments. Plasma cells were defined as B220^{lo}CD138⁺ lymphocytes. **A)** Representative flow plots of mLN plasma cells, pre-gated on lymphocytes. mLN plasma cells quantified by **B)** frequency, **C)** total. **D)** Representative flow plots of splenic plasma cells. Splenic plasma cells quantified by **E)** frequency, **F)** total. **G)** Representative flow plots of lung plasma cells. Lung plasma cells quantified by **H)** frequency, **I)** total. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. Experiment, n=1.

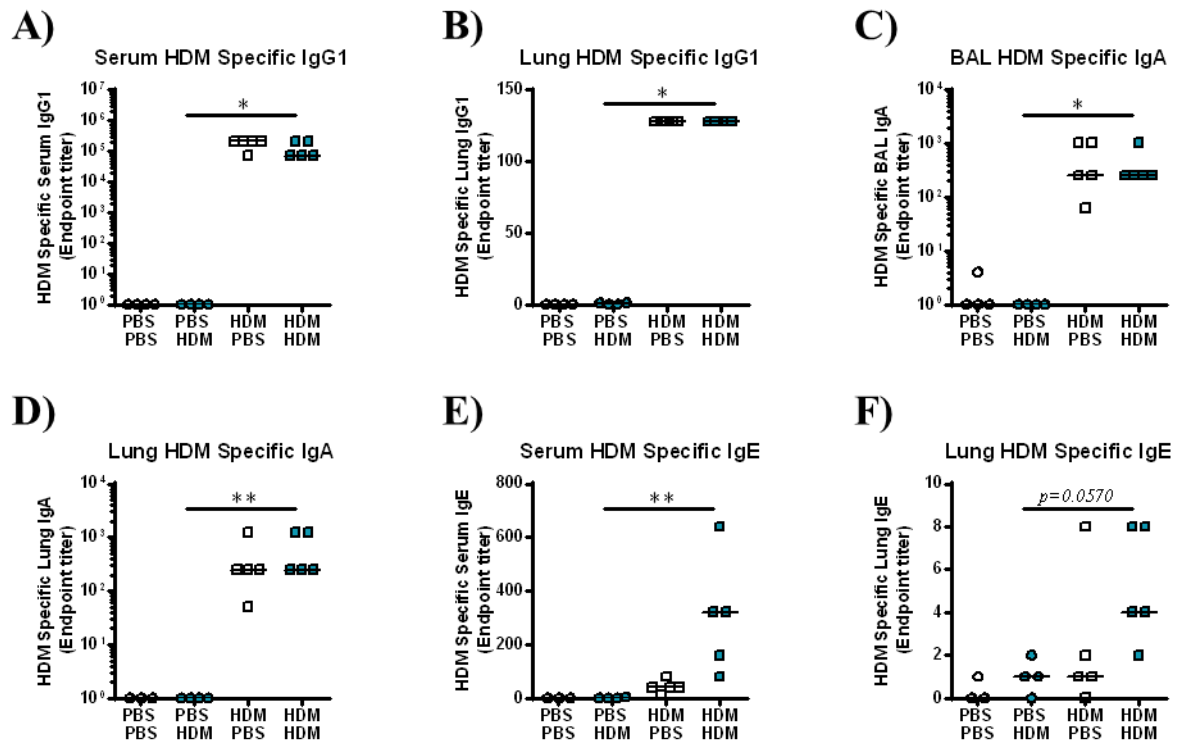


Figure 3.32: HDM specific IgE is increased after allergen re-exposure

HDM specific antibody was assessed by ELISA. Serum, lung homogenate and BAL was titrated, allergen specific antibody was measured and endpoint titer was calculated. **A)** Serum IgG1, **B)** Lung IgG1, **C)** BAL IgA, **D)** Lung IgA, **E)** Serum IgE, **F)** Lung IgE. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Kruskal Wallis test with Dunn's multiple comparison test. n=4-5 per group. Experiment, n=1.

3.4. Discussion

The aim of this chapter was to understand if the scale of the T_{FH} and GC response during AAD was determined by the length of allergen exposure. Initial experiments in this chapter sought to understand where and when the T_{FH} and GC responses occurred using two aeroallergen exposure models of AAD. Overall, it was clear that the T_{FH} and GC responses develop both systemically and locally, and the magnitude of the response generally increased with allergen exposure. The second part of the chapter aimed to characterise the secondary T_{FH} response and a strong local T_{FH} and IgE response was seen when mice were allergen re-challenged after disease resolution.

3.4.1. Primary T_{FH} responses

In both HDM and ALT driven allergic disease models, T_{FH} are present in SLOs and locally within the lung tissue, and overall responses are similar between the two models. T_{FH} first appear in the lung draining mLN and with further allergen exposure at other sites including the spleen and lungs. The fast expansion of T_{FH} within the mLN in this study, fits with data from Coquet and colleagues, whom identified mLN T_{FH} in mice given a single intranasal dose of HDM (Coquet et al., 2015). Allergen exposure restricts T_{FH} generation to the nearby lung draining lymph node, rather than appearing in all lymph nodes, therefore T_{FH} are absent from the skin draining ILN, even after 5 weeks of allergen exposure. Consistent with this allergen exposure induces T_{FH} populations depending on the route of sensitisation and T_{FH} are present in skin draining lymph nodes when mice are HDM sensitised via the skin (Coquet et al., 2015), similar to other model antigens (Fazilleau et al., 2007).

During AAD the mLN is the predominant site of T_{FH} generation, consistent with the SLOs representing the major site of humoral immunity. However, as allergen exposure continues T_{FH} are detectable locally within the lung tissue, indicating a novel requirement for chronic allergen exposure to generate local T_{FH} responses. Interestingly, local lung T_{FH} appear at time-

points where the key hallmark features of allergic disease are established (Johnson et al., 2004). Vroman and colleagues also detect lung T_{FH} in their chronic mouse model of HDM driven AAD (Vroman et al., 2017) but to date lung T_{FH} have not been identified in the asthmatic human lung. Given that multiple studies identifying local T_{FH} populations during chronic human diseases, including within the nasal polyps during rhinosinusitis (Zhang et al., 2016), the synovial fluid during RA (Chu et al., 2014) and the liver tissue of Hepatitis B (Jia et al., 2015) and C (Tripodo et al., 2012, Spaan et al., 2015) patients, it is plausible for T_{FH} to be present in the lungs of patients with allergic disease.

Multiple human studies now describe cT_{FH} within the peripheral blood of patients with allergic disease (Kamekura et al., 2015, Kim et al., 2016, Schulten et al., 2017, Gong et al., 2018) and in acute mouse models, 7 days after infection or immunisations, including LCMV Armstrong (Hale et al., 2013), NP-OVA (Sage et al., 2014a) and PR8 Influenza A (Sage et al., 2014a). However, in this study both cT_{FH} and cT_{FR} were absent in the circulation of allergen exposed animals, even after 5 weeks of chronic dosing. Human cT_{FH} represent disease biomarkers as they are present at low frequencies or absent in healthy individuals and increase in patients with diseases, including asthma (Gong et al., 2018), allergic rhinitis (Schulten et al., 2017), SLE (Simpson et al., 2010), Sjorgen syndrome (Simpson et al., 2010), chronic Hepatitis B (Hu et al., 2017) or following influenza vaccination (Bentebibel et al., 2013). During allergic asthma $IL-4^+$ or $IL-21^+$ cT_{FH} are capable of inducing B cell cultures to drive IgE and IgG1 production and their proportions positively correlate with serum IgE levels (Gong et al., 2018), thus indicating a potential role of T_{FH} in allergic asthma.

The literature widely suggests cT_{FH} to contain a memory-like population (Morita et al., 2011, He et al., 2013b, Ueno, 2016) possessing the ability to provide B cell help (Morita et al., 2011, He et al., 2013b) and consistent with this influenza A vaccination induces a cT_{FH} population which undergoes a rapid recall response in subsequent annual vaccinations and associates with a circulating plasmablast response (Herati et al., 2017). However, despite this knowledge, little is understood about how these cT_{FH} relate to “bona fide” T_{FH} populations in SLOs and the

ontogeny of these cells is a subject of great debate. It is known however that cT_{FH} are reliant on DC help (Sage et al., 2014a) but not B cell help for their generation (He et al., 2013b, Sage et al., 2014a) and thus appear to develop independently of the GC.

As the mouse model of AAD does not induce a cT_{FH} population it may not require a circulating early memory-like T_{FH} population to drive disease and consistent with this cT_{FH} do not appear after a period of allergen cessation nor do they increase upon allergen rechallenge (data not shown in this chapter). This implies that in this model all potential T_{FH} progress straight to “bona fide” T_{FH} found in tissues rather than entering the circulation. However, it cannot be ruled out that this cT_{FH} population are simply an element of human asthma that the mouse model is incapable of replicating.

Continuous high dose antigen and persistent TCR stimulation favours T_{FH} differentiation, as seen in repeat protein immunisations (Baumjohann et al., 2013) or chronic infections, including HIV (Lindqvist et al., 2012) and chronic LCMV (Fahey et al., 2011, Harker et al., 2011). In this study, T_{FH} accumulate with persistent allergen exposure, suggesting a link between T_{FH} numbers and the availability of antigen. This implies that T_{FH} numbers may continue to rise indefinitely if allergen exposure continues. Therefore, it would be interesting to evaluate whether a saturation point exists. Allergic asthma results from frequent and continuous allergen exposure which could partly account for why asthmatic individuals have higher proportions of cT_{FH} relative to healthy individuals (Gong et al., 2018). Therefore, it may be interesting to compare cT_{FH} proportions between allergic and non-allergic patients.

During chronic AAD, T_{FR} are present at the same sites as T_{FH} and therefore are detectable in the mLN, spleen and lungs, and absent from the ILN and the circulation. Although, there are variations in the T_{FR} kinetics between the two aeroallergen models, relatively few T_{FR} are evident throughout the study and T_{FH} and GC heavily outnumber T_{FR} at all time-points. This data aligns with the literature, where T_{FR} represent a relatively small population in comparison to T_{FH} and GC B cells and negatively regulate humoral immunity (Chung et al., 2011, Linterman et al., 2011). However, more significant increases in T_{FR} are seen during ALT driven

disease which supports ALT driving a more extreme model of chronic AAD (Snelgrove et al., 2014, Castanhinha et al., 2015) and thus may require more regulation than the HDM model.

The T_{FH} response precedes the GC B cell response beginning 1 week after allergen exposure while the GC response is only identifiable after 3 weeks, consistent with T_{FH} driving GC development (Crotty, 2014). Moreover, GC B cells are only present at sites where T_{FH} exist, and thus similarly reside predominantly in the mLN while also being evident in the spleen and locally within the lung tissue. Alongside this T_{FH} and GC B cells correlate tightly at all sites where both cells are present thus showing there is no dysregulation in the magnitude or kinetics of this highly dependent relationship during chronic AAD.

The observation of GC B cells in the lung tissue is particularly interesting because it suggests the presence of a tertiary lymphoid structure within the lungs of allergen exposed animals. Lung tertiary lymphoid organs, termed inducible bronchus associated lymphoid tissues (iBALT) develop in for a number of respiratory disorders (Randall and Mebius, 2014), including chronic obstructive pulmonary disease (COPD) (Hogg et al., 2004, Gosman et al., 2006, Demoor et al., 2009, Van Pottelberge et al., 2010), following influenza A infection (Moyron-Quiroz et al., 2004, GeurtsvanKessel et al., 2009, Neyt et al., 2016) and patients with RA with respiratory complications (Turesson et al., 2005, Rangel-Moreno et al., 2006). Structures suggestive of iBALT are also present in the bronchi tissue of asthmatic patients and these structures are more frequent and larger than those in healthy individuals (Elliot et al., 2004). Thus, indicating that during chronic AAD, antibody production can potentially occur in the lungs and thus outside of traditional SLOs. This fits with data from Takhar and colleagues who describe the presence of switch transcripts to IgE in bronchial biopsies of asthmatic patients (Takhar et al., 2007) indicating that local CSR can occur in the lungs.

Chronic allergen induces a dominant IgG1⁺ GC B cell response and results in IgG1 being the most detectable antibody isotype in both serum and lung tissue. Consistent with this the type 2 cytokine, IL-4 is a critical switch factor for CSR towards IgG1 as well as IgE (Snapper and Paul, 1987). The governance of IgG1⁺ is particularly true in the mLN where IgG1⁺ GC B cells

represent 80% of the GC B cells, but this isotype is less dominant in the spleen and the lung, indicating other isotypes to be important at these sites. IgG2a⁺ GC B cells account for less than 5% of GC B cells consistent with IgG2a being far more important during viral infections (Coutelier et al., 1987, Huber et al., 2006), as IFN- γ is the switch factor for IgG2a (Snapper and Paul, 1987). Interestingly, IgA is detectable in the BAL and lungs of allergen exposed animals consistent with its neutralising role at mucosal sites (Pilette et al., 2004, Mantis et al., 2011). This could explain the reduction in IgG1⁺ GC B cell proportions in the lung tissue however, the contribution of IgA⁺ GC B cells is not assessed in this part of the thesis.

Using flow cytometry, it was difficult to identify IgE⁺ GC B cells in this work (data not shown), especially given IgE⁺ GC B cells are rare and present at very low frequencies. Furthermore, IgE is often bound to Fc receptors making it tough to accurately stain on B cells. During this PhD, several techniques were used to try and stain IgE⁺ GC B cells for flow cytometry analysis. Firstly, surface IgE staining was performed by simply adding Fc block to the antibody cocktail. When this was deemed unsuccessful, the cells were put through an acid wash to strip IgE from Fc ϵ receptors and Fc block treatment was used prior to extracellular staining to identify IgE⁺ cells (Laffleur et al., 2015). Lastly, a high concentration of purified anti-IgE antibodies were used to block surface IgE, before permeabilising cells and staining intracellularly with fluorescent tagged anti-IgE antibodies (Yang et al., 2012). None of the trialled methods were successful. However, multiple groups show using reporter mice that B cells expressing the IgE B cell receptor exist in the GC (Talay et al., 2012, Yang et al., 2012, He et al., 2013a) but have a short lifespan and either rapidly progress towards plasma cells differentiation or undergo apoptosis following BCR signalling (Erazo et al., 2007, Talay et al., 2012, Yang et al., 2012, He et al., 2013a, Laffleur et al., 2015). Therefore, our inability to identify these cells by flow cytometry may simply reflect that fact that they are biologically rare.

The main cellular outputs of a GC are memory B cells and antibody secreting plasma cells which are both present in the lungs and SLOs after 3 weeks of allergen exposure. This fits with the emergence of antibody both systemically in the serum and locally in the BAL and lung

tissue from this time-point. This aligns with work by other groups showing total and HDM-specific IgG1 and IgE to be detectable in the serum after 3 weeks of exposure, remaining stable or increasing at 5 and 7 weeks of intranasal allergen treatment (Johnson et al., 2004). Therefore, by the 3-week time point, the humoral response is established, comprising of T_{FH} , GC B cells, memory B cells, plasma cells and antibody. After this point the response scales up with further allergen exposure (kinetics summarised in table 6).

Taken together, the data presented here demonstrates that AAD induces T_{FH} systemically and locally, and their generation precedes GC B cell induction at the same locations leading to the subsequent production of antibody. The length of allergen exposure dictates the scale of the humoral response and overall the timing of the T_{FH} response supports the kinetics of the emergence of allergen specific antibodies.

	Week 1	Week 3	Week 5
mLN T _{FH}	↑	↑	↑
Spleen T _{FH}	—	↑	↑
Lung T _{FH}	—	↑	↑
Blood T _{FH}	—	—	—
ILN T _{FH}	—	—	—
mLN GC B cells	—	↑	↑
Spleen GC B cells	—	↑	↑
Lung GC B cells	—	↑	↑
ILN GC B cells	—	—	—
Memory B cells	—	↑	↑
Plasma cells	—	↑	↑
Allergen specific antibody	—	↑	↑
Total antibody	—	↑	↑

— Equivalent to PBS ↑ Increased relative to PBS
 ↑ Increased only in HDM driven disease

Table 6: Summary of allergen induced humoral response kinetics

Summary of the timing of the T_{FH}, GC, memory B cell, plasma cell and antibody responses induced by either house dust mite (HDM) or alternaria alternate (ALT). T_{FH}, GC, memory B cells and plasma cell responses were assessed at multiple sites. Antibody responses were analysed in the serum, lung and BAL.

3.4.2. T_{FH} stability and allergen rechallenge responses

Asthmatic patients often go through periods where they are not exposed to their trigger allergen, but still mount substantial allergic responses when they are re-exposed. Thus, the stability of the HDM induced T_{FH} and GC response was assessed during allergic disease resolution, along with the impact of HDM rechallenge. Mice were exposed to HDM for 5 weeks, given no allergen for 4 weeks then re-challenged to either HDM or PBS. After the rest period, allergic inflammation was resolved, characterised by loss of lung and BAL inflammatory cells, including eosinophils, Th2 and Th17 cells, similar to other studies (Cates et al., 2004, Johnson et al., 2004). Although, AHR was not assessed in this study, resolution of inflammation is normally accompanied by loss or reduced AHR (Johnson et al., 2004, Kearley et al., 2009). In agreement with this, IL-13, a key driver of AHR (Wills-Karp et al., 1998) is dramatically down to control levels following rest. Lastly, in terms of the antibody response, IgE is dramatically lost over the rest period while both IgG1 and IgA levels remain stable and do not change. This aligns with knowledge that the half-life of IgE is shorter than other antibody isotypes (Waldmann et al., 1976). Consistent with observations in patients following the transfer of serum containing allergen-specific antibody, IgE dramatically declines over a 50 day period, while IgG levels are maintained (Lawrence et al., 2017).

T_{FH} persist in the mLN even after an extensive period of allergen withdrawal and disease resolution. Supporting these findings, other groups show T_{FH} retention in the lymph nodes of mice, more than 60 days after immunisation with *Schistosoma mansoni* egg extract or following LCMV infection (Hale et al., 2013, Fairfax et al., 2015). This is predominantly due to the continuous availability of antigen within the GC, present in tight complexes with complement receptors on FDCs creating an antigen reservoir critical for the GC maintenance (Hanna and Szakal, 1968, Nossal et al., 1968, Mandels et al., 1980, Aguzzi et al., 2014). This enables GC B cells to still provide antigen to T_{FH} long after disease resolution thus allowing T_{FH} to persist. Alongside this mLN GC B cells also linger long after antigen cessation in this model consistent with several disease models (Ise et al., 2014, Fairfax et al., 2015).

Interestingly, splenic and lung T_{FH} proportions and numbers return to baseline levels during the rest period, indicating that unlike their mLN counterparts they are reliant on a supply of fresh antigen for their maintenance. Despite the loss of T_{FH} at both sites, GC B cells can persist indicating that at these sites the retention of GC B cells occurs in the absence of T_{FH} . Therefore, the method of antigen maintenance at these sites may vary from the mLN which represent the predominant T_{FH} site in this model.

Upon allergen re-exposure, a strong allergic recall response ensues involving a dramatic increase in pulmonary inflammation and IL-13 cytokine secretion. Interestingly, mLN T_{FH} populations do not undergo a rapid induction following allergen re-exposure which contrasts with observations of strong secondary T_{FH} responses to infectious agents and protein immunisation models (Ise et al., 2014, Fairfax et al., 2015). Neither mLN GC B cells nor memory B cell populations increase after allergen re-challenge, indicating that the rechallenge protocol did not alter these mLN populations. Furthermore, allergen rechallenge did not alter splenic T_{FH} or GC B cell populations. In fact, the lung T_{FH} are the only allergen responsive T_{FH} population and they increase both by proportion and total cells, indicating that in contrast to the primary response where mLN cells are fast responders, lung resident immune cells react more rapidly to re-challenge. Given that the primary lung T_{FH} response develops slowly, this implies that the rapid recall mechanism differs from the primary response, particularly considering there is no retention of lung resident T_{FH} during the rest period. One explanation of this is the work by Fairfax and colleagues which show secondary T_{FH} to arise from resident non- T_{FH} memory populations rather than long lasting T_{FH} populations (Fairfax et al., 2015). Therefore, it is plausible that this fast T_{FH} response in the lungs results from other lung resident memory $CD4^+$ T cells differentiating into T_{FH} populations. Consistent with this theory, the lung retains a small population of $PD1^+CXCR5^-$ (T_{EFF}) cells during the rest period and this population, like the lung T_{FH} , responds rapidly to allergen re-exposure.

Following HDM rechallenge, HDM-specific IgE rapidly increases both systemically and locally in the lung. This IgE response mirrors the lung T_{FH} response, returning to baseline after

allergen cessation and substantially increasing with HDM rechallenge. In contrast IgG1 and IgA levels remain steady despite the re-introduction of allergen into the model. Whether, the rapid local T_{FH} response directs the fast IgE response would be interesting to evaluate. However, the T_{EFF} cell response also dramatically increases after allergen rechallenge and therefore could also be critical for these secondary IgE responses. It is worth noting that in this model despite the major changes in lung T cell populations the most responsive plasma cell population resides in the mLN, suggesting these plasma cells to be important for the rapid increase in both systemic and local IgE responses. The presence of traditional memory IgE⁺ B cells is controversial (Talay et al., 2012, He et al., 2013a) therefore it would be interesting to understand whether this plasma cell burst in the mLN gives rise to secondary IgE responses directly from memory IgE⁺ B cells or memory IgG1⁺ B cells present after allergen cessation undergoing sequential switch to IgE plasma cells (Erazo et al., 2007). Furthermore, the role that T_{FH} play in this secondary IgE response is important to understand.

3.4.3. Concluding remarks

This chapter demonstrates that during chronic AAD, T_{FH} not only reside in the lung draining lymph node and the spleen, but also locally within the lung tissue. T_{FH} are first found in the mLN and with persistent continuous allergen exposure they are also found at the site of inflammation. T_{FH} precede and are accompanied by GC B cells after 3 weeks of exposure at the same sites, alongside memory B cells, plasma cells and antibody. Consistent with the original hypothesis, the length of allergen exposure determines the scale and the magnitude of the T_{FH} and GC response. However, T_{FH} responses are short lived in the lung and spleen when antigen is no longer available, but are stable in the mLN. Lung T_{FH} are highly responsive to allergen rechallenge along with allergen specific IgE. Overall this chapter shows persistent allergen to generate a substantial local and systemic T_{FH} population and is the first study to analyse T_{FH} and GC responses over time using clinically relevant models of chronic AAD. In

the next chapter the therapeutic potential of targeting T_{FH} during ongoing disease will be examined.

Chapter 4: Exploring the impact of ICOS-L blockade on T_{FH} responses and established chronic allergic airway disease

4.1. Introduction

In the previous chapter, it was demonstrated that during chronic AAD, T_{FH} accumulated within the lungs and SLOs and these T_{FH} were accompanied by GC B cells, memory B cells, plasma cells and allergen specific antibody responses. Although, T_{FH} are critical for the generation of allergen specific IgE (Kobayashi et al., 2017, Noble and Zhao, 2016) and are important drivers of AAD (Coquet et al., 2015, Ballesteros-Tato et al., 2016), whether T_{FH} represent therapeutic targets has yet to be investigated.

ICOS is a co-stimulatory molecule which is part of the immunoglobulin superfamily, expressed on CD4⁺ T cells following TCR engagement (Hutloff et al., 1999) and is critical for T cell activation (Dong et al., 2001). ICOS engagement with its ligand, ICOS-L is important for expression of BCL-6, which in turn engages the T_{FH} transcriptional program (Choi et al., 2011). T_{FH} express high levels of ICOS and require continuous ICOS signalling to maintain their phenotype and thus fulfil their downstream functions (Weber et al., 2015). This is in contrast to other CD4⁺ T cells that only require ICOS signalling during initial DC priming. After DC priming, newly differentiated T_{FH} termed pre-T_{FH} migrate from the T cell zone within SLOs towards the T cell/B cell zone border where they interact with activated B cells which continue to supply T_{FH} with ICOS signalling in addition to TCR stimulation to sustain their differentiation and help maintain their phenotype (Nurieva et al., 2008, Weinstein et al., 2014). Hence, ICOS-L stimulation is critical to the survival of the T_{FH} lineage and patients with common variable immunodeficiency (CVID) due to genetic defects in ICOS, fail to generate T_{FH} (Salzer et al., 2004, Grimbacher et al., 2003) while T_{FH} accumulate in *Roquin* mutant mice which overexpress ICOS (Vinuesa et al., 2005, Pratama et al., 2013, Vogel et al., 2013).

Although ICOS expression is not restricted to T_{FH}, multiple studies have shown that administering mice with ICOS-L targeting antibodies, can selectively diminish T_{FH} without impacting other subsets of CD4⁺ T cells (Baumjohann et al., 2013, Choi et al., 2011, Weber et

al., 2015, Stone et al., 2015). This decrease can be rapid and has been observed as quickly as 20 hours after antibody administration (Weber et al., 2015). ICOS-L mediated T_{FH} reduction was associated with decreased GC B cells (Baumjohann et al., 2013, Choi et al., 2011) consistent with their reliance on T_{FH} signalling. Therefore, this represents a useful method of depleting T_{FH} during an ongoing immune response. However, this method has only been trialled during acute models of inflammation or infection rather than in a sustained antigen exposure setting.

The aim of this chapter was to determine whether interfering with ICOS/ICOS-L signalling after the establishment of AAD was capable of reducing T_{FH} populations and to assess the impact this had on pathology. In particular it was essential to understand if blocking ICOS signalling would be sufficient to deplete T_{FH} during chronic disease when antigen is readily available. To examine this mice were chronically exposed to HDM for 5 weeks and ICOS-L targeting antibodies were administered *in vivo* for the last 2 weeks. All mice were culled at the end of week 5.

4.1.1 Hypothesis

Blocking ICOS signalling to T_{FH} during chronic allergic disease will deplete T_{FH} and improve allergic disease.

4.1.2 Aims

- 1) To determine whether T_{FH} can be depleted after the establishment of chronic AAD and what impact this has on humoral immunity
- 2) To understand the effect of late ICOS-L blockade on hallmark allergic airway disease symptoms

4.2. Methods

4.2.1 ICOS-L blockade during HDM or ALT driven allergic disease

Female 7-8-week old BALB/c mice were exposed to intranasal (i.n) HDM (25 µg), ALT (10 µg) or a control of PBS (25 µl) 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS ligand antibody (α-ICOS-L) or isotype control (2A3) antibody (i.p) 3 times a week. Mice were culled at the end of week 5 (summarised in **Figure 4.1A**). Blood was collected, and the lungs and mLN were harvested. Lung function was assessed using the flexiVent system and cellular populations were determined by flow cytometry. Serum antibody levels were analysed by ELISA.

4.2.2. IL-13 GFP reporter mice exposed to HDM driven allergic disease

Male and female IL-13^{+/GFP} reporter (IL-13R) mice were exposed to intranasal (i.n) HDM (25 µg) or PBS (25 µl) 3 times a week for 3 weeks. Mice were culled at the end of week 3 (summarised in **Figure 4.1B**). Lungs and mLN were harvested.

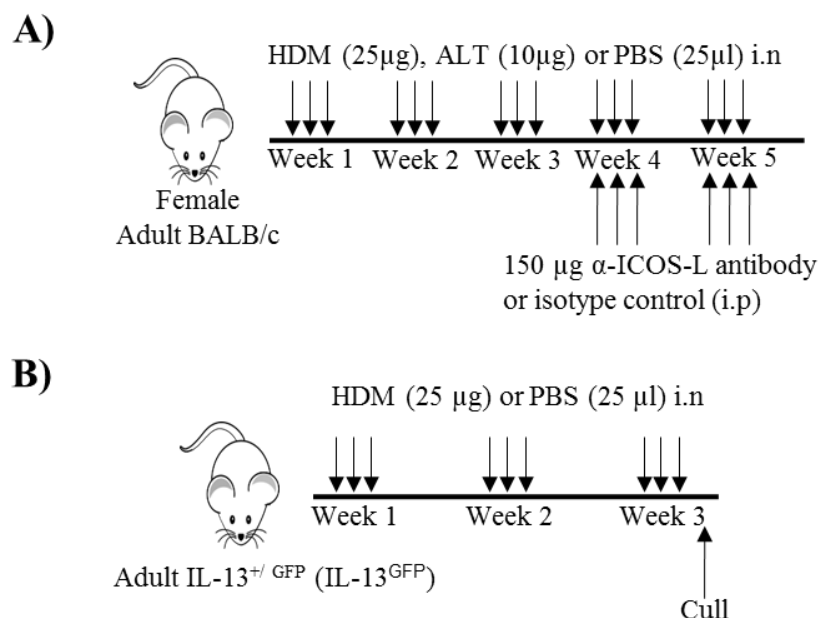


Figure 4.1: Method

A) ICOS-L blockade protocol during chronic allergic airway disease. Adult female BALB/c mice were exposed (i.n) to 25 µg HDM, 10 µg ALT or 25 µl PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg anti-ICOS-L (α-ICOS-L) or isotype control (2A3) antibody (i.p), 3 times a week. Mice were culled at the end of week 5. **B)** Adult IL-13^{+/GFP} (IL-13^{GFP}) mice were exposed to 25 µg HDM or 25 µl PBS, 3 times a week for 3 weeks. Mice were culled at the end of week 5. All experiments were culled 18 hours after the final dose.

4.3 Results

4.3.1 T_{FH} are depleted during chronic allergic airway disease by ICOS-L administration

T_{FH} are reliant on continuous ICOS-L signalling to be able to maintain their phenotype and have been successfully diminished *in vivo* in several acute models by administration of ICOS-L targeting antibodies (Baumjohann et al., 2013, Akiba et al., 2005, Choi et al., 2011, Stone et al., 2015, Weber et al., 2015). To determine if T_{FH} could be depleted during ongoing chronic AAD by ICOS-L intervention, mice were exposed to HDM or ALT for 5 weeks. Given that local and systemic T_{FH} responses and hallmarks AAD features were established after 3 weeks of allergen exposure, at the start of week 4, mice were concomitantly administered an ICOS-L blocking (α -ICOS-L) antibody or an isotype control (2A3) antibody. Samples were collected at the end of week 5 (**Figure 4.1A**).

After 5 weeks of HDM exposure T_{FH} were induced within the mLN and lungs of HDM and 2A3 treated animals (**Figure 4.2A-E**). Administration of α -ICOS-L significantly reduced T_{FH} proportions in the mLN and lungs of HDM exposed animals (**Figure 4.2A-C**). Consistent with this total numbers of T_{FH} in the lungs were significantly reduced after α -ICOS-L treatment compared to 2A3 controls, however the reduction of T_{FH} numbers in the mLN was not statistically significant (**Figure 4.2D, E**). Similarly, during ALT driven AAD, T_{FH} were induced within the mLN and lungs of ALT and 2A3 treated animals compared to PBS controls (**Figure 4.3A-E**). α -ICOS-L treatment successfully diminished T_{FH} frequency and numbers at both sites (**Figure 4.3A-E**). Taken together, this data showed that even after the establishment of chronic AAD, T_{FH} were effectively reduced by α -ICOS-L administration.

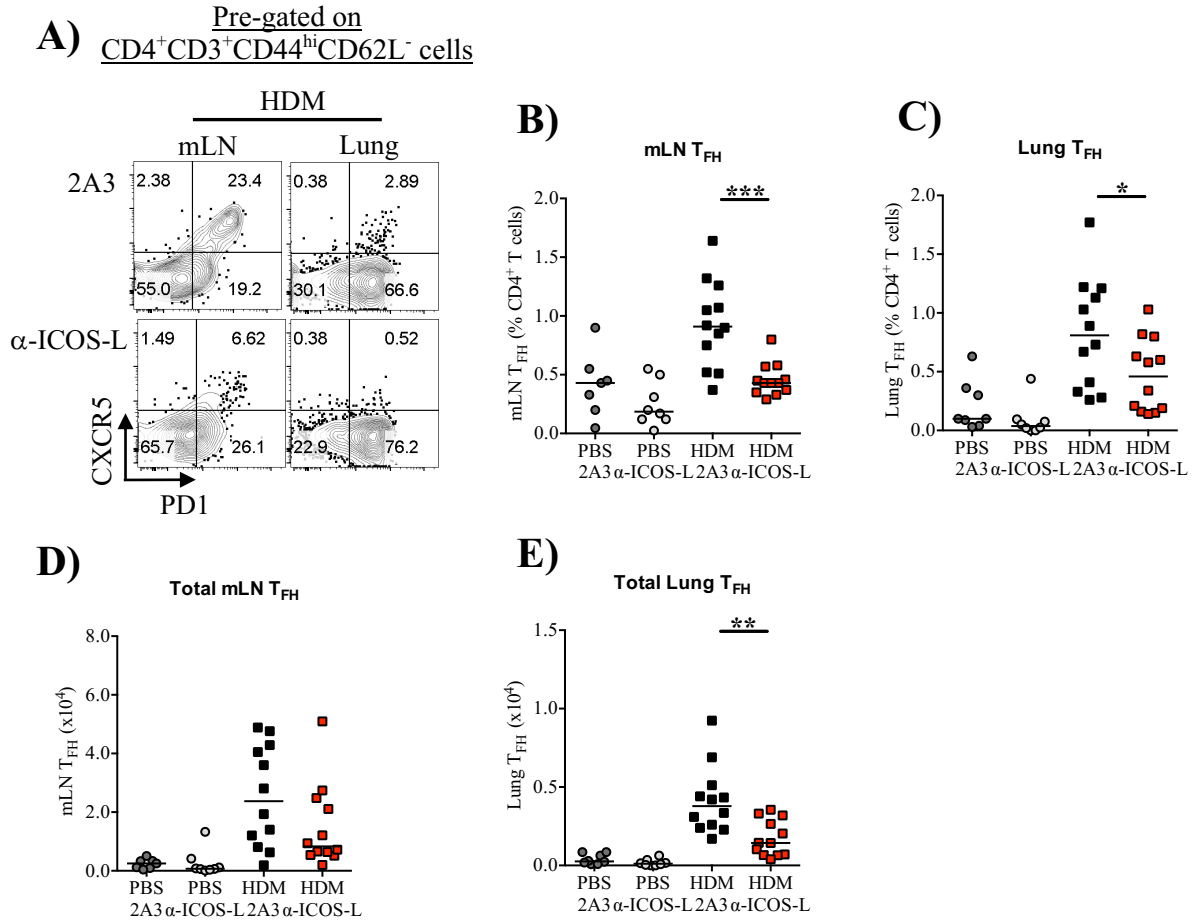


Figure 4.2: ICOS/ICOS-L interactions are required to sustain T_{FH} during chronic HDM driven AAD.

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the frequency and number of T_{FH} within cellular compartments. **A)** Representative flow plots of mLN and lung T_{FH} following HDM and 2A3 or α -ICOS-L treatment, quantification in **B)** mLN T_{FH} frequency, **C)** Lung T_{FH} frequency, **D)** mLN T_{FH} numbers, **E)** Lung T_{FH} numbers. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data shown is pooled from two independent experiments, n=8-12.

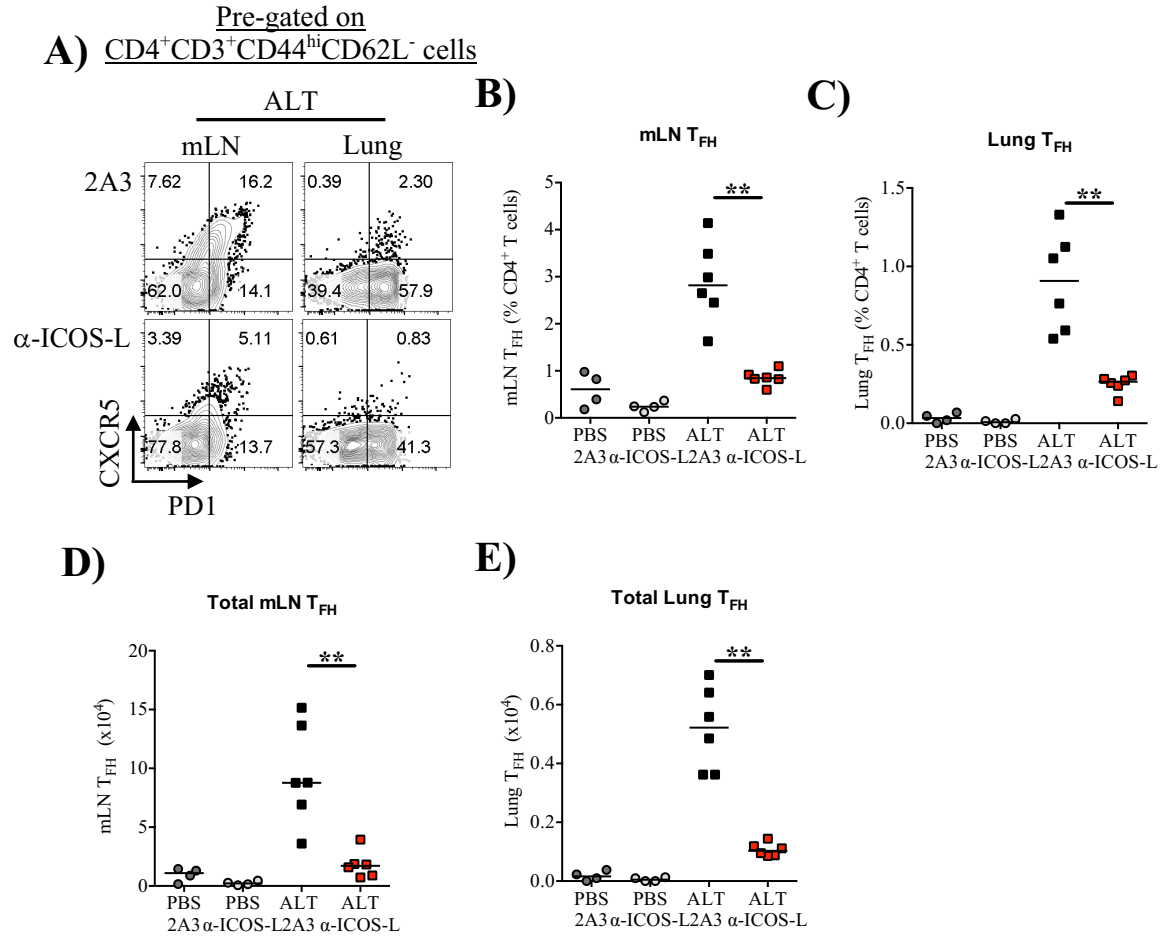


Figure 4.3: ICOS/ICOS-L interactions are required to sustain T_{FH} during chronic ALT driven AAD.

Adult female BALB/c mice were exposed (i.n) to 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the frequency and number of T_{FH} within cellular compartments. **A)** Representative flow plots of mLN and lung T_{FH} following ALT and 2A3 or α -ICOS-L treatment, quantification in **B)** mLN T_{FH} frequency, **C)** Lung T_{FH} frequency, **D)** mLN T_{FH} numbers, **E)** Lung T_{FH} numbers. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data is based on one experiment, $n = 4-6$.

4.3.2. Loss of T_{FH} impairs GC B cell responses

GC B cells are dependent on T_{FH} for their generation and rely on survival signals and cues from T_{FH} to proliferate and differentiate into memory cells and plasma cells (Linterman et al., 2010, Zotos et al., 2010, Weinstein et al., 2014). Consistent with this requirement, HDM exposed mice given 2A3 had increased mLN GC B cell proportions compared to PBS treated mice and this was dramatically decreased by α -ICOS-L intervention (**Figure 4.4A-B**). However, as was seen for mLN T_{FH} the reduction in mLN GC B cell number was not significant (**Figure 4.4C**). ALT exposure also induced a substantial GC B cell population within the mLN (**Figure 4.4D, E**). α -ICOS-L intervention did not alter the proportion of mLN GC B cells in ALT exposed animal but it did cause a significant reduction in the number (**Figure 4.4D, E**). In both aeroallergen models, allergen exposure induced GC B cell populations locally within the lung and these populations were depleted with α -ICOS-L intervention both by proportion and total number (**Figure 4.4A, F-I**). This data shows that along with reductions in T_{FH}, the T_{FH} dependent GC B cell population was also reduced by therapeutic α -ICOS-L administration.

A major output of the GC response is the generation of plasma cells therefore this population was also assessed. Plasma cells were identified in the mLN and lungs of aeroallergen treated animals but their proportions were similar in allergen exposed animals given α -ICOS-L compared to those given 2A3 (**Figure 4.5A**). The number of plasma cells in the mLN of mice given HDM and α -ICOS-L was similar to 2A3 treated controls (**Figure 4.5B**). Comparable results were observed in the ALT study (**Figure 4.5C**). However, α -ICOS-L intervention did reduced plasma cell numbers in the lungs of allergen treated animals compared to 2A3 treated controls (**Figure 4.5D, E**). Thus α -ICOS-L intervention was capable of reducing lung plasma cell numbers fitting with the reduction in GC B cell populations following ICOS-L blockade.

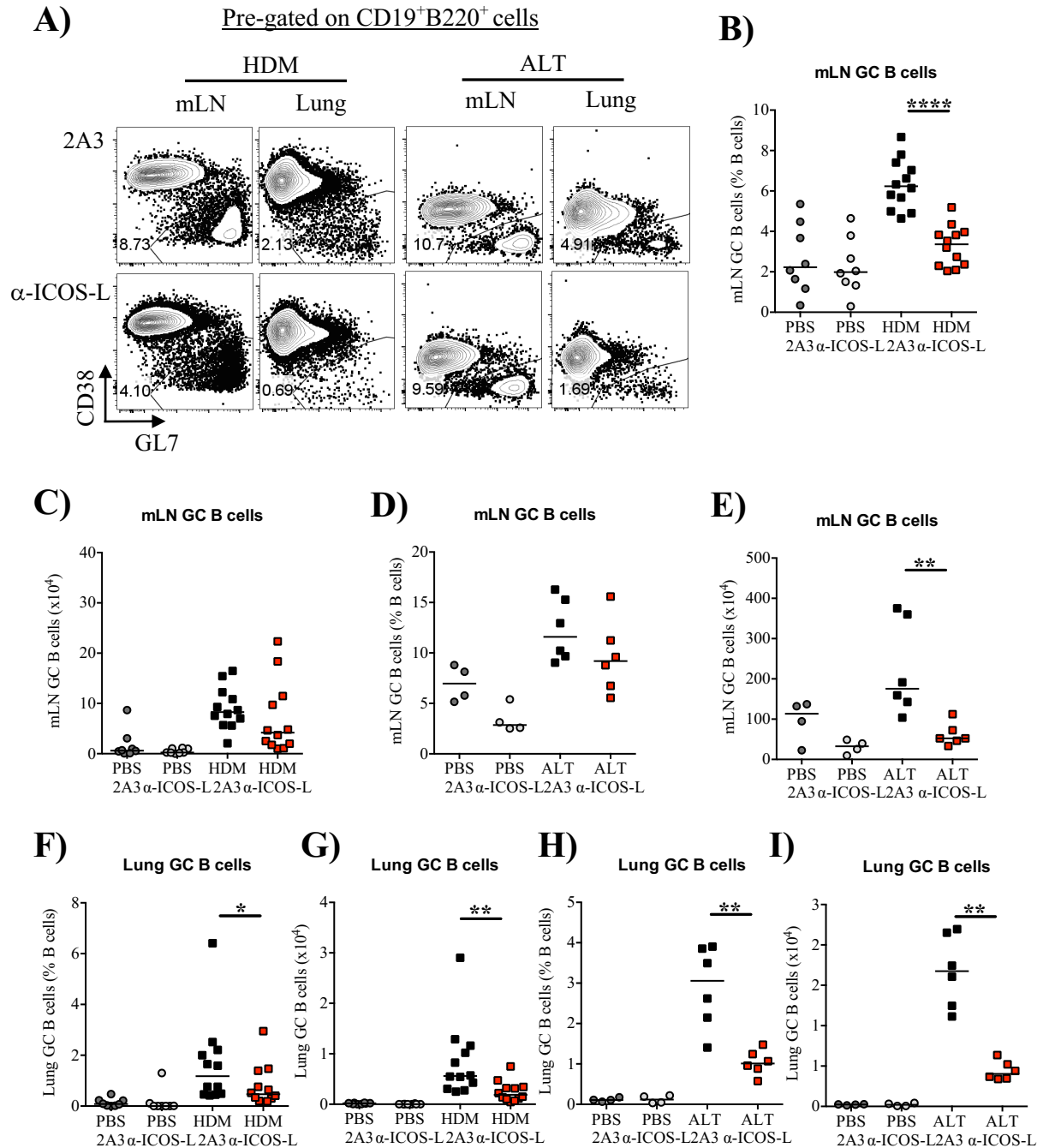


Figure 4.4: ICOS/ICOS-L interactions are required to sustain GC B cell responses

Adult female BALB/c mice were exposed (i.n) to 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the frequency and number of GC B cells within cellular compartments. **A)** Representative flow plots of mLN and lung GC B cells following allergen and 2A3 or α -ICOS-L treatment, quantification in **B-C)** HDM study mLN GC B cells, **B)** frequency, **C)** number, **D-E)** ALT study mLN GC B cells, **D)** frequency, **E)** number, **F-G)** HDM study lung GC B cells, **F)** frequency, **G)** number, **H-I)** ALT study Lung GC B cells number, **H)** frequency, **I)** number. Statistical significance was determined using a Mann Whitney U test. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT experiment was performed once, $n = 4-6$.

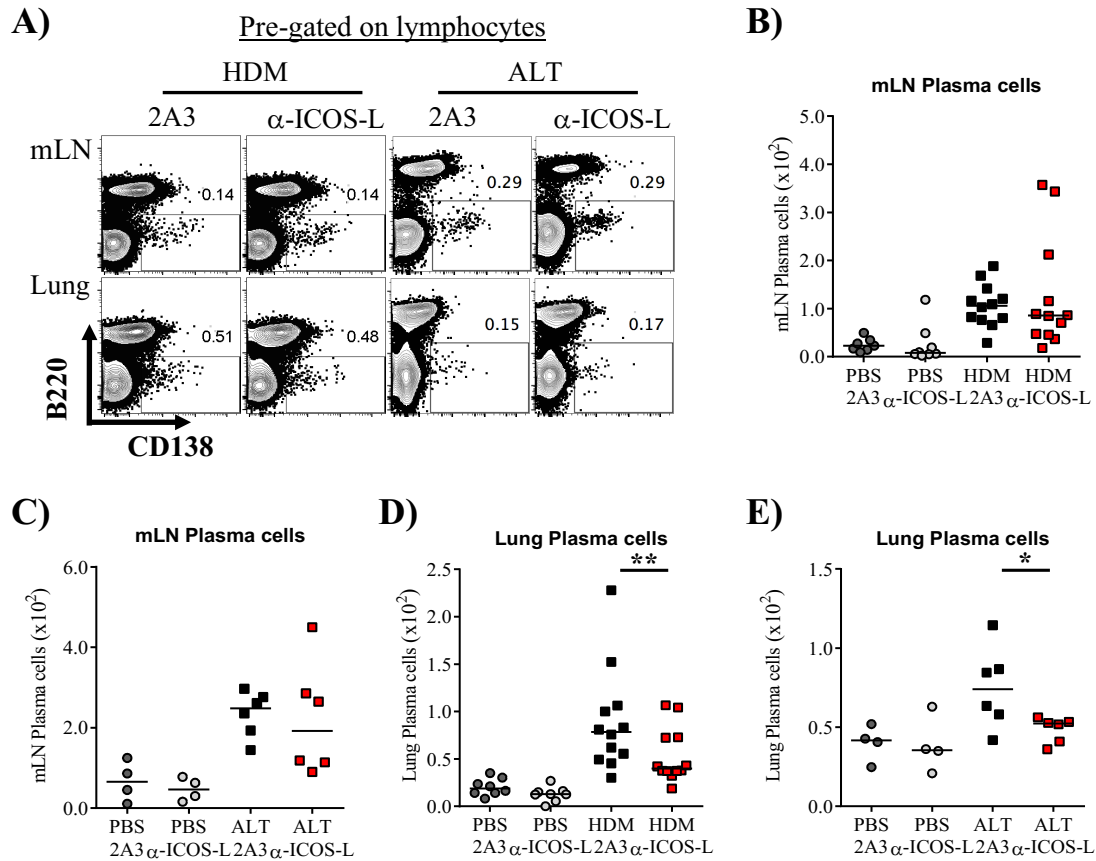


Figure 4.5: Blocking ICOS/ICOS-L interactions reduces lung plasma cell numbers
 Adult female BALB/c mice were exposed (i.n) to 25 µg HDM, 10 µg ALT or 25 µl PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg α-ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A)** Representative flow plots of mLN and lung plasma cells frequencies following HDM or ALT and 2A3 or α-ICOS-L treatment. **B-E)** Plasma cells numbers are quantified, **B)** mLN plasma cells – HDM study, **C)** mLN plasma cells – ALT study, **D)** Lung plasma cells – HDM study, **E)** Lung plasma cells – ALT study. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT experiment was performed once, $n = 4-6$.

4.3.3. T_{FH} depletion during chronic AAD alters IgE

Allergen specific antibody, in particular IgE, is a hallmark feature of AAD (Lambrecht and Hammad, 2015). T_{FH} are critical for IgE production during AAD (Kobayashi et al., 2017, Vijayanand et al., 2012), therefore the impact of α-ICOS-L on allergen specific antibody was

assessed. HDM and ALT specific IgG1 were not altered by α -ICOS-L treatment and were comparable to 2A3 treated animals (**Figure 4.6A, B**). In contrast HDM specific IgE was significantly reduced by α -ICOS-L intervention (**Figure 4.6C**), while ALT specific IgE showed a trend to decrease (**Figure 4.6D**). In accordance with this mast cell protease 1 (MCPT1) which was detectable in the serum of HDM exposed animals given 2A3 was reduced by α -ICOS-L treatment (**Figure 4.6E**). This difference was not observed in the ALT treated animals (**Figure 4.6F**). Taken together, this data implies that α -ICOS-L treatment was able to deplete T_{FH} and GC B cells and resulted in reductions in allergen specific IgE.

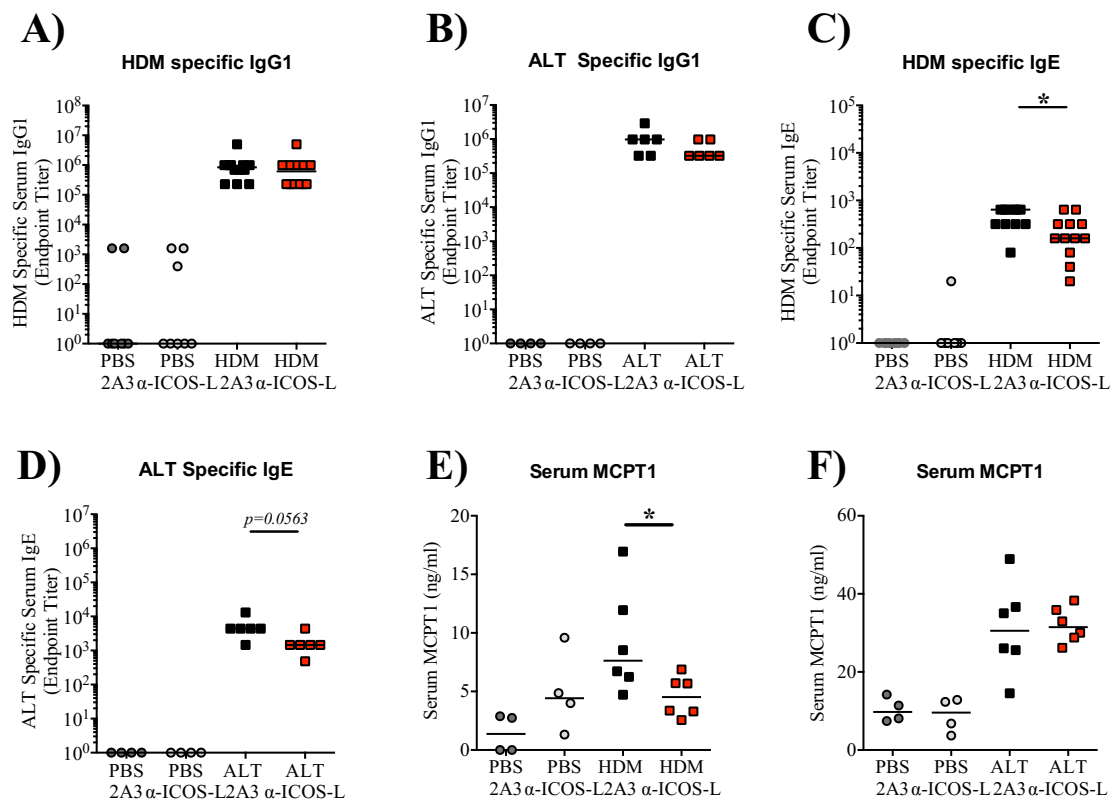


Figure 4.6: Depleting T_{FH} results in reduced allergen specific IgE.

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A-D**) Allergen specific antibody was detected by ELISA by serially diluting serum and endpoint titers were calculated. **A)** HDM specific IgG1, **B)** ALT specific IgG1, **C)** HDM specific IgE, **D)** ALT specific IgE. **E-F**) Mast cell protease 1 (MCPT1) in the serum was analysed by ELISA **E)** HDM driven allergic disease, **F)** ALT driven allergic disease. Statistical significance was determined using a Mann Whitney U test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. HDM data is pooled from two independent experiments, $n=8-12$. ALT experiment was performed once, $n=4-6$.

4.3.4. ICOS-L blockade does not affect lung IL-4 or IL-21 concentrations

The two major cytokines produced by T_{FH} are IL-4 and IL-21, therefore given the fall in T_{FH} populations after α -ICOS-L intervention, the levels of both cytokines was also measured. Despite the reductions in T_{FH} in the lungs and mLN, levels of lung IL-4 and IL-21 were unchanged in allergen and α -ICOS-L treated animals compared to 2A3 treated controls (**Figure 4.7A-D**). The type 2 cytokine, IL-4 was increased in the lungs of allergen treated animals compared to PBS controls but was unchanged between the two allergen treated groups (**Figure 4.7A, B**). Similar results were observed for lung IL-21 (**Figure 4.7C, D**). Taken together this suggests that despite the loss of T_{FH} within the model, other cells may be capable of maintaining IL-4 and IL-21 levels in the lung tissue.

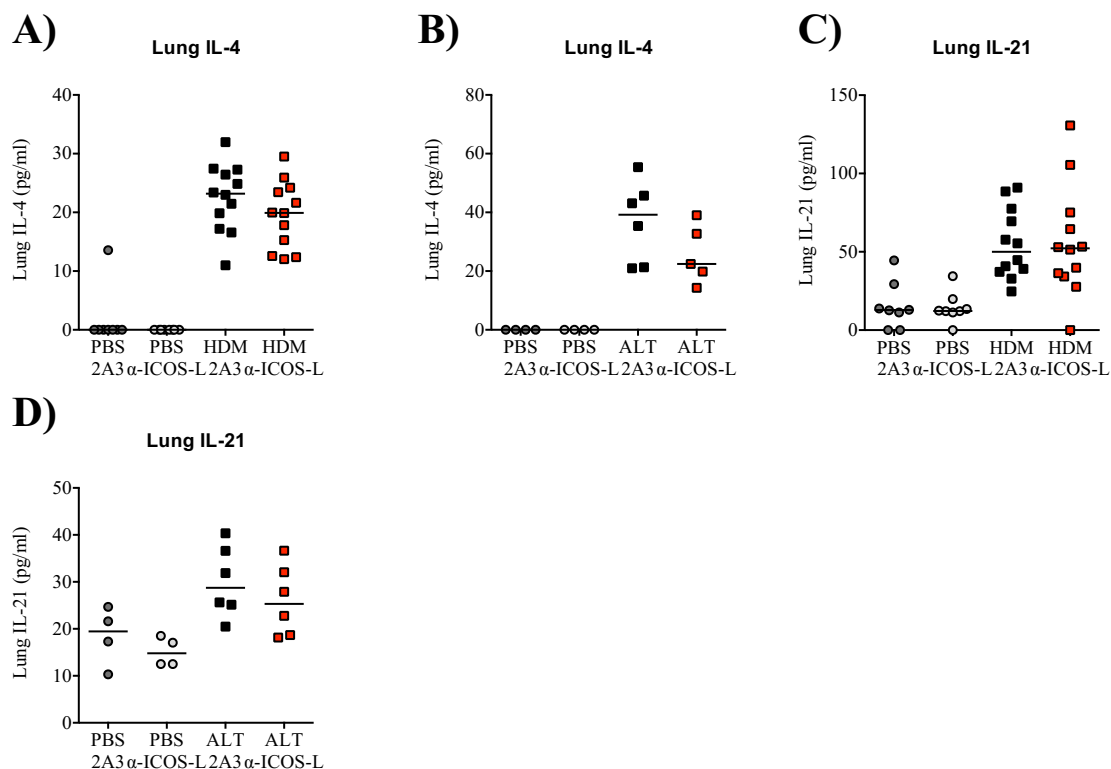


Figure 4.7: Pulmonary IL-4 and IL-21 are unchanged by interfering with ICOS/ICOS-L interactions

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. IL-4 and IL-21 were measured in lung homogenate by ELISA. **A)** IL-4 – HDM study, **B)** IL-4 – ALT study, **C)** IL-21– HDM study, **D)** IL-21 – ALT study. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT data is based on one experiment, $n = 4-6$.

4.3.5. ICOS-L blockade reduced pulmonary inflammation

Airway inflammation is a classical feature of AAD, therefore the impact of therapeutic α -ICOS-L intervention was assessed. Allergen exposure increased the number of cells in the lungs and airways of mice given the isotype control, 2A3 compared to PBS controls (**Figure 4.8A-D**). While, α -ICOS-L administration reduced aeroallergen induced cellular infiltration into the lungs (**Figure 4.8A, B**). Additionally, α -ICOS-L also decreased the influx of cells into the airways of HDM exposed animals compared to HDM and 2A3 treated controls (**Figure 4.8C**). However, α -ICOS-L did not alter the number of cells in the BAL of ALT exposed animals (**Figure 4.8D**).

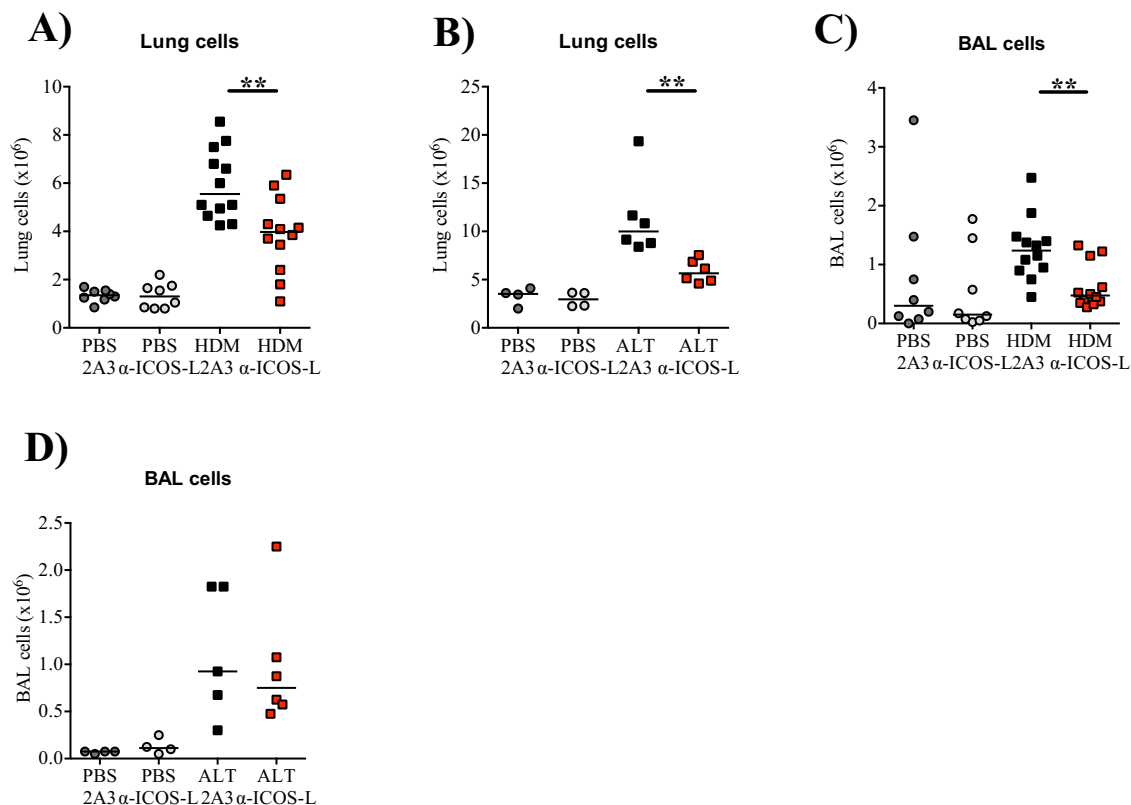


Figure 4.8: Interfering with ICOS/ICOS-L interactions reduces pulmonary inflammation. Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A-D**) Total number of cells were counted within the lungs and BAL. **A**) Lung cells in HDM study, **B**) Lung cells in ALT study, **C**) BAL cells in HDM study, **D**) BAL cells in ALT study. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data shown is pooled from two independent experiments, $n = 8-12$. ALT data is based on one experiment, $n = 4-6$.

Another hallmark of allergic disease is the influx of eosinophils in the lungs and the airways. Together with the fall in cellular influx into the lungs, the number of lung eosinophils was reduced in aeroallergen and α -ICOS-L treated mice compared to 2A3 controls (**Figure 4.9A, B**). This appeared to be due to an overall reduction in inflammation as eosinophil proportions were similar between allergen and 2A3 or α -ICOS-L treated mice (**Figure 4.9C, D**). IL-5 and eotaxin-2 are important for the survival and recruitment of eosinophils into the lungs and airways (Lambrecht and Hammad, 2015, Forssmann et al., 1997, Foster et al., 1996). Both mediators were increased by aeroallergen exposure and were unchanged by α -ICOS-L intervention (**Figure 4.9E-H**). Therefore, α -ICOS-L was able to improve aeroallergen induced pulmonary inflammation, characterised by a fall in eosinophils, but this did not appear to be due to a direct impairment of eosinophil recruitment into the lungs.

4.3.6. ICOS-L blockade does not affect mucus hyper-secretion, collagen deposition or airway smooth muscle thickening

Mucus hyper-secretion, collagen deposition and smooth muscle layer thickening are hallmark features of AAD. To determine if ICOS-L blockade affected mucus production goblet cells around the airways were PAS stained and quantified using an arbitrary scoring system. PAS positive cells could be visualised around the airways of allergen treated animals given either 2A3 (**Figure 4.10A**) or α -ICOS-L (**Figure 4.10 B**). The PAS score was increased in HDM exposed animals compared to PBS treated controls but no difference was observed between HDM and 2A3 or α -ICOS-L treated animals (**Figure 4.10C**). Next, the impact of α -ICOS-L intervention on collagen deposition was assessed. Similar collagen deposition was observed around the airways between HDM and 2A3 or α -ICOS-L administered mice (**Figure 4.10D, E**) and no difference in total collagen within the lungs was observed between the two HDM treated groups (**Figure 4.10F**). Moreover, in the asthmatic lung ASM cell hypertrophy and hyperplasia results in ASM layer thickening around the airways. ASM staining around the airways was similar between the HDM and 2A3 and HDM and α -ICOS-L treated groups

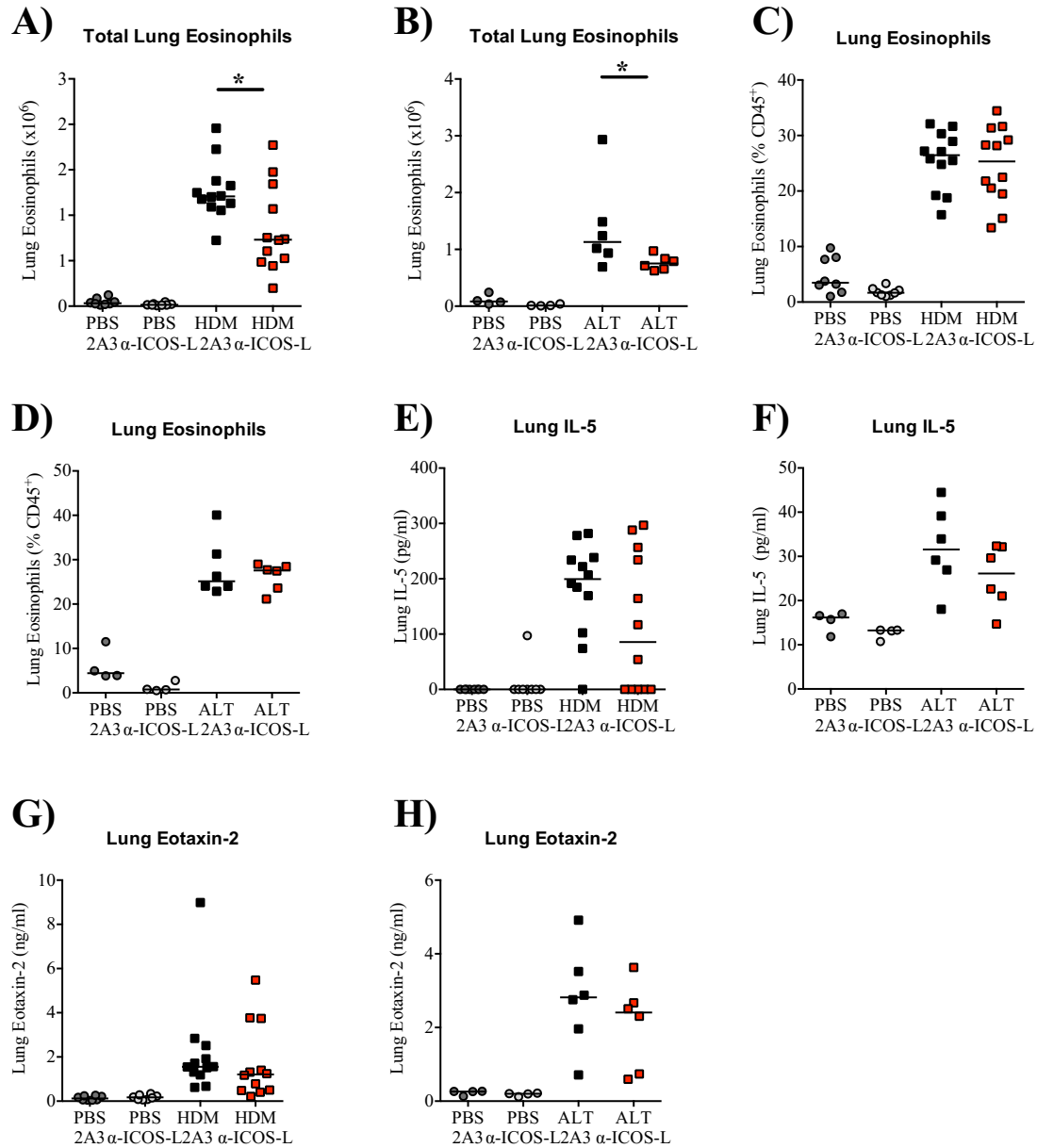


Figure 4.9: Blocking ICOS/ICOS-L interactions does not directly impair eosinophil recruitment to the lungs.

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A-D**) Flow cytometry was used to assess the frequency and number of lung eosinophils. Total number of lung eosinophils are assessed in the **A**) HDM study, **B**) ALT study. Proportions of lung eosinophils are assessed in the **C**) HDM study, **D**) ALT study. **E-H**) IL-5 and Eotaxin-2 concentrations within lung homogenate were assessed by ELISA. **E-F**) Lung IL-5, **E**) HDM study, **F**) ALT study, **G-H**) Eotaxin-2, **G**) HDM study, **H**) ALT study. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT data is based on one experiment, $n = 4-6$.

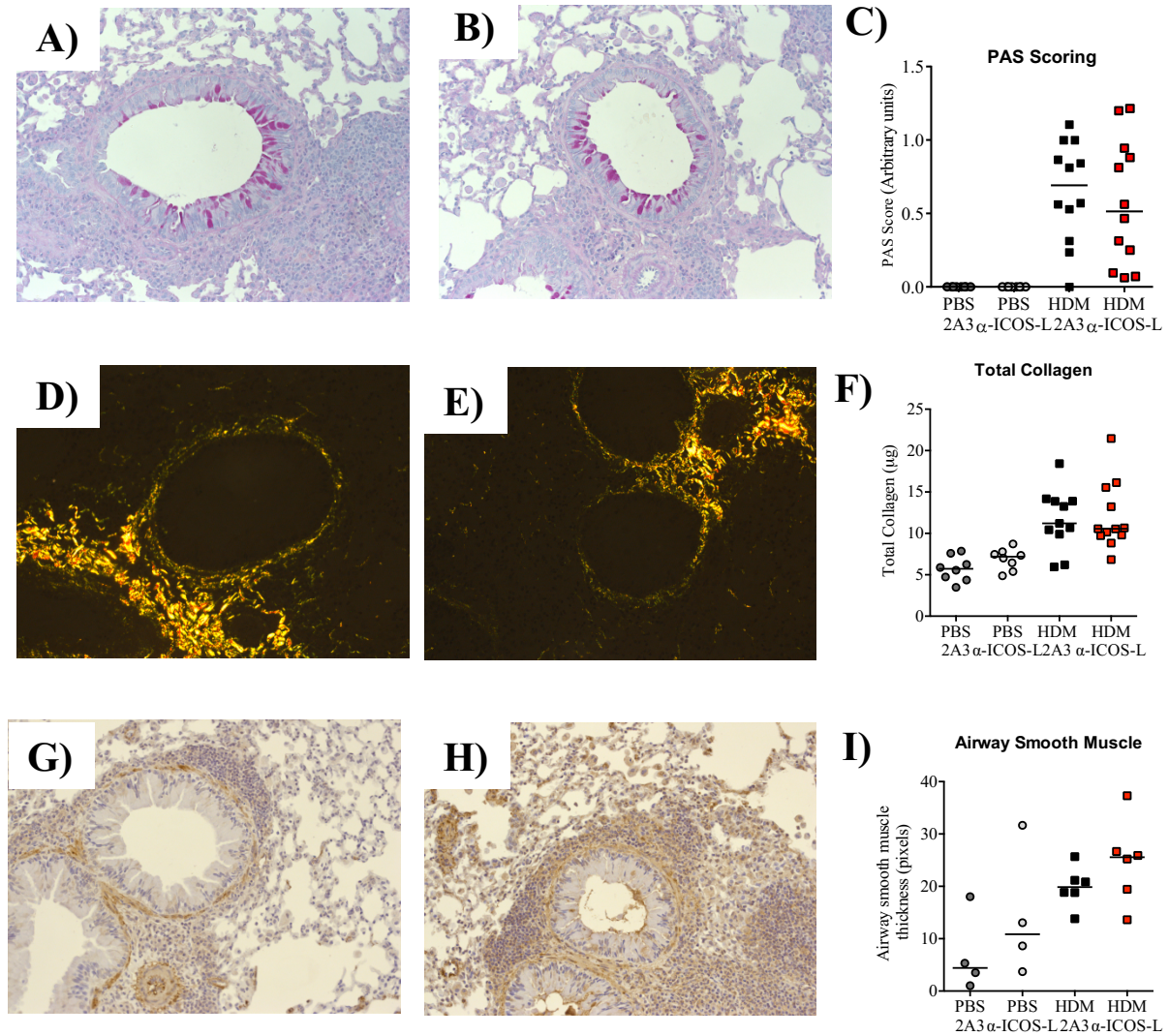


Figure 4.10: Therapeutic ICOS-L blockade does not alter mucus hyper-secretion, collagen deposition or airway smooth muscle thickening.

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A-B)** Representative images of Periodic Acid-Schiff (PAS) staining on paraffin embedded formalin fixed (PEFF) lung sections used to score mucus hyper-secretion. **A)** HDM+2A3, **B)** HDM+ α -ICOS-L, **C)** Quantification of PAS scoring. **D-E)** Representative staining of Pico-Sirus Red on PEFF lung used to determine collagen I and III deposition. **D)** HDM+2A3, **E)** HDM+ α -ICOS-L, **F)** Total collagen in the lungs determined by Sircol soluble collagen assay. **G-H)** Representative staining of airway smooth muscle (ASM) cells and myofibroblasts identified by immunohistochemical staining of anti-smooth muscle actin antibody (α -SMA). **G)** HDM+2A3, **H)** HDM+ α -ICOS-L, **I)** ASM layer thickness was measured from α -SMA stained sections. Images were taken at x 20 magnification. HDM data is pooled from two independent experiments, n=8-12.

(**Figure 4.10G, H**), and no significant difference was observed in the size of the ASM layer between the two allergen treated groups (**Figure 4.10I**). Therefore, although therapeutic α -ICOS-L administration altered humoral immunity and pulmonary inflammation it had no obvious impact on ASM thickening, total collagen deposition or mucus hyper-secretion.

4.3.7. ICOS-L blockade improves allergen induced airway hyper-responsiveness

AHR is another hallmark feature of AAD, therefore the effect of interfering with ICOS/ICOS-L interactions on AHR was assessed. HDM exposed animals given 2A3 had increased airway resistance and elastance and decreased airway compliance compared to PBS controls (**Figure 4.11A**). α -ICOS-L treatment decreased HDM induced airway resistance and elastance and increased airway compliance (**Figure 4.11A**). Similar results were observed for ALT driven allergic airway disease where α -ICOS-L administration reduced airway resistance and elastance (**Figure 4.11B**). However, α -ICOS-L treatment failed to alter ALT driven airway compliance (**Figure 4.11B**). Overall, therapeutic α -ICOS-L treatment was able to dampen aeroallergen induced AHR resulting in improved lung function.

4.3.8. ICOS-L blockade reduced pulmonary IL-13

IL-13 has been shown to be an important driver of AHR (Grünig et al., 1998, Wills-Karp et al., 1998), therefore we assessed whether the level of pulmonary IL-13 was altered by α -ICOS-L intervention. HDM exposure increased lung and BAL concentrations of IL-13 (**Figure 4.12A, B**). Mice administered α -ICOS-L in combination with HDM had dramatically decreased IL-13 within the lungs compared to 2A3 treated animals (**Figure 4.12A**). However, α -ICOS-L did not alter IL-13 concentrations in the BAL of HDM exposed animals (**Figure 4.11B**). In ALT treated animals, the concentration of IL-13 was increased in the lungs and airways compared to PBS controls, but was unaffected by α -ICOS-L treatment (**Figure 4.12C, D**), however α -ICOS-L treatment significantly reduced IL-13 within the BAL compared to isotype treated controls

(Figure 4.12D). Overall, this data suggests that α -ICOS-L treatment was sufficient to decrease pulmonary IL-13 induced in both aeroallergen models of AAD.

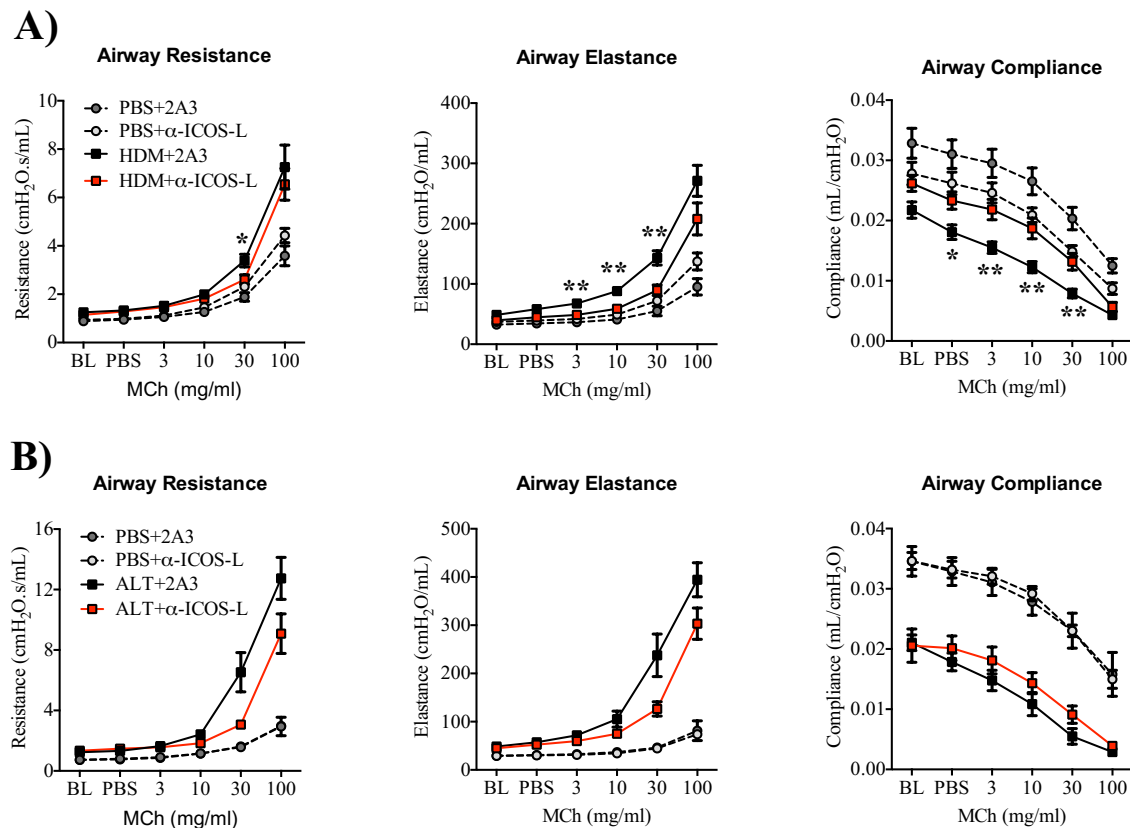


Figure 4.11: Blocking ICOS/ICOS-L interactions improves allergen induced airway hyperresponsiveness

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Airway hyperresponsiveness was determined by exposing mice to 0-100mg/ml methacholine (MCh). Airway resistance, elastance and compliance were then measured by flexiVent assessment. **A)** HDM study, **B)** ALT study. Curves display mean $\pm$ SEM. * = statistical difference between allergen+2A3 and allergen+ α -ICOS-L. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT data is based on one experiment, $n = 4-6$.

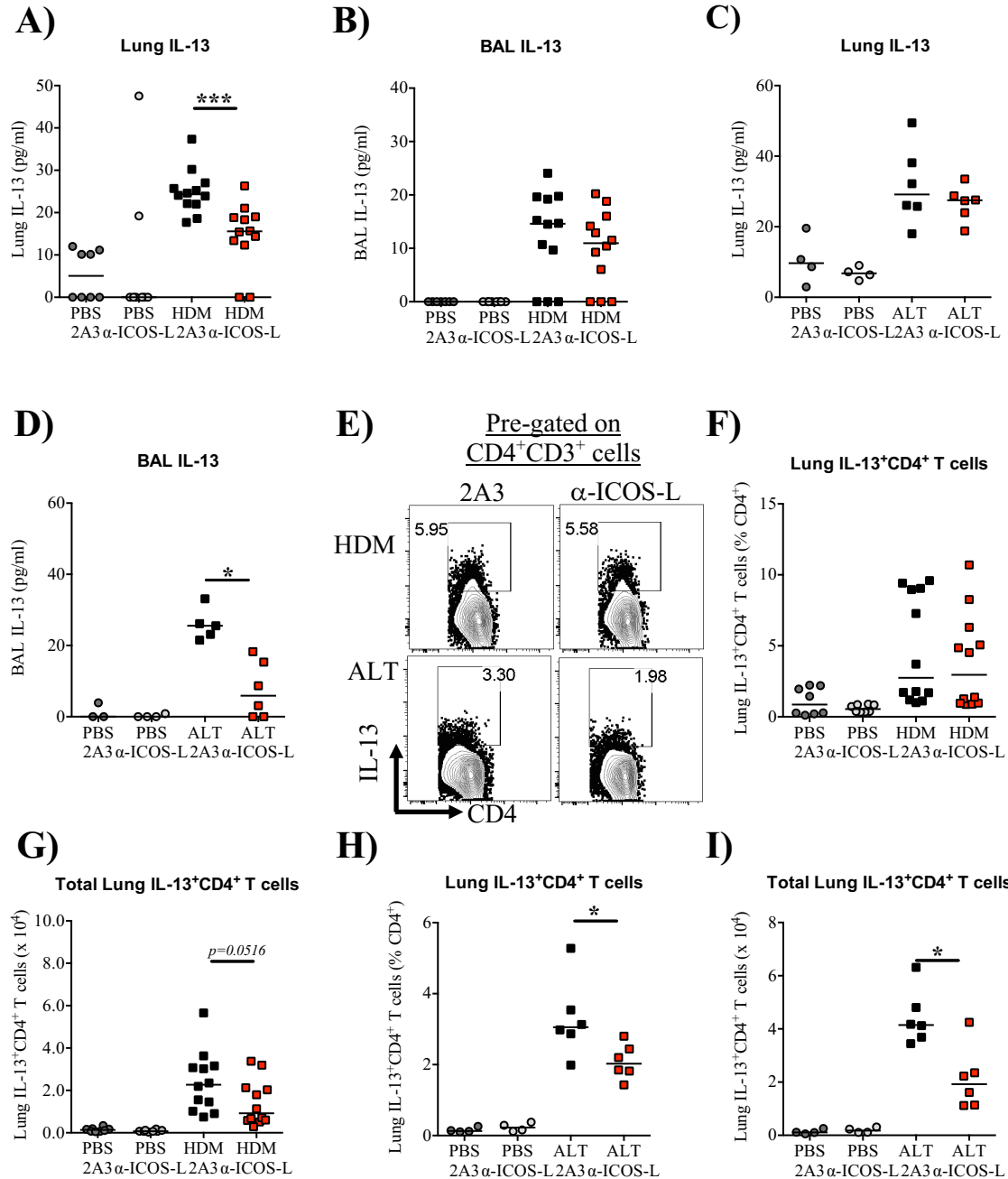


Figure 4.12: Blocking ICOS/ICOS-L interactions reduces pulmonary IL-13

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A-D)** IL-13 concentration within lung homogenate and BAL were assessed by ELISA. **A)** Lung IL-13 - HDM study, **B)** BAL IL-13 - HDM study. **C)** Lung IL-13 – ALT study, **D)** BAL IL-13 – ALT study. **E-I)** Flow cytometry was used to determine the frequency and number of IL-13⁺ CD4⁺ T cells within the lungs. **E)** Representative flow plots of IL-13⁺ CD4⁺ T cells in the lungs following allergen and 2A3 or α -ICOS-L treatment, data is quantified in **F-G)** HDM study, **F)** frequency, **G)** number, **H-I)** ALT study, **H)** frequency, **I)** number. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT data is based on one experiment, $n = 4-6$.

Given that pulmonary IL-13 was reduced, we investigated whether IL-13⁺ CD4⁺ T cells represented direct or indirect targets of α -ICOS-L administration. HDM exposure increased IL-13⁺ CD4⁺ T cells within the lungs and α -ICOS-L treatment did not alter the proportion of these cells within the lungs compared to 2A3 controls (**Figure 4.12E, F**). But the total number of IL-13⁺ CD4⁺ T cells showed a trend towards near significant decrease following α -ICOS-L treatment (**Figure 4.12G**). Interestingly, in the ALT study, mice exposed to ALT in combination with α -ICOS-L had decreased proportions and numbers of IL-13⁺ CD4⁺ T cells compared to 2A3 treated controls (**Figure 4.12E, H-I**). Taken together, this data shows that α -ICOS-L intervention during chronic AAD altered IL-13 production.

4.3.9. Late ICOS/ICOS-L administration decreases pulmonary IL-17A

IL-17A was increased in the lungs of HDM and ALT treated mice given 2A3 compared to PBS controls (**Figure 4.13A, B**). α -ICOS-L intervention reduced HDM induced IL-17A (**Figure 4.13A**), but had no effect on ALT induced disease (**Figure 4.13B**). The proportion of IL-17A⁺ CD4⁺ T cells was increased after HDM treatment but this was unaltered by therapeutic α -ICOS-L (**Figure 4.13C, D**). Consistent with IL-13⁺ CD4⁺ T cells, the total number of IL-17A⁺ CD4⁺ T cells was significantly reduced along with the overall fall in pulmonary inflammation (**Figure 4.13E**). ALT did not induce a strong IL-17A⁺CD4⁺ T cell response and this was unchanged by proportion or number (**Figure 4.13C, F-G**). Therefore, α -ICOS-L treatment did not directly target Th17 cells but resulted in a fall in IL-17A levels due to the overall decrease in lung inflammation.

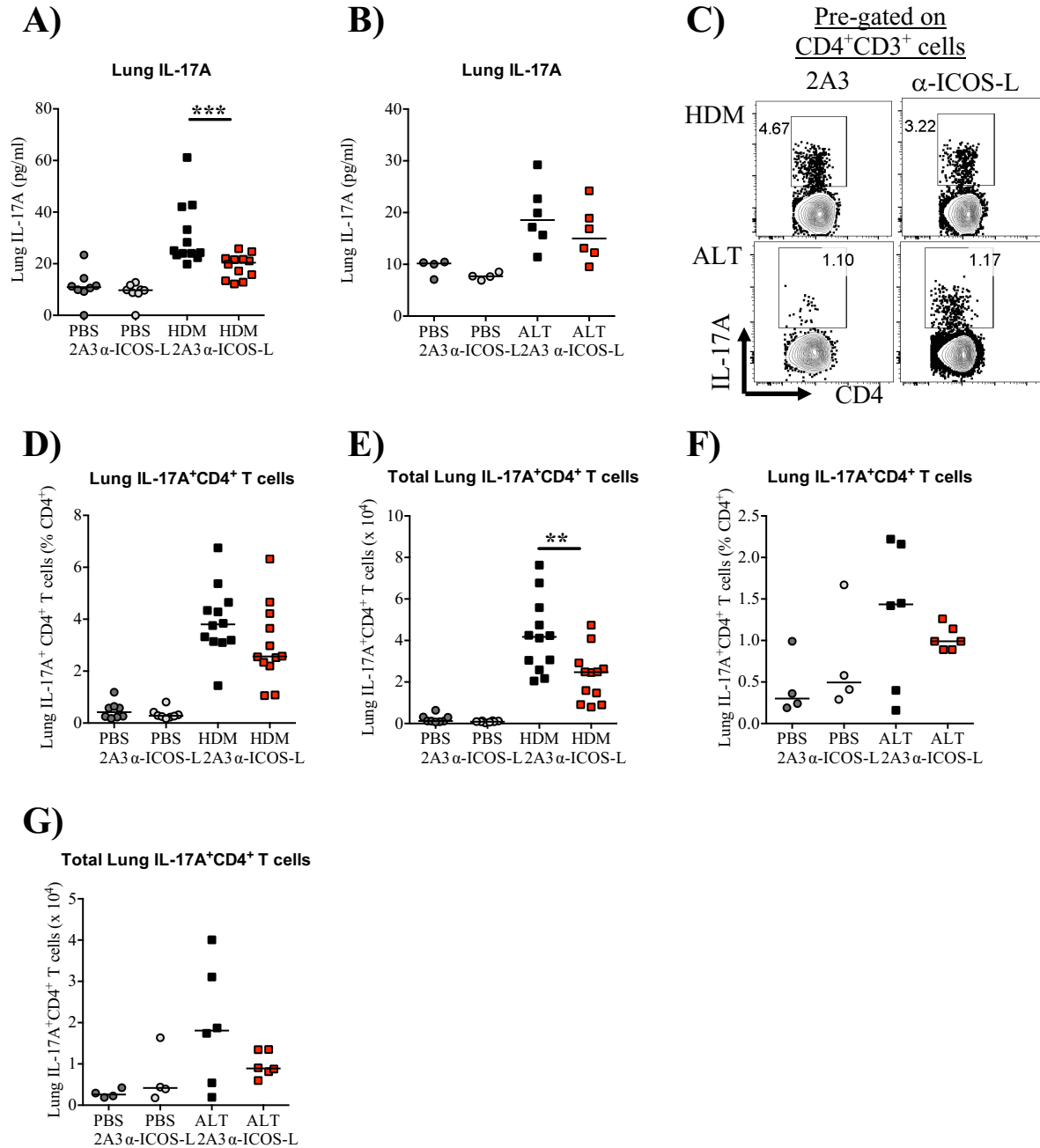


Figure 4.13: Blocking ICOS/ICOS-L reduces pulmonary IL-17A

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. **A-B)** Lung IL-17A concentration was measured within lung homogenate by ELISA. **A)** HDM study, **B)** ALT study. **C-G)** Flow cytometry was used to determine the frequency and number of IL-17A⁺ CD4⁺ T cells within the lungs. **C)** Representative flow plots of IL-17A⁺ CD4⁺ T cells in the lungs following allergen and 2A3 or α -ICOS-L treatment, data is quantified in **D-E)** HDM study, **D)** frequency, **E)** number, **F-G)** ALT, **F)** frequency, **G)** number. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT data is based on one experiment, $n = 4-6$.

4.3.10. ILCs are not direct targets of ICOS-L blockade

ICOS signalling has been demonstrated to be critical for ILC survival during homeostasis and disease (Maazi et al., 2015, Paclik et al., 2015, Kamachi et al., 2015). Furthermore, ILCs are a major producer of IL-13 during AAD and have been shown to be capable of driving AHR (Barlow et al., 2012), therefore ILCs were assessed after α -ICOS-L treatment. The proportion of IL-13⁺ ILCs was increased within the lungs of aeroallergen and 2A3 exposed animals compared to PBS controls and this was unchanged by the addition of α -ICOS-L (**Figure 4.14A-C**). Similar results, were observed with the proportion of IL-17A⁺ ILCs and α -ICOS-L intervention did not alter allergen induced cellular populations (**Figure 4.14A, D-E**). The number of lung IL-13⁺ ILCs and IL-17A⁺ ILCs were not significantly decreased by the α -ICOS-L intervention (**Figure 4.15A-D**), although there was a trend to reduction of both in HDM exposed animals after α -ICOS-L treatment fitting with the reduced overall cell count in the lungs of these mice (**Figure 4.15A, C**). Thus, ILCs did not appear to be direct targets of ICOS-

4.3.11. ICOS-L blockade reduces T regulatory cells

Tregs are anti-inflammatory cells, able to suppress AAD (Lewkowich et al., 2005) and ICOS expression is known to increase on Tregs during type 2 pathology (Redpath et al., 2013). Therefore, the impact of ICOS-L blockade on Tregs was also assessed. Following HDM exposure the proportion and number of mLN Foxp3⁺CD4⁺ T cells was unaffected by ICOS-L blockade compared to 2A3 treated controls (**Figure 4.16A, B-C**), while in the lungs both the number and proportion of Foxp3⁺CD4⁺ T cells was reduced (**Figure 4.16A, D-E**). In ALT driven allergic disease, the proportion and number of both mLN and lung Foxp3⁺ CD4⁺ T cells were reduced by ICOS-L treatment (**Figure 4.16A, F-I**). Taken together, this data shows ICOS-L intervention to affect Treg populations induced during chronic AAD but despite this the intervention was still capable of reducing hallmark features of the disorder.

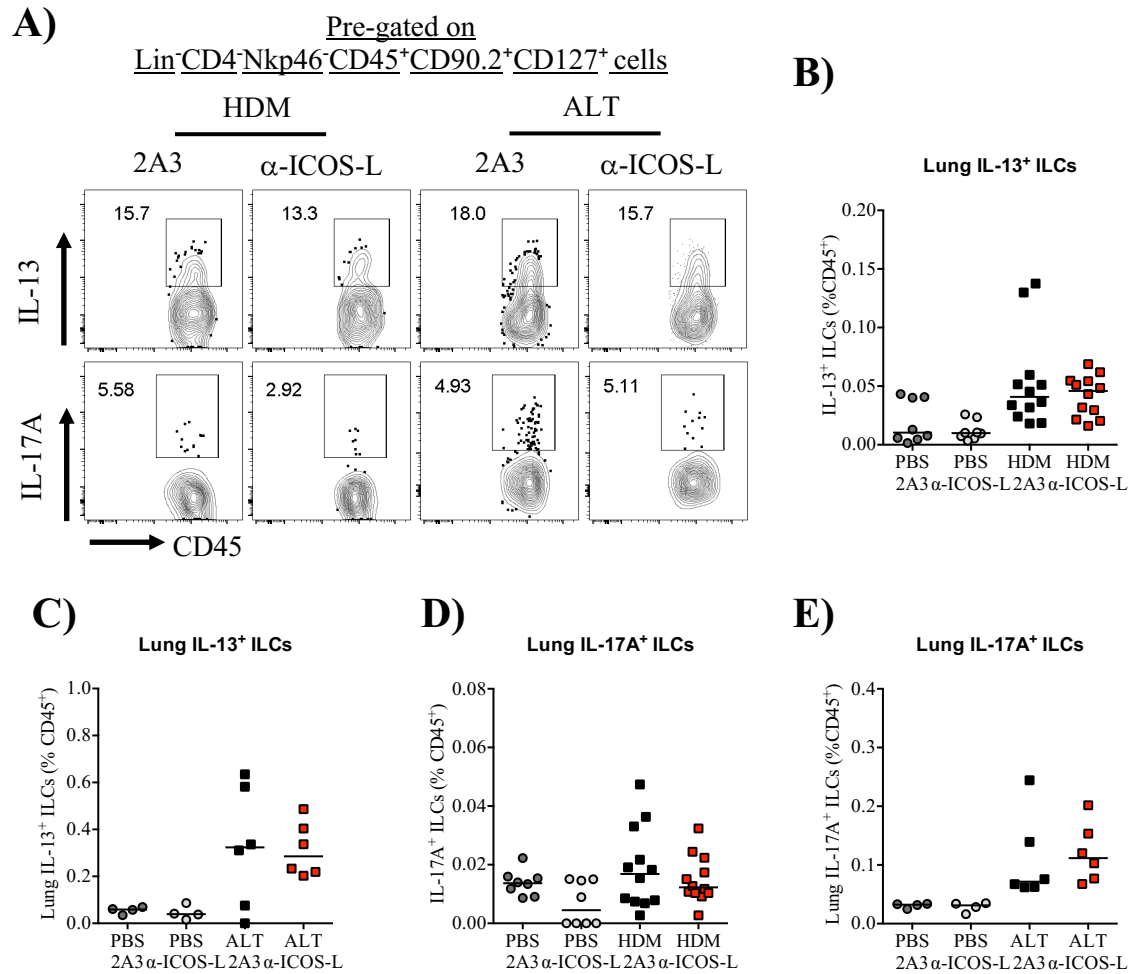


Figure 4.14: ILCs are not direct targets of ICOS-L blockade

Adult female BALB/c mice were exposed (i.n) to 25 μg HDM, 10 μg ALT or 25 μl PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μg α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the frequency IL-13⁺ and IL-17A⁺ ILCs within the lungs. **A)** Representative flow cytometry of IL-13⁺ and IL-17A⁺ ILCs in the lungs following allergen and 2A3 or α -ICOS-L treatment, data is quantified in **B-C**) IL-13⁺ ILCs, **B)** HDM study, **C)** ALT study, **D-E**) IL-17A⁺ ILCs, **D)** HDM study, **E)** ALT study. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT data is based on one experiment, $n = 4-6$.

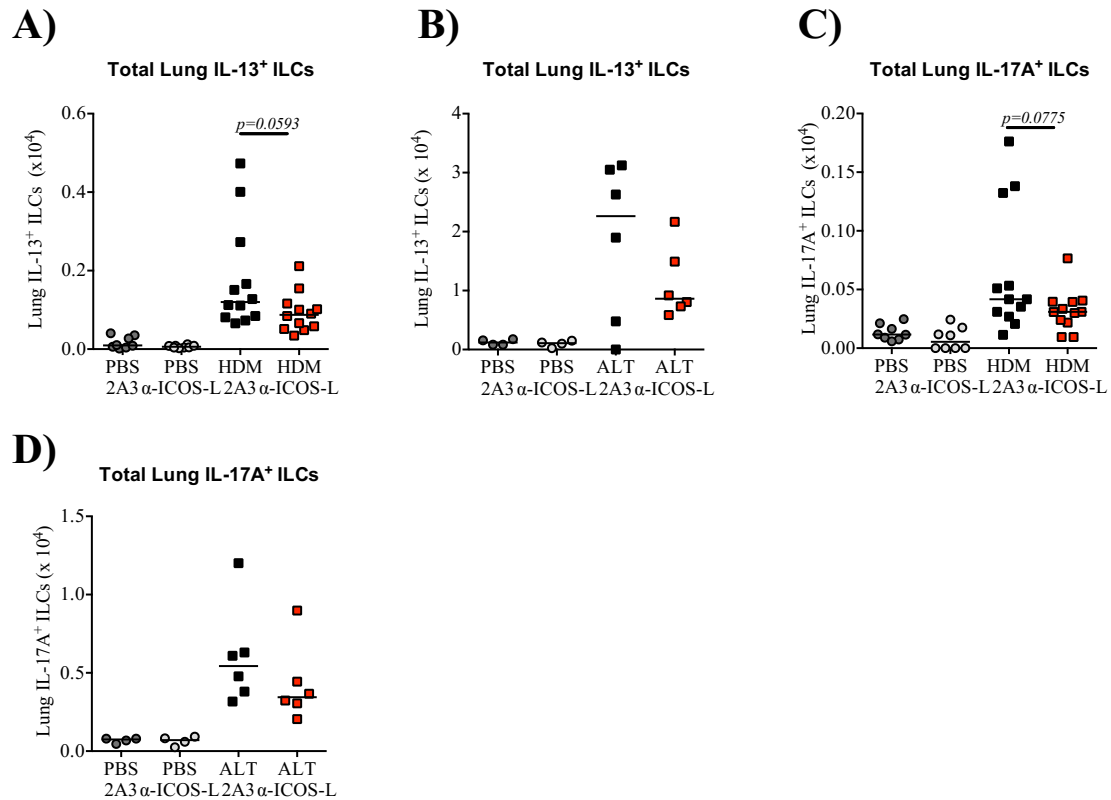


Figure 4.15: Blocking ICOS/ICOS-L does not alter IL-13⁺ or IL-17A⁺ ILC numbers

Adult female BALB/c mice were exposed (i.n) to 25 µg HDM, 10 µg ALT or 25 µl PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 µg α-ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the total number of IL-13⁺ and IL-17A⁺ ILCs within the lungs. ILC numbers are quantified in **A-B**) IL-13⁺ ILCs, **A**) HDM study, **B**) ALT study, **C-D**) IL-17A⁺ ILCs **C**) HDM study, **D**) ALT study. Statistical significance was determined using a Mann Whitney U test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. HDM data is pooled from two independent experiments, $n=8-12$. ALT data is based on one experiment, $n=4-6$.

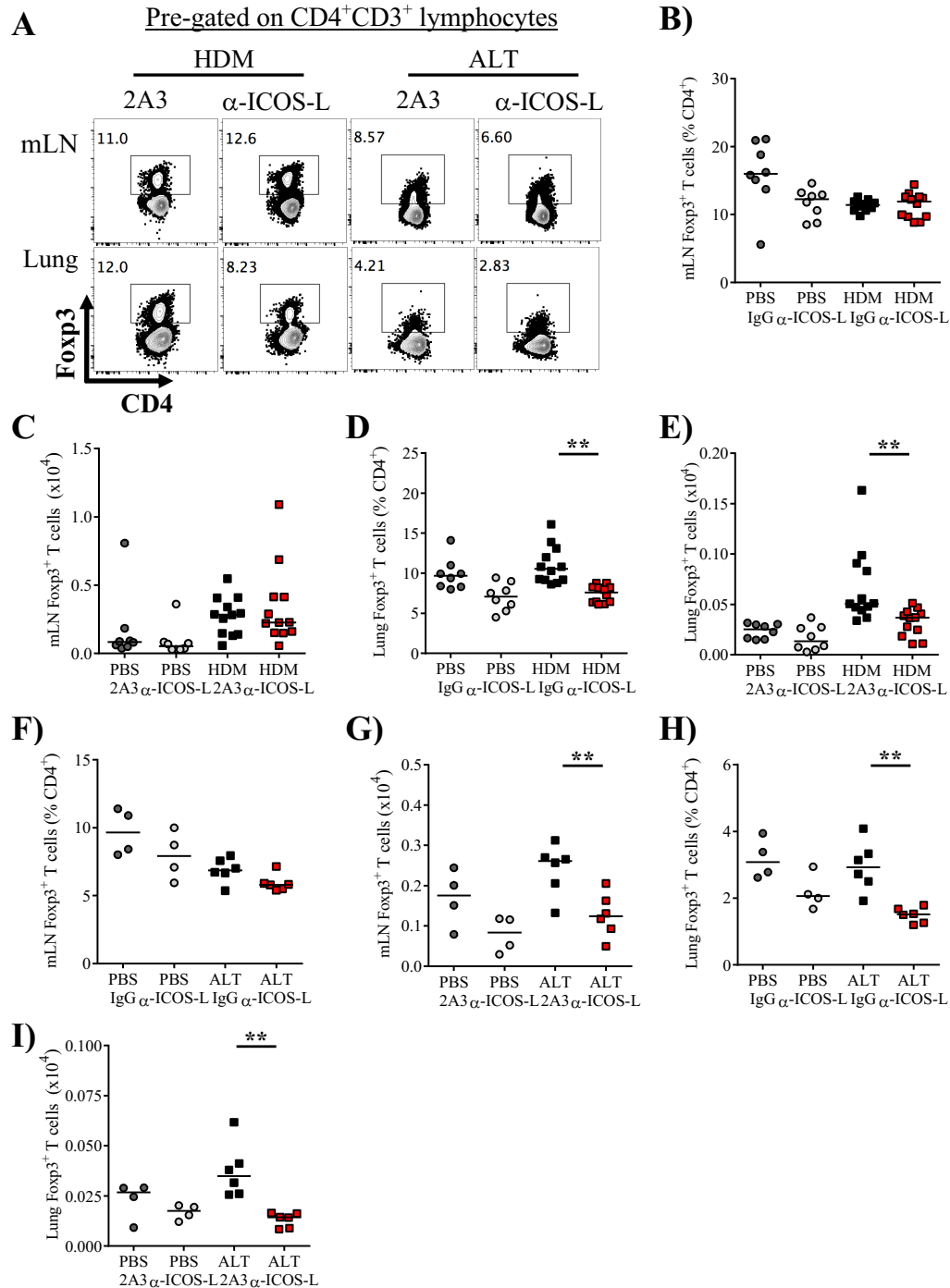


Figure 4.16: Therapeutic ICOS-L blockade reduces T regulatory cells

Adult female BALB/c mice were exposed (i.n) to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 3 times a week for 5 weeks. From the start of week 4 mice were also administered 150 μ g α -ICOS-L or 2A3 antibody (i.p) 3 times a week. Mice were culled at the end of week 5. Flow cytometry was used to determine the number of Foxp3⁺ CD4⁺ T cells within the mLN and lungs. **A)** Representative flow cytometry of Foxp3⁺ cells in allergen treated mice given 2A3 or α -ICOS-L. Pre-gated on CD4⁺CD3⁺ T cells. Foxp3⁺CD4⁺ T cell proportion and numbers were assessed **B-E)** HDM study, **B)** mLN proportion **C)** mLN total, **D)** Lung proportion, **E)** Lung total. **F-I)** ALT study, **F)** mLN proportion **G)** mLN total, **H)** Lung proportion, **I)** Lung total. Statistical significance was determined using a Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. HDM data is pooled from two independent experiments, $n = 8-12$. ALT experiment was performed once, $n = 4-6$.

4.3.12. T_{FH} do not produce IL-13

Given that T_{FH} appeared to be the major pro-inflammatory T cell population directly targeted by α -ICOS-L intervention in both aeroallergen models and a large reduction in IL-13 was observed, whether T_{FH} directly produce IL-13 was assessed. To do this IL-13^{+/GFP} reporter (IL-13^{GFP}) mice were utilised, where IL-13⁺ cells were reported as GFP⁺ cells. In HDM exposed IL-13^{GFP} mice, CXCR5 expression was segregated from IL-13^{GFP} expression in both the mLN and lungs (**Figure 4.17A**). Consistent with this IL-13^{GFP} expression was predominately identified within the non-T_{FH} effector (T_{EFF}) population rather than the T_{FH} compartment within the HDM treated animals (**Figure 4.17B-E**). Taken together, this shows that even during chronic AAD, T_{FH} are not dysregulated to produce IL-13 and drive AAD. Therefore, the observed fall in IL-13 was due to the overall decrease in pulmonary inflammation caused by α -ICOS-L intervention.

The results of this data are summarised in **Figure 4.18** (Uwadiae et al., 2018). Briefly, exposure to aeroallergens drives chronic AAD, characterised by a humoral immune response consisting of local and systemic T_{FH}, GC B cell responses and allergen specific antibody. In addition to other hallmarks of AAD, including AHR and airway inflammation. α -ICOS-L treatment targeted both local and systemic T_{FH} populations impairing GC B cells leading to reduced IgE. This intervention additionally improved AHR and pulmonary inflammation. The observed improvements appeared to be due to a drop in pulmonary IL-13 from Th2 cells and ILC2s which were indirect targets of α -ICOS-L intervention. Overall, IL-13 was the major type 2 cytokine reduced following ICOS-L blockade and thus may be responsible for improved AAD phenotype.

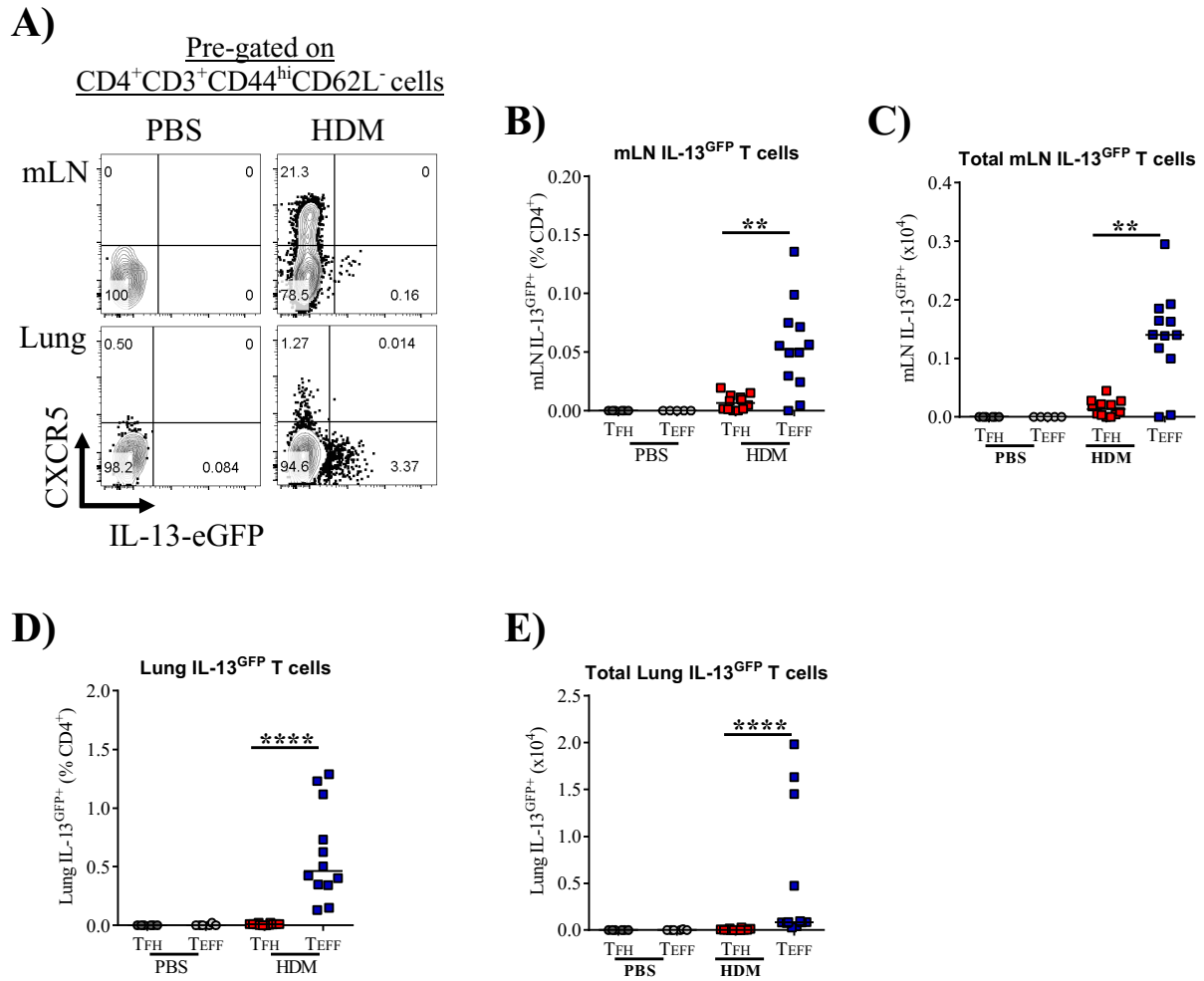


Figure 4.17: T_{FH} do not produce IL-13 during chronic allergic airway disease

Adult male and female IL-13^{GFP} reporter mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 3 times a week for 3 weeks. Flow cytometry was used to determine the frequency of total number of IL-13^{GFP} cells. **A)** Representative flow plots of CXCR5⁺ T cells and IL-13^{GFP} cells in PBS and HDM treated animals, pre-gated on $CD4^+CD3^+CD44^{hi}CD62L^-$ lymphocytes. IL-13^{GFP} T_{FH} (CXCR5⁺PD1⁺CD4⁺CD44^{hi}CD62L⁻) and T_{EFF} cells (CXCR5⁺CD4⁺CD44^{hi}CD62L⁻) were quantified. **B)** Frequency of mLN IL-13^{GFP} cells, **C)** Total number of mLN IL-13^{GFP} cells, **D)** Frequency of lung IL-13^{GFP} cells, **E)** Total number of IL-13^{GFP} cells. Data shown is pooled from two independent experiments, n=6-12.

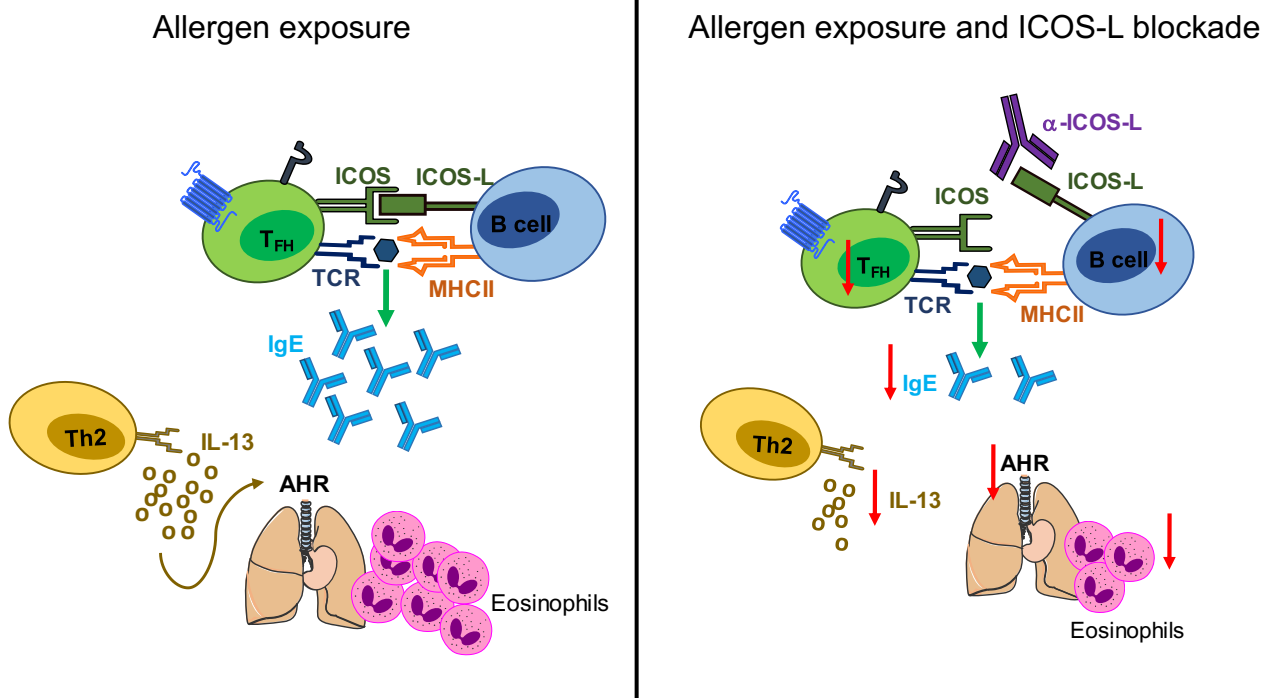


Figure 4.18: Graphical summary of the effects of blocking ICOS/ICOS-L interactions on established chronic allergic airway disease.

Airway hyperresponsiveness (AHR), Inducible T cell co-stimulator (ICOS), Inducible T cell co-stimulator ligand (ICOS-L), Major histocompatibility complex (MHC), T cell receptor (TCR), T follicular helper cell (T_{FH}), T helper 2 cell (Th2). Recently published in Uwadiae et al., 2018 Allergy.

4.4 Discussion

The aim of this chapter was to understand the therapeutic potential of depleting T_{FH} during chronic allergic disease. Given that T_{FH} express high levels of ICOS and can be depleted in acute models by interfering with ICOS/ICOS-L interactions, the impact of T_{FH} loss during established allergic disease was examined. To investigate this mice were exposed to aeroallergen for 5 weeks and from the start of week 4 were additionally administered α -ICOS-L antibodies. Therapeutic administration of α -ICOS-L was found to reduce both mLN and lung T_{FH} populations and the subsequent humoral responses including GC B cells, plasma cells and allergen specific IgE, while additionally improving hallmark features of allergic disease.

4.4.1. ICOS-L blockade targets T_{FH} and the subsequent humoral response

ICOS-L blockade significantly reduces T_{FH} responses both within the SLOs and locally within the lungs. This blockade alters T_{FH} responses during chronic allergen exposure indicating that even when antigen is readily available, ICOS-L blockade is still sufficient and effective to impair T_{FH} . The effect appears to be general and thus occurs in two aeroallergen models of AAD. Interestingly, although T_{FH} are targets of ICOS-L intervention levels of their hallmark cytokine, IL-21 are unchanged by the α -ICOS-L treatment. Furthermore, the other canonical T_{FH} cytokine IL-4 also remains at the same level. Despite both being produced in large quantities by T_{FH} , NKT cells and Th17 cells can also both produce IL-21 (Nurieva et al., 2007, Korn et al., 2007, Coquet et al., 2007, Harada et al., 2006), while Th2 cells, ILCs, NKT cells, basophils and many other cells can produce IL-4 (Lambrecht and Hammad, 2015, Akbari et al., 2003, Brunner et al., 1993, Miyake and Karasuyama, 2017, Arock et al., 1993). Therefore, some redundancy may exist. In this study, neither IL-21⁺ nor IL-4⁺ T_{FH} are examined, but given the reduction in the T_{FH} population as a whole it is expected that these cytokine positive populations will also fall with α -ICOS-L intervention.

Consistent with the fall in T_{FH} populations, reductions in GC B cell and lung plasma cell occurs and this decrease is sufficient to reduce HDM specific IgE and temper the levels of ALT

specific IgE. ALT drives a much stronger IgE response than HDM (Castanhinha et al., 2015) fitting with data from chapter 3 showing ALT to drive approximately 50-fold more IgE than HDM (**Figure 3.22B, 3.23B**). Thus, modifying levels of fungal induced IgE may require longer periods of ICOS-L blockade. Interestingly, this short 2-week intervention is inadequate to alter the level of allergen-specific IgG1 despite the fall in IgE. The half-life of IgE is known to be considerably shorter than other antibody isotypes (Waldmann et al., 1976), and chapter 3 clearly demonstrates this as allergen driven IgE returns to PBS control levels after a 4-week rest period while IgG1 and IgA levels remain steady (**Figure 3.32A-E**). Furthermore, in humans the transfer of serum containing either HDM specific Der p1 or grass pollen specific Lol p 1 into patients with hypogammaglobulinemia or healthy individuals, shows allergen specific IgE to rapidly decline over a 50-day period while IgG is relatively stable (Lawrence et al., 2017). The drop in IgE levels over time did not result from changes in the number IgE bound cells (Lawrence et al., 2017). Therefore, to observe substantial declines in IgG1 concentrations or IgE in fungal models may require a longer therapeutic α -ICOS-L administration protocol.

Allergen sensitisation generates allergen specific IgE which binds to mast cells or basophils via Fc ϵ R1. Upon allergen exposure cross-linking of Fc ϵ R1 bound IgE results in degranulation of mast cells and basophils and the release of several pro-inflammatory mediators. In patients, allergen exposure and sudden mediator release results in asthma exacerbations (Lambrecht and Hammad, 2015). Here consistent with reductions in HDM specific IgE, the level of the mast cell protease, MCPT-1 within the serum also decreases, suggesting ICOS-L blockade and thus T_{FH} reduction to decrease mast cell activation. Fitting with this MCPT1 does not diminish within the serum of ALT and α -ICOS-L mice in comparison to ALT and 2A3 controls, consistent with the non-significant reduction in IgE. Taken together this data implies T_{FH} to be important in the maintenance of GC B cell responses and IgE during chronic AAD and

effectively demonstrates that blocking ICOS-L interactions during disease is capable of disrupting the humoral immune response.

4.4.2. ICOS-L blockade improves chronic AAD

ICOS-L blockade improves hallmark features of allergen driven chronic AAD, including AHR and pulmonary inflammation, such as eosinophils. This reduction in eosinophilia results from a fall in cellular inflammation rather than the direct action of ICOS-L intervention on IL-5 or eotaxin-2 secreting populations leading to defective eosinophil survival or recruitment. Furthermore, following ICOS-L blockade, IL-13 levels decrease in the lungs of HDM exposed animals and in the BAL of ALT treated mice. Given both T_{FH} and IL-13 decline in this model and IL-13 is a powerful driver of AHR and airway inflammation (Wills-Karp et al., 1998, Grünig et al., 1998), it seemed possible that T_{FH} could be contributing to IL-13 secretion in the model. However, upon examination during chronic HDM driven AAD, T_{FH} could not produce IL-13 and thus loss of T_{FH} could not be directly responsible for declining IL-13 levels following ICOS-L intervention. Furthermore, this data shows that during chronic AAD T_{FH} do not acquire a T_{FH2} phenotype and secrete IL-13 to drive disease (Morita et al., 2011).

Th2 cells are the major adaptive producer of IL-13, while ILC2s are the main innate cell population secreting IL-13 (Lambrecht and Hammad, 2015). In the HDM model, neither IL-13⁺ CD4⁺ T cells nor ILC2s are direct targets of the ICOS-L blockade but instead both cellular populations trend towards a decrease in numbers, in line with an overall drop in gross pulmonary inflammation. ALT driven ILC2s display a similar trend to the HDM model. However, in the ALT model, following α -ICOS-L administration, both the proportion and number of IL-13⁺ CD4⁺ T cells decrease in the lungs, indicating that ICOS-L blockade may have slightly different effects depending on the type of aeroallergen. Overall, the reduction in IL-13 appears to be due to a decrease in the number of IL-13 producing cells in the lungs, rather than the specific targeting of an IL-13 secreting subset by ICOS-L blockade.

Interestingly, in addition to pro-inflammatory T cell populations, α -ICOS-L intervention also appears to cause a fall in the number of anti-inflammatory Tregs in the lungs. Despite this fall in Treg populations locally, α -ICOS-L intervention is still capable of impacting both the humoral immune response leading to reductions in allergic specific IgE, while still reducing cellular inflammation and AHR. Given that α -ICOS-L intervention is also reducing Treg populations it is possible that α -ICOS-L is also facilitating its effects by targeting other cellular populations. For example reductions in lung IL-17A also occur in the HDM driven model of AAD and similarly to IL-13, the number of IL-17A⁺CD4⁺ T cells also falls. Therefore, reduction in IL-17A may also be contributing to better AAD phenotype, especially given IL-17A can drive chronic AAD (McKinley et al., 2008). However, it is worth noting that the contribution of IL-17A to allergic disease is controversial and is generally dependent on the cellular source (McKinley et al., 2008, Schnyder-Candrian et al., 2006, Murdoch and Lloyd, 2010). IL-17A will be discussed in more depth in later chapters.

This investigation is not the first to block ICOS or ICOS-L during AAD. However, this study uniquely utilises clinically relevant allergens in a chronic model of AAD and blocking antibody in a therapeutic fashion. Previous studies administer ICOS or ICOS-L blocking antibodies prophylactically (Coyle et al., 2000, Gonzalo et al., 2001) or during an allergic exacerbation model (Wiley et al., 2003). In general these studies observe improvements in AAD phenotype, including a drop in eosinophil numbers, reductions in allergen specific IgE and allergen driven AHR (Coyle et al., 2000, Gonzalo et al., 2001), however importantly these studies pre-date the definition of T_{FH} and the understanding of their contribution to antibody mediated immunity. In this study, ICOS-L blockade replicates these results and ameliorates disease when given in a therapeutic manner after establishment of chronic AAD, despite a relatively short 2-week intervention.

ICOS-L blocking antibodies are safe and effective to administer to humans as proven by two phase I clinical trials for systemic lupus erythematosus (SLE) (Sullivan et al., 2016). Therefore,

it is possible that blocking ICOS signalling can be a beneficial treatment for asthma patients. The expression of ICOS on T cells only after activation limits the number of potential side-effects making it more attractive as an intervention over other co-stimulatory molecules such as CD28, which all CD4⁺ T cells including naïve T cells constitutively express. Furthermore, while CD40L is important for T cells to mediate help to B cells, blockade of this molecule during a SLE Phase 1 clinical trial led to life threatening complications due to expression of the molecule on platelets in addition to T cells (Boumpas et al., 2003, Yazdany and Davis, 2004). ICOS-L blockade targets multiple facets of allergic disease and therefore may be complimentary or advantageous to approved biological therapies, such as omalizumab (anti-IgE mAb) (Humbert et al., 2014), reslizumab (anti-IL-5 mAb) (Castro et al., 2015) and mepolizumab (IL-5 antagonist) (Ortega et al., 2014), which generally favour one arm of the allergic response over another. Therefore, blocking ICOS/ICOS-L signalling has the potential to provide therapeutic benefit to asthma patients, especially for severe patients whom are unable to control their symptoms even with high dose corticosteroids.

4.4.3. Concluding remarks

This chapter further increases understanding of the role of T_{FH} during chronic AAD, showing T_{FH} to be important for the ongoing maintenance of GC B cell responses and allergen specific IgE. It also shows that even in the chronic setting interfering with ICOS/ICOS-L interactions can impair both local and systemic T_{FH} responses. Moreover, the chapter efficiently demonstrates ICOS-L blockade can successfully ameliorate AAD hallmarks including AHR, eosinophilia and allergen specific IgE showing that this pathway could represent a therapeutic target in the future. Taken together this data suggests that T_{FH} may have a pathogenic role in existing allergic asthma. In the next chapter, the phenotype of T_{FH} generated during chronic AAD will be examined.

Chapter 5: Phenotyping CXCR5⁺ PD1⁺ CD4⁺ T cells induced during chronic allergic airway disease

5.1. Introduction

In the previous chapter, it was shown that mLN and lung T_{FH} could be targeted during ongoing chronic AAD and this was associated with improved pathology. Although, this implies that T_{FH} are important in allergic disease pathogenesis, it is not known whether the CXCR5⁺PD1⁺CD4⁺ T cells (T_{FH}) induced during allergic disease are prototypical T_{FH} or dysregulated. Furthermore, it is unclear whether site specific differences exist in the phenotype between local lung T_{FH} and systemic mLN T_{FH}.

The fundamental role of T_{FH} is to help B cells to proliferate, differentiate and thus help B cells to secrete high affinity, isotype switched antibody. Traditional T_{FH} express several markers, including CXCR5, PD1, Bcl-6 and high levels of ICOS. They supply B cells with co-stimulation through CD40-L and cytokine signalling via production of IL-4 and IL-21 (Vinuesa et al., 2016). Therefore, it is critical to understand if the lung CXCR5⁺PD1⁺CD4⁺ T cells possess the machinery to help B cells and determine the impact of the local tissue microenvironment on T_{FH} phenotype.

Furthermore, both T_{FH} and Th2 cells can produce IL-4 and it has been suggested that the two cellular populations are strongly linked during AAD. T_{FH} have been implicated as Th2 cell precursors generated in the mLN upon allergen sensitisation, which differentiate into Th2 cells and drive disease after allergen challenge (Ballesteros-Tato et al., 2016). In contrast, T_{FH} have also been shown to fail to become Th2 cells after adoptive transfer into non-sensitised animals (Coquet et al., 2015, Hondowicz et al., 2016). Furthermore, T_{FH} have been shown to express *Gata3* and potentially differentiate out of the Th2 lineage during helminth driven type 2 pathology (Zaretsky et al., 2009). Moreover, others have suggested T_{FH} and Th2 cells to be distinct cell types with separate transcription factor expression and functional roles (Liang et al., 2011, Kobayashi et al., 2017). Therefore, it is critical to understand whether the T_{FH} induced during chronic AAD share similarities with Th2 cells. In particular it is important to evaluate whether T_{FH} induced during chronic AAD are dysregulated to produce type 2 cytokines other than IL-4 and therefore may contribute to pathogenesis by means other than

driving a humoral response. Therefore, the aim of this chapter was to further phenotype CXCR5⁺PD1⁺CD4⁺ T cells induced during chronic AAD.

5.1.1. Hypothesis

CXCR5⁺PD1⁺CD4⁺ T cells induced during chronic allergic airway disease are bona fide T_{FH} and lung T_{FH} are comparable to their mLN counterparts.

5.1.2. Aims

- 1) To phenotype T_{FH} induced during chronic allergic airway disease
- 2) To understand the relationship between T_{FH} and Th2 cells
- 3) To determine the similarities and difference between lung and mLN T_{FH}

5.2. Methods

5.2.1. 5 week HDM and ALT model of allergic airway disease

Female adult BALB/c mice were exposed to intranasal (i.n) HDM (25 µg) or ALT (10 µg) 3 times a week for 5 weeks. To examine allergen induced allergic airway disease mice were culled at the end of week 5 and the lungs and mLN were harvested. Cellular populations were determined by flow cytometry (**Figure 5.1A**).

5.2.2. IL-13 reporter and knockout studies

To determine which cellular populations produce IL-13 during chronic allergic airway disease adult male and female IL-13-gfp/+ reporter mice (IL-13^{GFP}) were exposed to intranasal (i.n) HDM (25 µg) 3 times a week for 3 weeks. Cellular populations were determined by flow cytometry (**Figure 5.1B**). To determine the importance of IL-13 to T_{FH} generation during allergic disease, IL-13-gfp/gfp knockout mice (IL-13KO) and BALB/c mice were exposed to intranasal (i.n) HDM (25 µg) or PBS (25 µl), 3 times a week for 3 weeks. Cellular populations were determined by flow cytometry (**Figure 5.1C**).

5.2.3. Mouse strain comparisons

Adult female BALB/c, 129/S2J and C57BL/6 mice were exposed (i.n) to HDM (25 µg) 3 times a week for 3 weeks. To assess strain differences mice were culled at the end of week 3 and the lungs and mLN were harvested. Cellular populations were determined by flow cytometry (**Figure 5.2A**).

5.2.4. Intravascular discrimination of cellular populations

Adult female C57BL/6 mice were exposed (i.n) to HDM (25 µg) or PBS (25 µl), 5 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v) mice via the tail vein with FITC-labelled CD45 antibody, 3-5 mins before culling. Tissue resident cells were defined as CD45⁻ cells. Cellular populations were assessed by flow cytometry (**Figure 5.2B**).

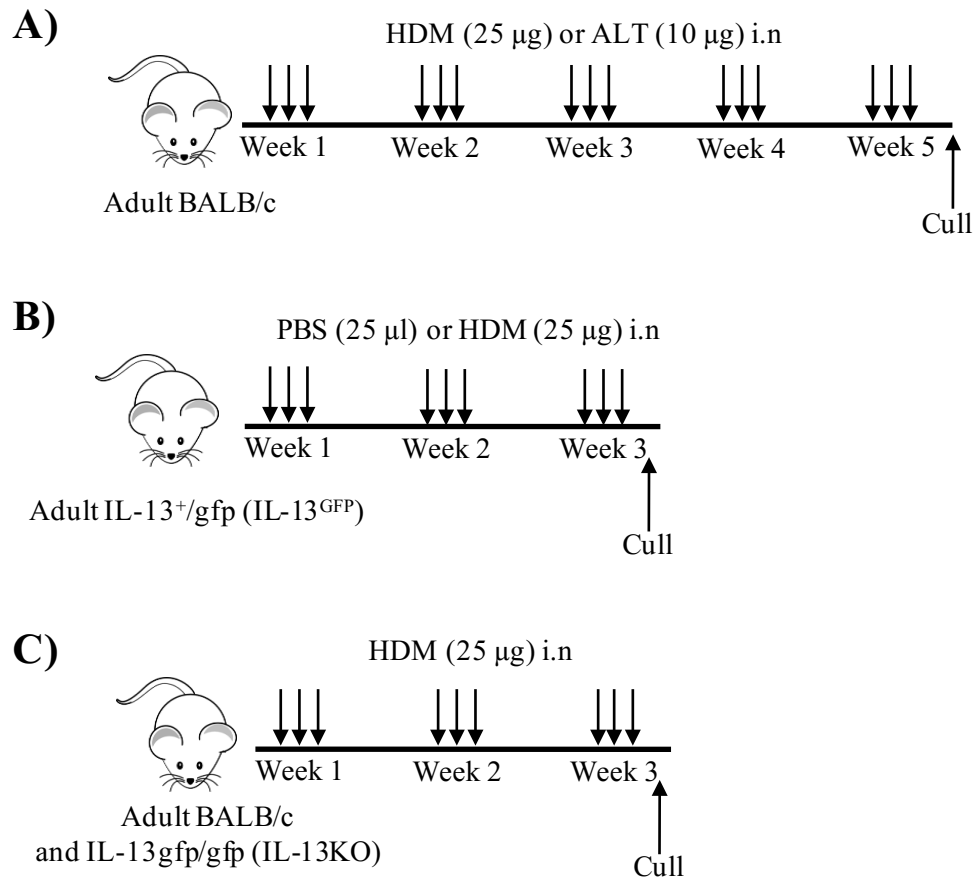


Figure 5.1: Methods 1

A) Adult female BALB/c mice were exposed to either 25 µg HDM or 10 µg ALT, 3 times a week for 5 weeks. Mice were culled at the end of week 5. **B)** Adult IL-13^{+/GFP} (IL-13^{GFP}) mice were exposed to 25 µg HDM or 25 µl PBS, 3 times a week for 3 weeks. Mice were culled at the end of week 3. **C)** Adult BALB/c mice and IL-13^{GFP/GFP} (IL-13KO) mice were exposed to 25 µg HDM, 3 times a week for 3 weeks. Mice were culled at the end of week 3. All mice, regardless of study design were culled 18 hours after the final dose.

5.2.5. T_{FH} sorting experiments

For sorting experiments, adult female C57BL/6 mice were exposed (i.n) to HDM (25 µg) or PBS (25 µl), 5 times a week for 5 weeks before being intravascularly injected as described in Figure 5.2B. The spleen, lungs and mLN were harvested and processed then enriched for CD4⁺ T cells using a STEMCell CD4⁺ T cell negative selection kit. Tissue resident (CD45⁻) T_{FH} and non-T_{FH} effector cells (T_{EFF}) were sorted from the mLN and lung tissue of HDM treated animals, while naïve tissue resident CD4⁺ T cells were sorted from the spleen of PBS treated

animals. RNA was extracted from the sorted cells and qPCR was used to assess gene expression.

5.2.5. IL-17A reporter studies

Adult female and male *il-17a^{cre}rosa26-rfp^{fl/fl}* (IL-17A-RFP) and *rosa26-rfp^{fl/fl}* (WT) mice were exposed (i.n.) to HDM (25 µg) or PBS (25 µl) 5 times a week for 5 weeks. To examine IL-17A producing populations during chronic allergic airway disease mice were culled at the end of week 5 and the lungs and mLN were harvested. Cellular populations were determined by flow cytometry (**Figure 5.2C**).

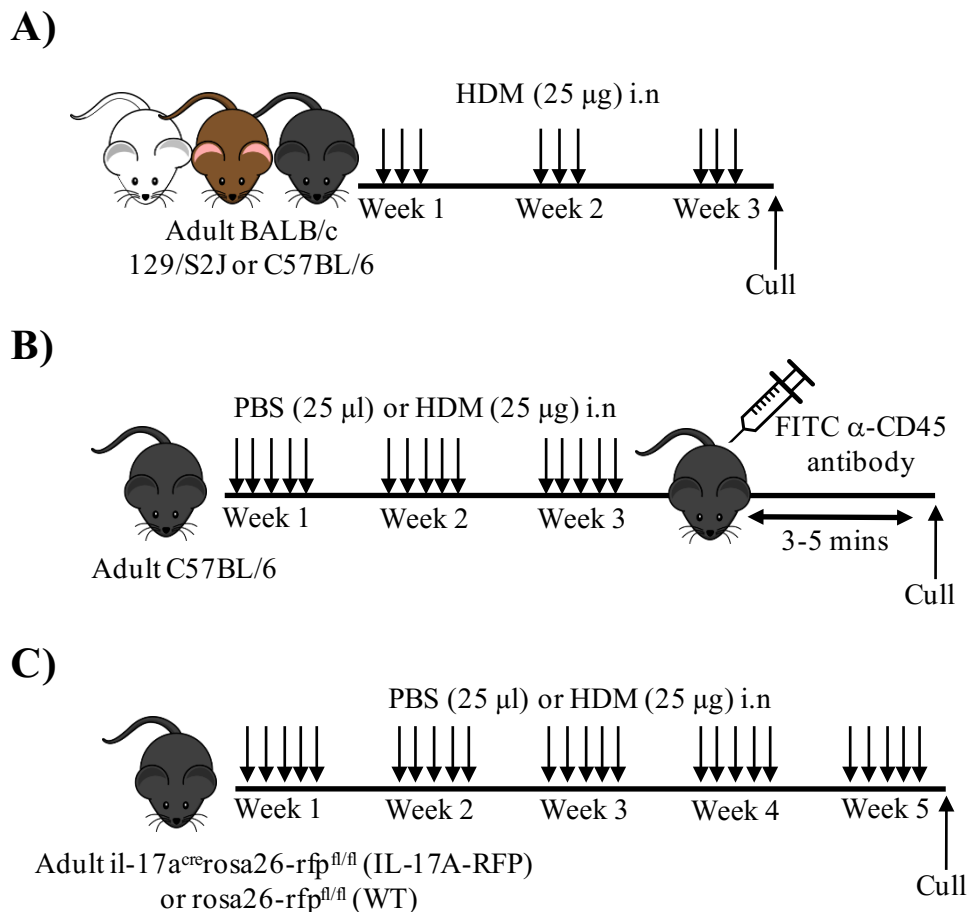


Figure 5.2: Methods 2

A) Adult female BALB/c, 129/S2J and C57BL/6 mice were exposed (i.n.) to HDM 3 times a week for 3 weeks. Mice were culled at the end of week 3. **B)** Adult female C57BL/6 mice were exposed to 25 µg HDM or 25 µl PBS 3 times a week for 3 weeks. At the end of week 3 mice were intravenously injected via the tail vein with 3 µg of FITC labelled CD45 antibody. Mice were culled 3-5 mins later. For some studies mice were exposed to HDM for 5 weeks. Mice were culled at the end of week 3 or 5. **C)** Adult *il-17a^{cre}rosa26-rfp^{fl/fl}* (IL-17A-RFP) or littermate controls *rosa26-rfp^{fl/fl}* (WT) mice were exposed (i.n.) to HDM 5 times a week for 5 weeks. Mice were culled at the end of week 5. All mice regardless of study design were culled 18 hours after the final dose.

5.3. Results

5.3.1. T_{FH} induced during allergic airway disease produce IL-4 and IL-21

The prototypical cytokines produced by T_{FH} are IL-4 and IL-21 which are both important for B cell differentiation, proliferation and antibody class switch recombination (Vinuesa et al., 2016). Therefore, it was important to assess whether T_{FH} induced within the lungs and the mLN of allergen exposed mice could produce these signature cytokines. To assess this, BALB/c mice were exposed to ALT, 3 times a week for 5 weeks. In the mLN and lungs of ALT treated animals IL-4⁺ T_{FH} accounted for approximately 20% of the T_{FH} population (**Figure 5.3A**). Therefore, T_{FH} at both sites were capable of producing IL-4.

Furthermore, in the mLN and lung tissue a small proportion of T_{FH} were IL-21⁺ (**Figure 5.3B**). In the mLN, approximately 20% of T_{FH} were IL-21⁺ (**Figure 5.3A**) and therefore represented a similar proportion to those that were IL-4⁺ (**Figure 5.3A, B**). However, in the lungs a lower proportion of T_{FH} appeared to be IL-21⁺ (**Figure 5.3B**). Taken together, this data shows that during chronic allergic disease, both lung and mLN T_{FH} are capable of producing IL-4 and IL-21.

5.3.2. T_{FH} do not express GATA3 during chronic AAD

T_{FH} have been suggested to have a close phenotypic relationship with Th2 cells. T_{FH} have been suggested to be precursors to Th2 cells, which go on to drive allergic disease (Ballesteros-Tato et al., 2016), while others have shown T_{FH} to express *Gata3* to similar levels to Th2 cells and differentiate out of the Th2 lineage during type 2 pathology (Zaretsky et al., 2009). Therefore, whether T_{FH} induced during chronic AAD, express GATA3 and share similarities with Th2 cells was examined. To understand this adult BALB/c female mice were exposed to HDM or ALT, 3 times a week for 5 weeks and T_{FH} and Th2 cell population were examined by flow cytometry (**Figure 5.4A**). On examination of mLN antigen experienced CD4⁺ T cells (CD44^{hi}CD62L⁺Foxp3⁻), CXCR5 and GATA3 expression was found to be mutually exclusive (**Figure 5.4A**). Consistent with this gating, mLN T_{FH} populations within both HDM

and ALT driven disease showed T_{FH} to lack GATA3 expression, while Th2 cells expressed high levels of the transcription factor (**Figure 5.4B**). Comparable results were observed for T_{FH} within the lungs of allergen treated mice (**Figure 5.4C, D**). Similarly, the T_{FH} lineage defining transcription factor, Bcl-6 was found highly expressed in both lung and mLN T_{FH} during chronic aeroallergen driven AAD, rather than in Th2 cells (**Figure 5.4E, F**). Taken together, this data demonstrates that during chronic AAD, T_{FH} (CXCR5⁺ PD1⁺ CD4⁺ T cells) in both the lungs and mLN do not express GATA3 but do express key markers of the T_{FH} lineage including, CXCR5 and Bcl-6.

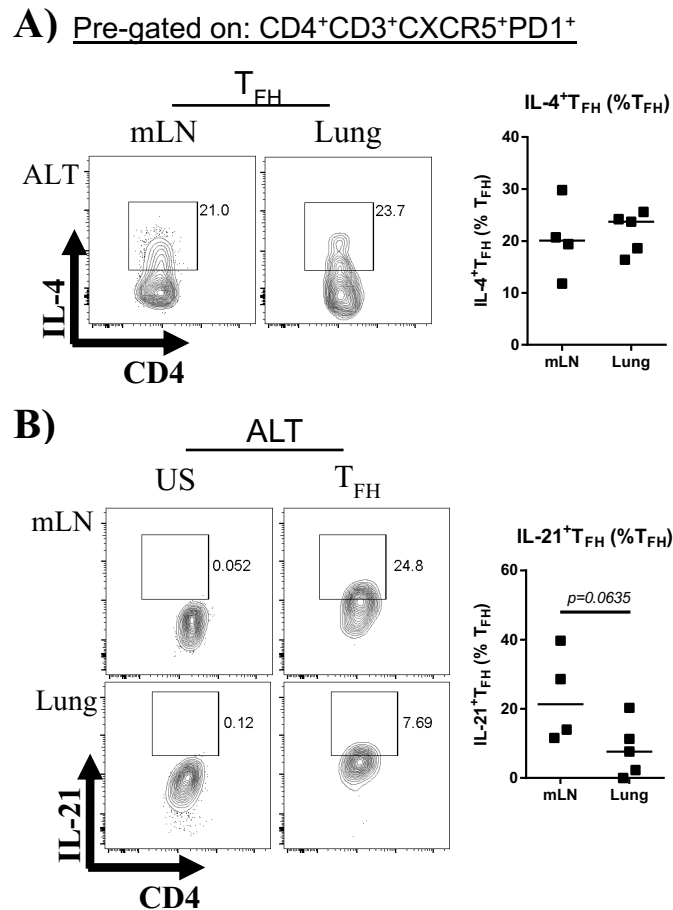


Figure 5.3: T_{FH} induced during chronic allergic airway disease produce IL-4 and IL-21

Adult female BALB/c mice were exposed to ALT, 3 times a week for 5 weeks. Flow cytometry was used to determine the frequency IL-4 T_{FH} (CXCR5⁺PD1⁺CD4⁺) as a proportion of T_{FH} in the mLN and lungs of ALT treated animals. **A)** Representative flow plots and quantification of IL-4⁺ T_{FH} within the mLN and lungs, **B)** Representative flow plots and quantification of IL-21⁺ T_{FH} within the mLN and lungs. Unstimulated (US) are also displayed. Median data is displayed. $n=4-5$ per group. Statistical significance was determined using a Mann Whitney U test. Data is representative of 2 independent experiments.

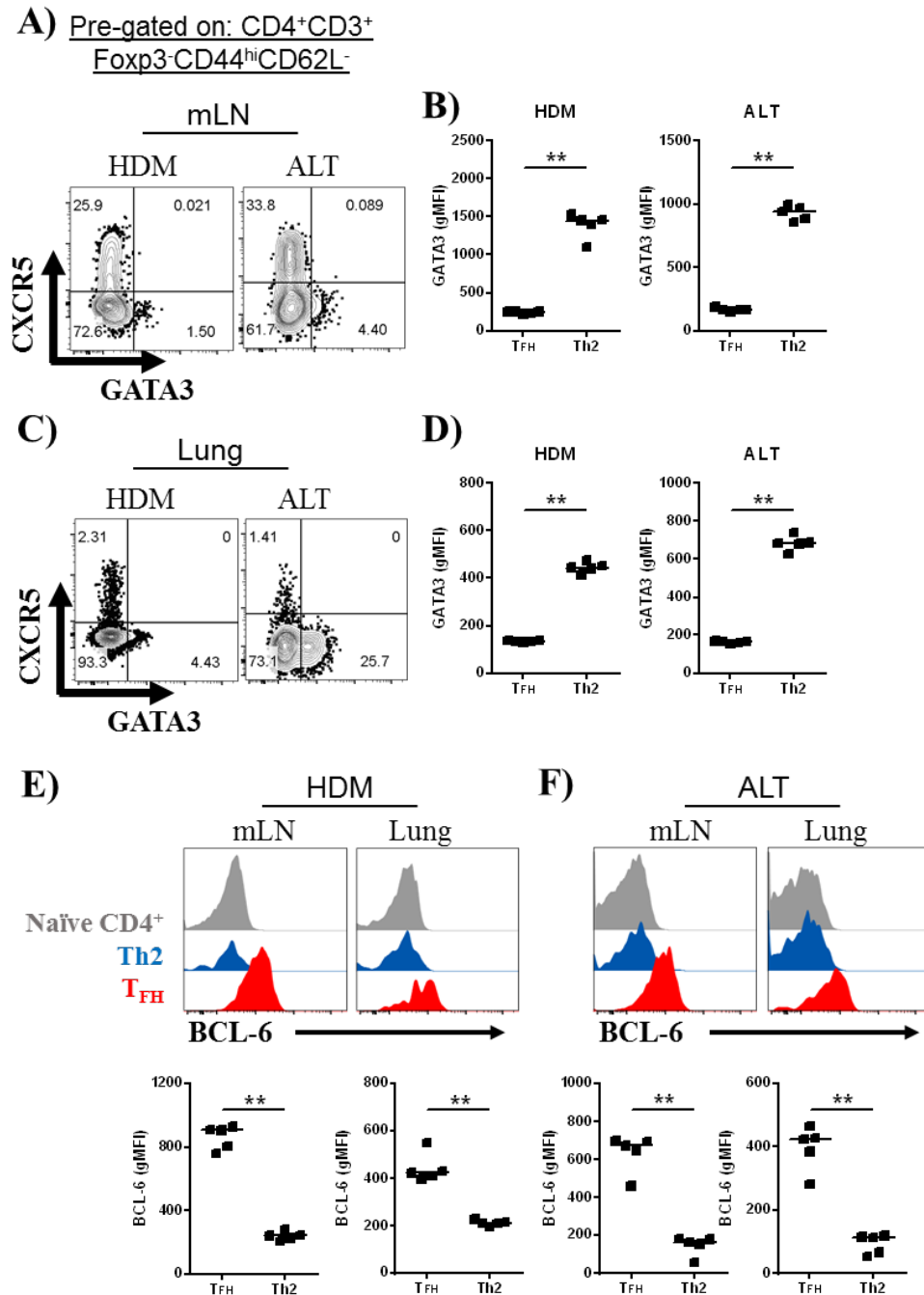


Figure 5.4: T_{FH} do not express GATA3

Adult BALB/c mice were exposed to 25 µg HDM or 10 µg ALT, 3 times a week for 5 weeks. Mice were culled at the end of week 5. **A)** Representative flow plots of CXCR5⁺ and GATA3⁺ T cells in the mLN of allergen treated animals. Pre-gated on CD4⁺CD3⁺Foxp3⁻CD44^{hi}CD62L⁻ cells. **B)** GATA3 geometric mean (gMFI) in mLN T_{FH} (CXCR5⁺PD1⁺) and Th2 cells (CXCR5⁻GATA3⁺) in HDM and ALT treated animals, **C)** Representative flow plots of CXCR5⁺ and GATA3⁺ T cells in the lung tissue of allergen treated animals. Pre-gated on CD4⁺CD3⁺Foxp3⁻CD44^{hi}CD62L⁻ cells. **D)** GATA3 gMFI in lung T_{FH} and Th2 cells in HDM and ALT treated animals. **E)** BCL-6 gMFI on T_{FH} and Th2 cells in the mLN and lungs of HDM treated animals compared to naïve CD4⁺ T cells (CD44^{lo}CD62L⁺), **F)** BCL-6 gMFI in T_{FH}, Th2 cells and naïve CD4⁺ T cells in the mLN and lungs of ALT treated animals. Median data is displayed. n=5 per group. Statistical significance was determined using a Mann Whitney U test. Data is representative of 2 independent experiments.

5.3.3. T_{FH} do not produce IL-13 during chronic AAD

Next, the type 2 cytokine repertoire of T_{FH} was explored. To examine IL-13 production, IL-13-gfp/+ reporter mice (IL-13^{GFP}) mice were exposed to PBS or HDM 3 times a week for 3 weeks (**Figure 5.5A**). Consistent with previous chapters PBS treated animals lacked CXCR5⁺ (T_{FH}) cells in their mLN and lung tissue, but following allergen exposure CXCR5⁺ cells were easily identified at both sites (**Figure 5.5B**). In HDM treated animals, IL-13-eGFP expression was distinct from CXCR5 and was instead restricted to CXCR5⁻ cells (**Figure 5.5B, C**). After, PMA/ionomycin stimulation, T_{FH} and non-T_{FH} effector cells (T_{EFF}) within the mLN and lungs were examined. T_{FH} failed to produce IL-13 after stimulation, which was produced instead by T_{EFF} cells (**Figure 5.5E-G**). Taken together, the data showed T_{FH} were incapable of producing IL-13 during chronic AAD.

5.3.4. IL-13 is not required for T_{FH} generation during allergic disease

IL-13 is a critical cytokine produced by Th2 cells and ILCs during allergic disease and is vital for the development of chronic AAD, primarily by driving AHR and mucus secretion (Lambrecht and Hammad, 2015, Grünig et al., 1998, Wills-Karp et al., 1998). Furthermore, IL-13 has been implicated to play an important role in human IgE switch (Punnonen et al., 1997, Punnonen et al., 1993) therefore the impact of this cytokine on T_{FH} generation during allergen exposure needed to be understood. Given that T_{FH} failed to produce IL-13, dependence on IL-13 for their generation and function was next examined. Thus, IL-13 deficient, IL-13gfp/gfp mice (IL-13KO) were exposed to HDM 3 times a week for 3 weeks and compared to BALB/c controls (WT) (**Figure 5.6A**). T_{FH} were induced in the mLN and lungs of allergen treated WT and IL-13KO mice (**Figure 5.6B**) and were found at similar proportions and numbers at both sites (**Figure 5.6C, D**). Concomitant with the lack of impact on T_{FH}, IL-13 deficiency had no impact on GC formation in the mLN (**Figure 5.7A-C**) and lungs (**Figure 5.7A, D-E**) or serum IgG1 and IgE (**Figure 5.7F, G**). Therefore, T_{FH} neither produce nor are they or the overall allergen specific antibody response dependent on IL-13 for their development.

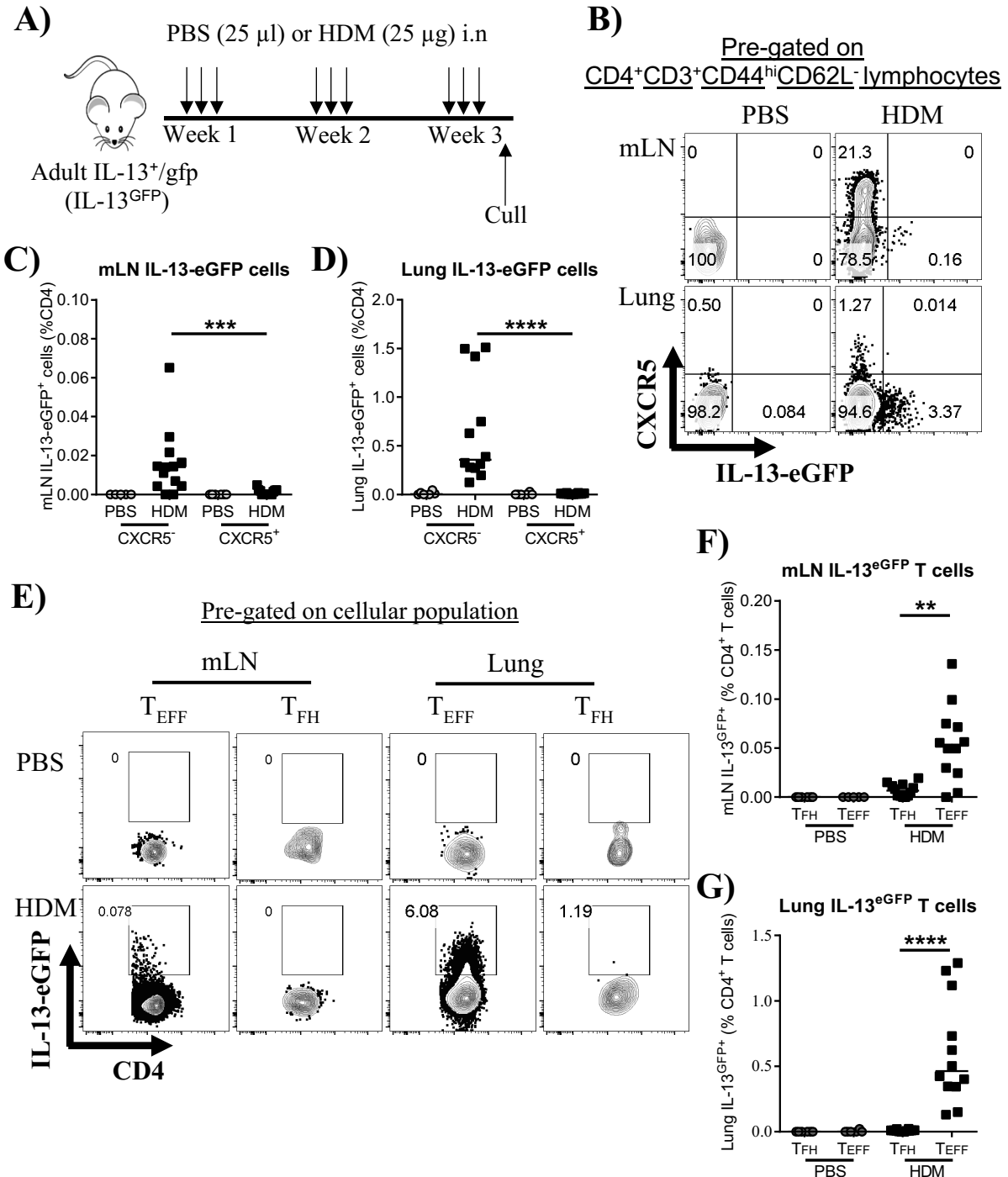


Figure 5.5: T_{FH} do not produce IL-13 during chronic allergic airway disease

Adult female IL-13^{+/GFP} reporter mice (IL-13^{GFP}) were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 3 times a week for 3 weeks. Flow cytometry was used to determine the frequency of IL-13^{GFP} cells. **A)** Schematic of experimental design, **B)** Representative flow plots of CXCR5⁺ and IL-13-eGFP⁺ cells in PBS and HDM treated animals without stimulation, pre-gated on CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes. Quantification of IL-13-GFP⁺ cells in CXCR5⁺ and CXCR5⁻ cells **C)** mLN, **D)** Lung, **E)** Representative flow plots of IL-13-eGFP⁺ T_{FH} and T_{EH} cells after PMA/ionomycin stimulation, **F)** Proportions of IL-13eGFP⁺ cells in the mLN, **G)** Proportions of IL-13eGFP⁺ cells in the lung tissue. PBS n=6, HDM n=12. Median data is displayed. Statistical significance was determined using a Mann Whitney U test. Data is pooled from two independent experiments.

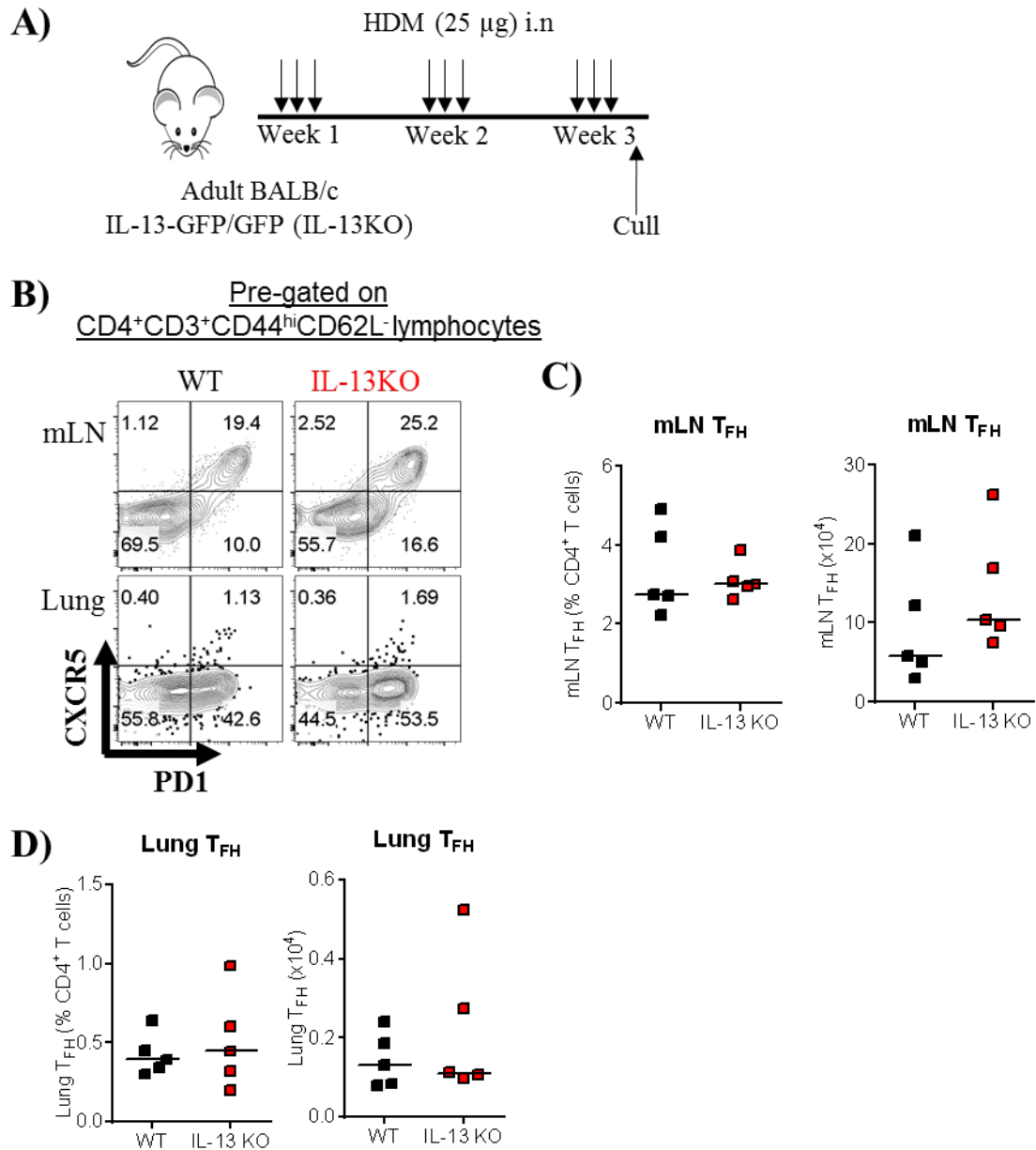


Figure 5.6: IL-13 is not required for T_{FH} generation during chronic allergic airway disease

Adult female IL-13^{GFP/GFP} knockout mice (IL-13KO) and wild type BALB/c (WT) were exposed (i.n) to 25 μ g HDM, 3 times a week for 3 weeks. Flow cytometry was used to determine the frequency of T cells within the lungs and mLN of adult BALB/c (WT) and IL-13^{GFP/GFP} mice (IL-13KO) treated with HDM. **A)** Schematic of experimental design, **B)** Representative flow plots of T_{FH} in the mLN and lungs of HDM treated WT and IL-13 KO mice. Pre gated on CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes. **C)** Quantification of mLN T_{FH} frequency and number, **D)** Quantification of lung T_{FH} frequency and number T_{FH}. n=5 per group. Statistical significance was determined using a Mann Whitney U test. Data is representative of 2 independent experiments.

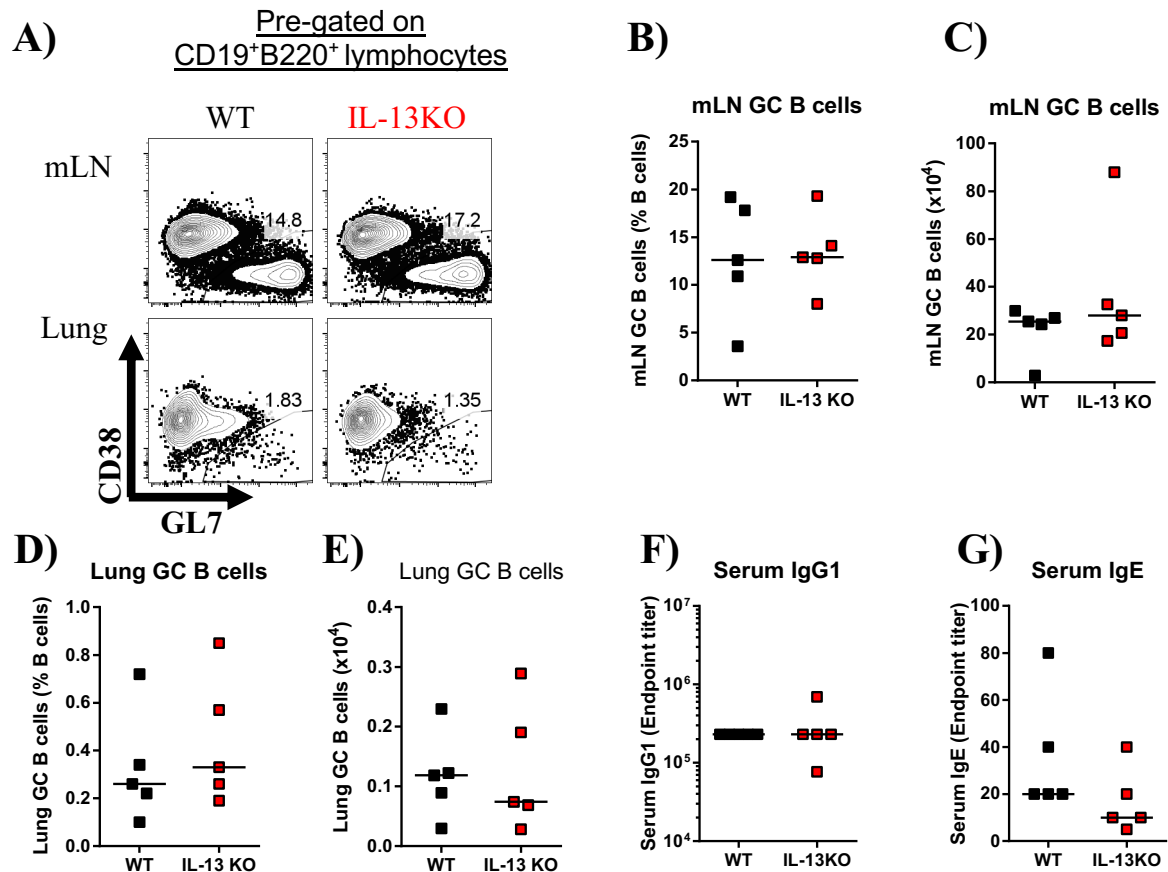


Figure 5.7: IL-13 does not impact GC B cell formation

Adult female IL-13^{GFP/GFP} knockout mice (IL-13KO) and wild type BALB/c (WT) were exposed (i.n) to 25 µg HDM, 3 times a week for 3 weeks. Flow cytometry was used to determine the frequency of germinal centre (GC) B cells within the lungs and mLN of adult WT and IL-13 KO treated with HDM. GC B cell were defined as CD38⁻GL7⁺ FAS⁺CD19⁺B220⁺ cells. **A)** Representative flow plots of GC B cells in the mLN and lungs of HDM treated WT and IL-13 KO mice. Pre gated on CD19⁺B220⁺ lymphocytes, **B-E)** GC B cells were quantified **B)** mLN frequency, **C)** mLN total, **D)** Lung frequency, **E)** Lung total. **F-G)** Serum HDM specific antibody, **F)** IgG1, **G)** IgE. Median data is displayed. n=5 per group. Statistical significance was determined using a Mann Whitney U test. Data is representative of 2 independent experiments.

5.3.5. C57BL/6 mice generate a strong T_{FH} response

The extent at which AAD develops in mice varies depending on mouse strain. Although, both BALB/C mice and C57BL/6 have been widely used to study AAD, BALB/c mice are known to develop stronger disease upon allergen exposure, characterised by higher levels of type 2 cytokines and AHR compared to their more allergen resistant C57BL/6 counterparts (Atochina et al., 2003, Gueders et al., 2009, Whitehead et al., 2003, Herz et al., 2004). Similarly, 129/S2J

animals develop heavily eosinophilic allergic disease compared to the other 2 strains (Whitehead et al., 2003). Although, many aspects of strain difference during AAD induction have been explored, the impact of strain on T_{FH} has yet to be studied. To examine whether T_{FH} generation varied between mouse strains, adult female BALB/c, 129/S2J (129) and C57BL/6 (BL/6) mice were exposed to HDM 3 times a week for 3 weeks (**Figure 5.8A**). The level of mLN inflammation was similar between the 3 mouse strains (**Figure 5.8B**). However, while BALB/c and 129 mice had comparable lung inflammation, total lung counts were lower in BL/6 mice when compared to their BALB/c and 129 counterparts (**Figure 5.8C**). Proportions of CD4⁺ T cells were also altered with mouse strain and BL/6 mice had the lowest frequency of CD4⁺ T cells (**Figure 5.8D, E**). Thus, the level of cellular inflammation and the CD4⁺ T cell population was affected by the genetic background of the strain.

T_{FH} populations were induced in the mLN and lungs of all 3 strains of mice following 3 weeks of aeroallergen exposure (**Figure 5.9A**). However, T_{FH} populations were found at the greatest frequency in the mLN of 129 and BL/6 animals, approximately 8-fold greater than observed in the BALB/c mice (**Figure 5.9B**). Similarly, total numbers of mLN T_{FH} were higher in 129 and BL/6 compared to BALB/c, being found highest in the 129 (**Figure 5.9C**). Despite, T_{FH} being identified at similar proportions in the mLN of the 129 and BL/6 mice, BL/6 mice had a lower number of mLN T_{FH} overall, consistent with the lower proportion of CD4⁺ T cells in BL/6 mice compared to the other two groups (**Figure 5.8D**). Lung T_{FH} were found at a greater frequency and number in the 129 and BL/6 mice compared to BALB/c and were highest overall in the BL/6 animals (**Figure 5.9D, E**). However, it was difficult to gate a clear distinct lung T_{FH} population in the 129 mice (**Figure 5.9A**).

Comparing the phenotype of mLN and lung T_{FH} within the different mouse strains, all populations expressed CXCR5, PD1 and Bcl-6 (**Figure 5.10A-F**). mLN and lung T_{FH} from BALB/c and 129 mice expressed similar levels of CXCR5, while BL/6 T_{FH} expressed a greater level of CXCR5 (**Figure 5.10A, B**). BALB/c and BL/6 mice expressed similar levels of PD1 (**Figure 5.10C, D**), while 129 mice possessed comparable level on their lung T_{FH} (**Figure 5.10D**), but not on their mLN population (**Figure 5.10C**). Lastly, Bcl-6 was expressed at a

similar level in mLN T_{FH} from all 3 groups, while in the lungs 129 mice had reduced Bcl-6 expression (**Figure 5.10E, F**). This data suggests that BALB/c and BL/6 mice generate both mLN and lung resident T_{FH} populations, while 129 mice generate mLN T_{FH} but potentially not lung resident T_{FH} , acknowledging difficulty in gating lung $CXCR5^+PD1^+$ cells and the low expression of Bcl-6.

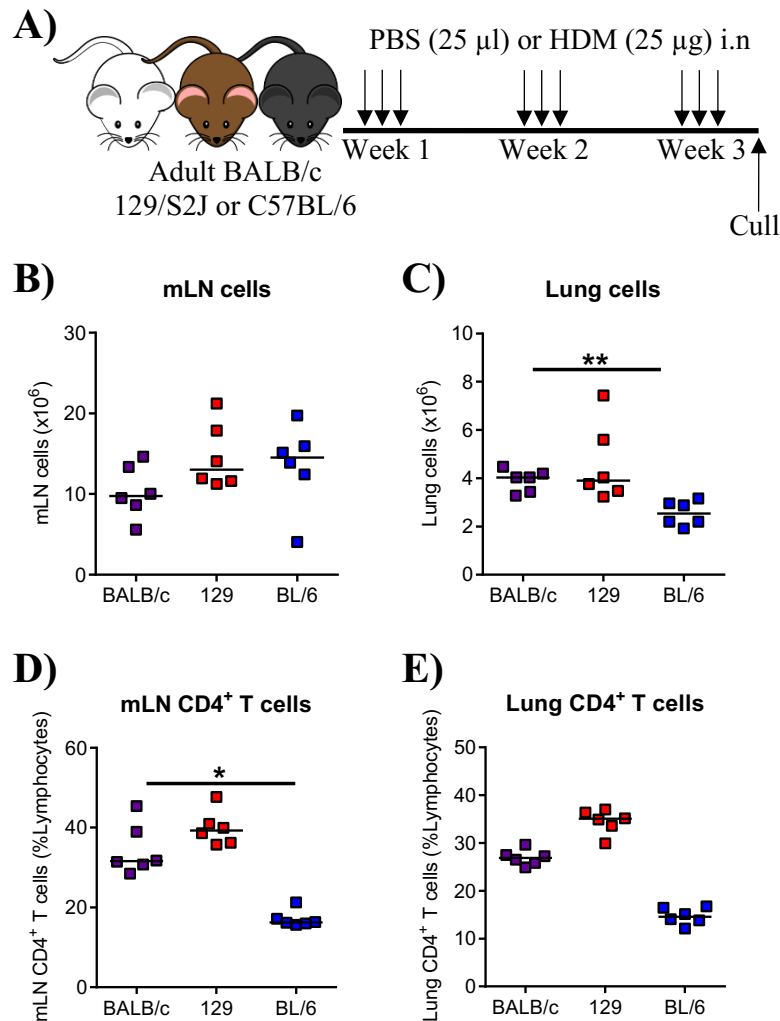


Figure 5.8: Allergen induced lung inflammation is lowest in C57BL/6 mice

Adult female BALB/c, 129/S2J (129) and C57BL/6 (BL/6) mice were exposed (i.n) to HDM 3 times a week for 3 weeks. **A)** Schematic of experimental design, **B)** mLN total cell counts, **C)** Lung total cell counts, **D)** Proportions of mLN CD4⁺ T cells, **E)** Proportions of lung CD4⁺ T cells. Median data is displayed. n=5 per group. Statistical significance was determined using a Kruskal Wallis with Dunn Multiple Comparisons test. Data is representative of 2 independent experiments.

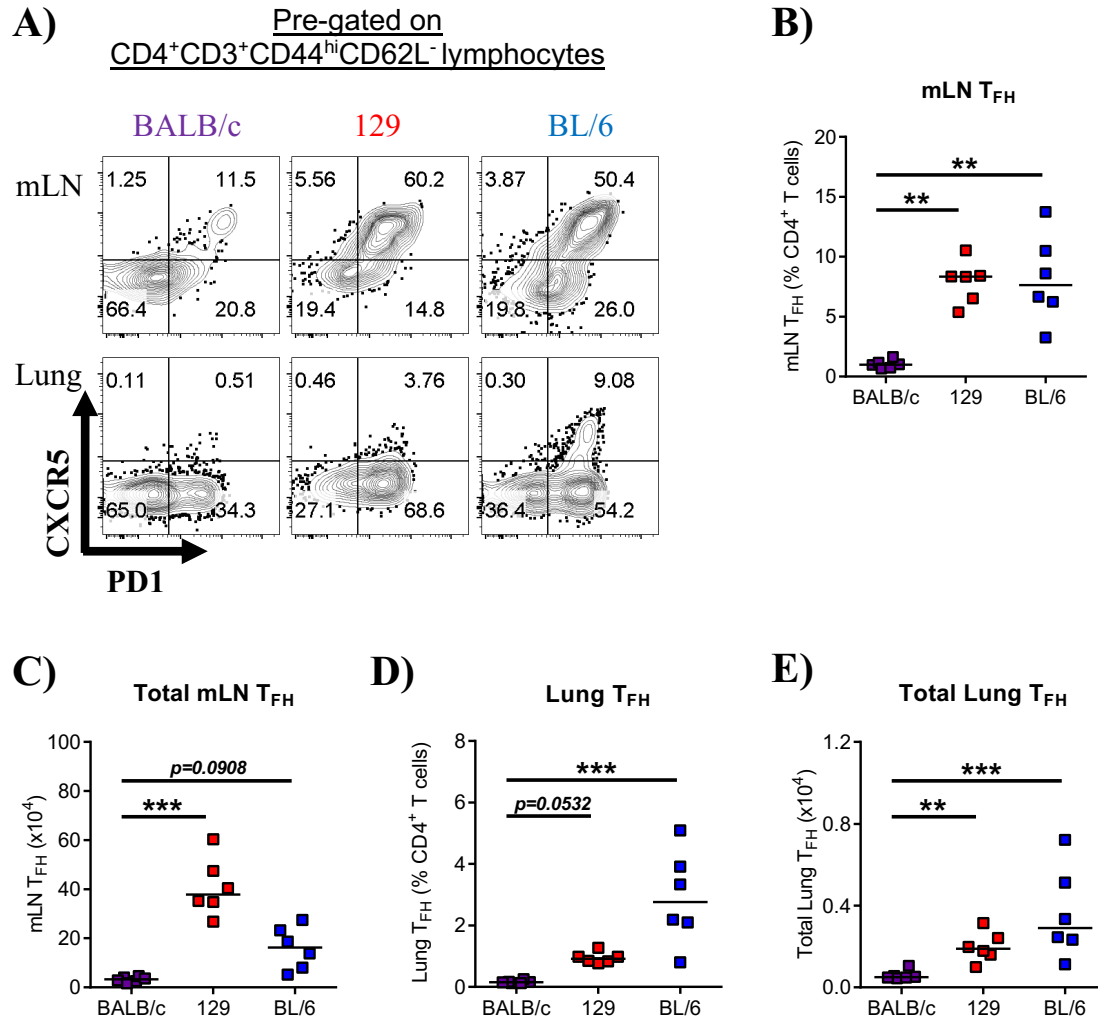


Figure 5.9: T_{FH} proportions and numbers vary depending on the strain of mouse
Adult female BALB/c, 129/S2J (129) and C57BL/6 (BL/6) mice were exposed (i.n) to HDM 3 times a week for 3 weeks. Flow cytometry was used to determine the frequency and number of T_{FH} following HDM exposure. T_{FH} were defined as $CXCR5^+PD1^+Foxp3^+CD4^+$, **A)** Representative flow plots of T_{FH} , pre-gated on $CD4^+CD3^+CD44^{hi}CD62L^-$ lymphocytes. Data was quantified **B)** Frequency of mLN T_{FH} , **C)** Total number of mLN T_{FH} , **D)** Frequency of lung T_{FH} , **E)** Total number of lung T_{FH} . Median data is displayed. $n=5$ per group. Experiment, $n=1$. Statistical significance was determined using a Kruskal Wallis with Dunn Multiple Comparisons test.

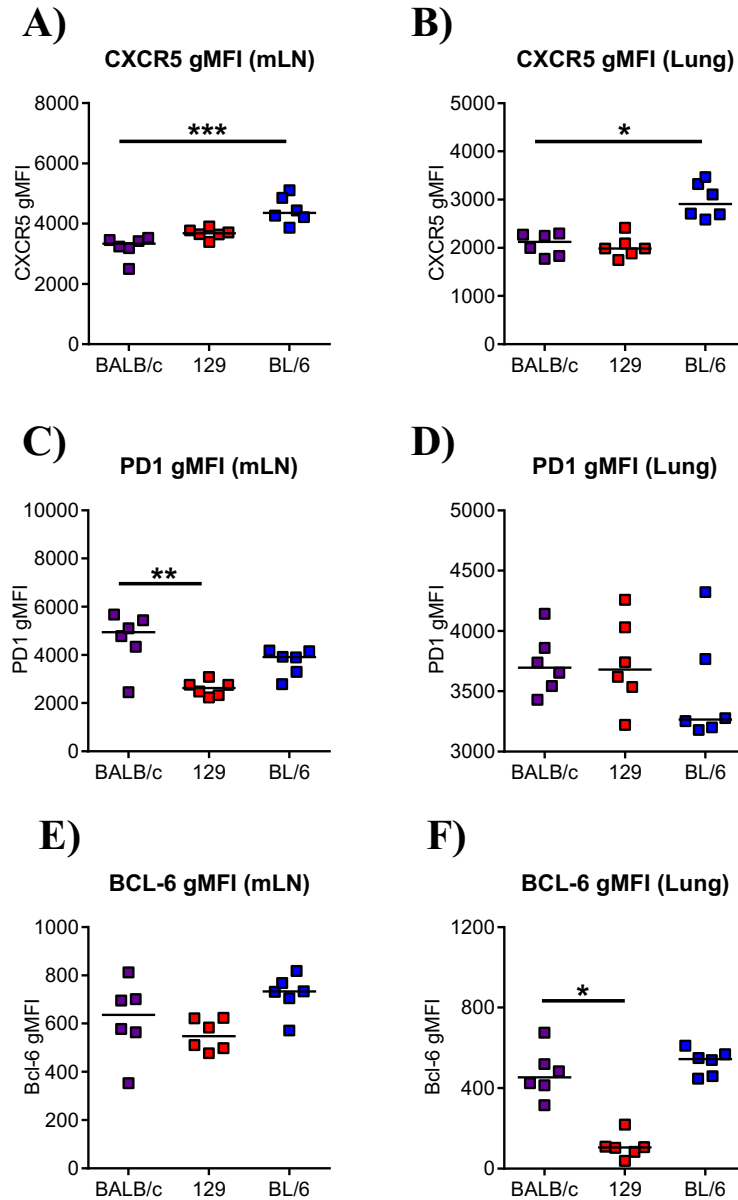


Figure 5.10: Expression of T_{FH} markers varies with mouse strain

Adult female BALB/c, 129/S2J (129) and C57BL/6 (BL/6) mice were exposed (i.n) to HDM 3 times a week for 3 weeks. Geometric mean fluorescence intensity (gMFI) was calculated for T_{FH} related markers on T_{FH} populations ($CXCR5^{+}PD1^{+}Foxp3^{-}CD4^{+}$). **A)** CXCR5 gMFI on mLN T_{FH} , **B)** CXCR5 gMFI on lung T_{FH} , **C)** PD1 gMFI on mLN T_{FH} , **D)** PD1 gMFI on lung T_{FH} , **E)** BCL-6 gMFI on mLN T_{FH} , **F)** BCL-6 gMFI on lung T_{FH} . Median data is displayed. n=5 per group. Experiment, n=1. Statistical significance was determined using a Kruskal Wallis with Dunn Multiple Comparisons test.

Taken together, this data shows that T_{FH} were found at higher levels in 129 and BL/6 mice than BALB/c animals. Both 129 and BL/6 induced large T_{FH} populations, however mLN and lung T_{FH} population in BL/6 mice appeared to express similar or higher levels of CXCR5 and Bcl-6, and had a proportionally greater ability to generate T_{FH} in the lungs, compared to their 129 and BALB/c counterparts. Therefore, all subsequent studies in this PhD were performed using C57BL/6 mice that had a greater and more mature T_{FH} population than the previously used BALB/c mouse.

5.3.6. Lung T_{FH} are tissue resident cells

As the lung is a highly vascularised tissue it was important to understand if the lung T_{FH} identified were within the lung tissue or the circulation. To understand this C57BL/6 mice were exposed to HDM or PBS 5 times a week for 3 weeks (**Figure 5.11A**). The 5 times a week protocol was used as it is the published chronic protocol used to drive full allergic disease phenotype in C57BL/6 mice (Johnson et al., 2004). After 3 weeks of exposure mice were intravenously injected via the tail vein with FITC labelled anti-CD45 (pan leukocyte marker) antibody 3-5 mins before sacrifice (**Figure 5.11A**). This short period allows sufficient time for leukocytes within the blood to be stained with anti-CD45 antibody without impacting tissue resident cells. Therefore, tissue resident (CD45⁻) and circulating (CD45⁺) cells can be distinguished using flow cytometry (Anderson et al., 2014, Galkina et al., 2005).

Confirming that the intravenously injected antibody successfully stained cells within the vascular system, the majority of CD4⁺ T cells within the blood were CD45⁺ in both PBS and HDM treated animals (**Figure 5.11B**). Consistent with the mLN being a minimally vascularised tissue, in both PBS and HDM treated animals very few cells were CD45⁺ and more than 99% were tissue resident CD45⁻ cells (**Figure 5.11B, C**). In the lungs of PBS treated animals, the majority of CD4⁺ T cells were within the circulation rather than within the tissue, while in HDM exposed animals the proportion of tissue resident CD4⁺ T cells was greatly increased to

around 80%, indicating a vast increase of cells in the lung tissue upon allergen exposure (Figure 5.11B, C).

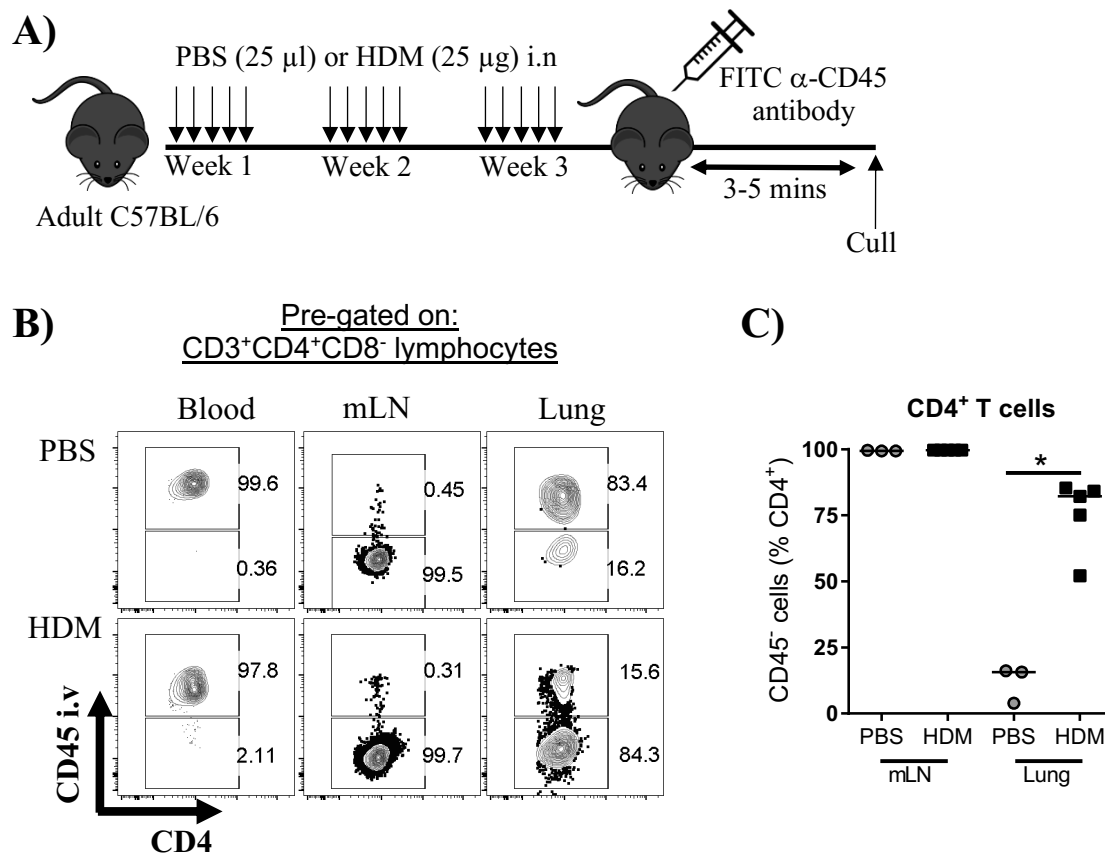


Figure 5.11: The majority of CD4⁺ T cells reside in the lung tissue of allergen exposed mice

Adult female C57BL/6 mice were exposed (i.n) to PBS and HDM 5 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v) with FITC-labelled CD45 antibody, 3-5 mins before culling. Tissue resident cells were defined as CD45⁻ cells. **A)** Schematic of experimental design, **B)** Representative flow plots of CD45 i.v. staining in the blood, mLN and lung CD4⁺ T cells in PBS and HDM treated animals, **C)** Quantification of CD45⁺ and CD45⁻ cellular populations in lung and mLN within PBS and HDM treated animals. Median data is displayed. n=3-5 per group. Statistical significance was determined using a Mann Whitney U test. Data is representative of 2 independent experiments.

Next, mLN and lung T_{FH} populations were examined within the CD45⁻ and CD45⁺ CD4⁺ T cell groups. Consistent with mLN cells being largely absent from the circulating pool, T_{FH} were not identified within the vascular and were instead found resident within the tissue (Figure 5.12A,

B). Similarly, lung T_{FH} were found in the tissue rather than in the circulation, indicating these cells were indeed truly within the lung (**Figure 5.12A, B**). The same trend was observed with mLN and lung T_{EFF} cells which were predominately identified as a tissue resident population (**Figure 5.12C**). Taken together, these data show that following allergen challenge the majority of $CD4^+$ T cells are found within the tissue and lung T_{FH} represent a tissue resident population.

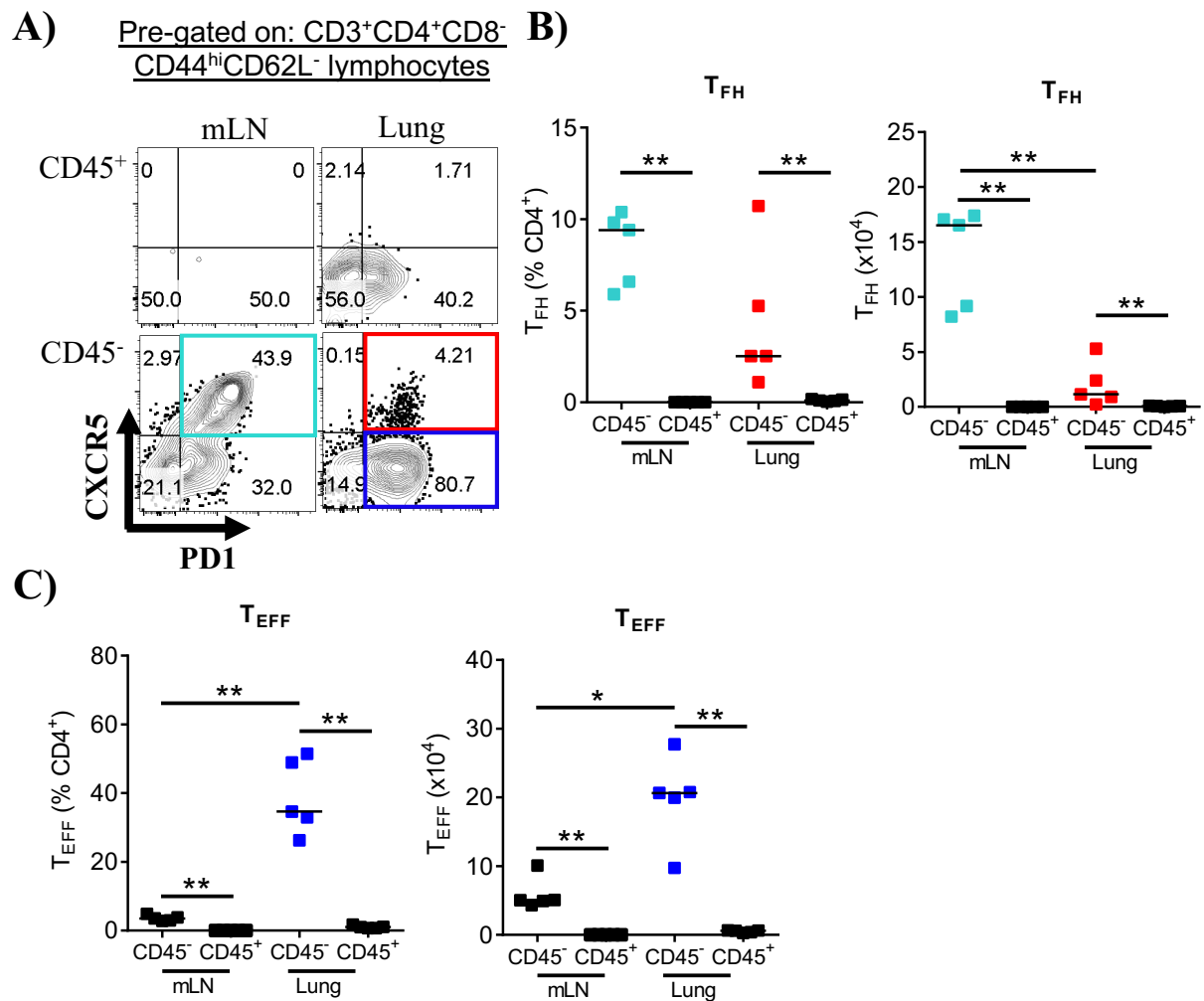


Figure 5.12: Lung T_{FH} are tissue resident cells

Adult female C57BL/6 mice were exposed (i.n) HDM 5 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v) with FITC-labelled $CD45$ antibody, 3-5 mins before culling. Tissue resident cells were defined as $CD45^-$ cells. **A)** Representative flow cytometry of T_{FH} and T_{EFF} populations in the lungs and mLN within the $CD45^+$ and $CD45^-$ populations, **B)** Quantified frequency and total number of T_{FH} populations in the mLN and lungs of HDM treated animals. **C)** Quantified frequency and total number of T_{EFF} populations in the mLN and lungs of HDM treated animals. Median data is displayed. $n=5$ per group. Statistical significance was determined using a Kruskal Wallis with Dunn Multiple Comparisons test. Data is representative of 2 independent experiments.

5.3.7. Overlapping and distinct features of lung and mLN T_{FH}

To further phenotype lung tissue T_{FH} (CD45⁺CD4⁺CXCR5⁺PD1⁺Foxp3⁻), they were compared to their mLN T_{FH} counterparts, other lung T_{EFF} cells (CD45⁺CXCR5⁺PD1⁺) and naïve mLN CD4⁺ T cells (CD45⁺CD4⁺Foxp3⁻) using flow cytometry (**Figure 5.13**). Consistent with T_{FH} populations being gated as CXCR5⁺PD1⁺ and T_{EFF} cells as CXCR5⁺PD1⁺, CXCR5 was expressed on the two T_{FH} population but absent from the lung T_{EFF} cells. Interestingly, mLN T_{FH} expressed higher levels of CXCR5 than lung T_{FH} (**Figure 5.13A**). Furthermore, both T_{FH} populations expressed Bcl-6, with the lung T_{FH} populations expressing lower levels of Bcl-6 than their mLN counterparts. (**Figure 5.13A**). Consistent with mLN T_{FH}, lung T_{FH} and lung T_{EFF} cells being gated as PD1⁺ cells, PD1 expression was seen on all 3 populations, with the two T_{FH} populations expressing more PD1 than lung T_{EFF} cells. No difference was found in the level of PD1 expression between the two T_{FH} groups (**Figure 5.13A**). ICOS expression was found on all 3 activated T cell populations and no difference in expression was found between the two T_{FH} populations (**Figure 5.13A**). P selectin glycoprotein ligand 1 (PSGL1) which is known to be downregulated on T_{FH} (Poholek et al., 2010) was barely expressed on the T_{FH} populations, but well expressed on lung T_{EFF} cells (**Figure 5.13A**). CXCR4 is associated with GC light and dark zone organisation (Allen et al., 2004) and was expressed by both mLN and lung T_{FH}, with the mLN T_{FH} expressing more CXCR4 than the lung T_{FH} (**Figure 5.13A**). Taken together these data show that lung CXCR5⁺PD1⁺CD4⁺ T cells express high levels of CXCR5, PD1, Bcl-6, ICOS, CXCR4 and low PSGL1 expression, indicating a phenotype indicative of a bona fide T_{FH}.

The T cells were then further analysed for other T cell associated markers. All 3 activated populations had a proportion of cells that were CD44^{hi}, with the lung T_{FH} cells having the lowest proportion of CD44^{hi} cells (**Figure 5.13B**). Consistent with antigen experience, all 3 activated cells had reduced proportions of CD62L⁺ cells, with the lowest frequency of CD62L⁺ cells being in the two lung populations (**Figure 5.13B**). Ly6C was expressed at a low level on all 3 effector T cell populations (**Figure 5.13B**). A small proportion of the 3 activated T cell populations were

also CD127⁺, with the two T_{FH} populations possessing the lowest frequency of CD127⁺ cells (**Figure 5.13B**). Lung and mLN T_{FH} populations and T_{EFF} cells expressed the activation marker CD69, however fewer lung T_{FH} expressed CD69 than their mLN counterparts (**Figure 5.13B**). The tissue residency marker, CD103 was only expressed by a subset of the two lung CD4⁺ T cell populations, and not by the T_{FH} in the mLN (**Figure 5.13B**). Thus, lung T_{FH} were fairly similar to their mLN counterparts, and appeared to be antigen experienced, activated cells. However, the mLN appeared to represent a larger antigen experienced and activated T_{FH} population than the lungs.

Human T_{FH} can be subdivided into T_{FH1}, T_{FH2} and T_{FH17} based on the expression of the chemokine receptors, CXCR3, CCR4 and CCR6. CXCR3⁺CCR6⁻ cells are T_{FH1}, CCR4⁺CXCR3⁻CCR6⁻ cells are T_{FH2}, while CXCR3⁻CCR6⁺ are termed T_{FH17} cells (Morita et al., 2011). In the mouse, CXCR3 was poorly expressed on all analysed populations (**Figure 5.14A**). Few CXCR3⁺CCR6⁻ populations were identified in any of the analysed T cell populations, although a greater proportion of mLN T_{FH} appeared to be CXCR3⁺CCR6⁻ than in lungs but, no significant difference was observed between the two T_{FH} populations (**Figure 5.14A, B**). CXCR3⁻CCR6⁺ cells were found in the 3 activated T cell populations and a greater proportion of lung T_{FH} compared to mLN T_{FH} appeared to possess this T_{FH17} phenotype (**Figure 5.14A, C**). In fact, around 25% of lung T_{FH} had a T_{FH17}-like phenotype compared to only 8% of mLN T_{FH} population (**Figure 5.14C**). Lastly, the expression of T_{FH2} phenotype was assessed. Very few cells from each group were CCR4⁺CCR6⁻CXCR3⁻ cells and no differences were found between the analysed groups (**Figure 5.14D, E**). Taken together these data showed that the lung T_{FH} population expressed typical T_{FH} markers and a larger proportion of these cells displayed a T_{FH17} cell phenotype compared to their mLN counterparts.

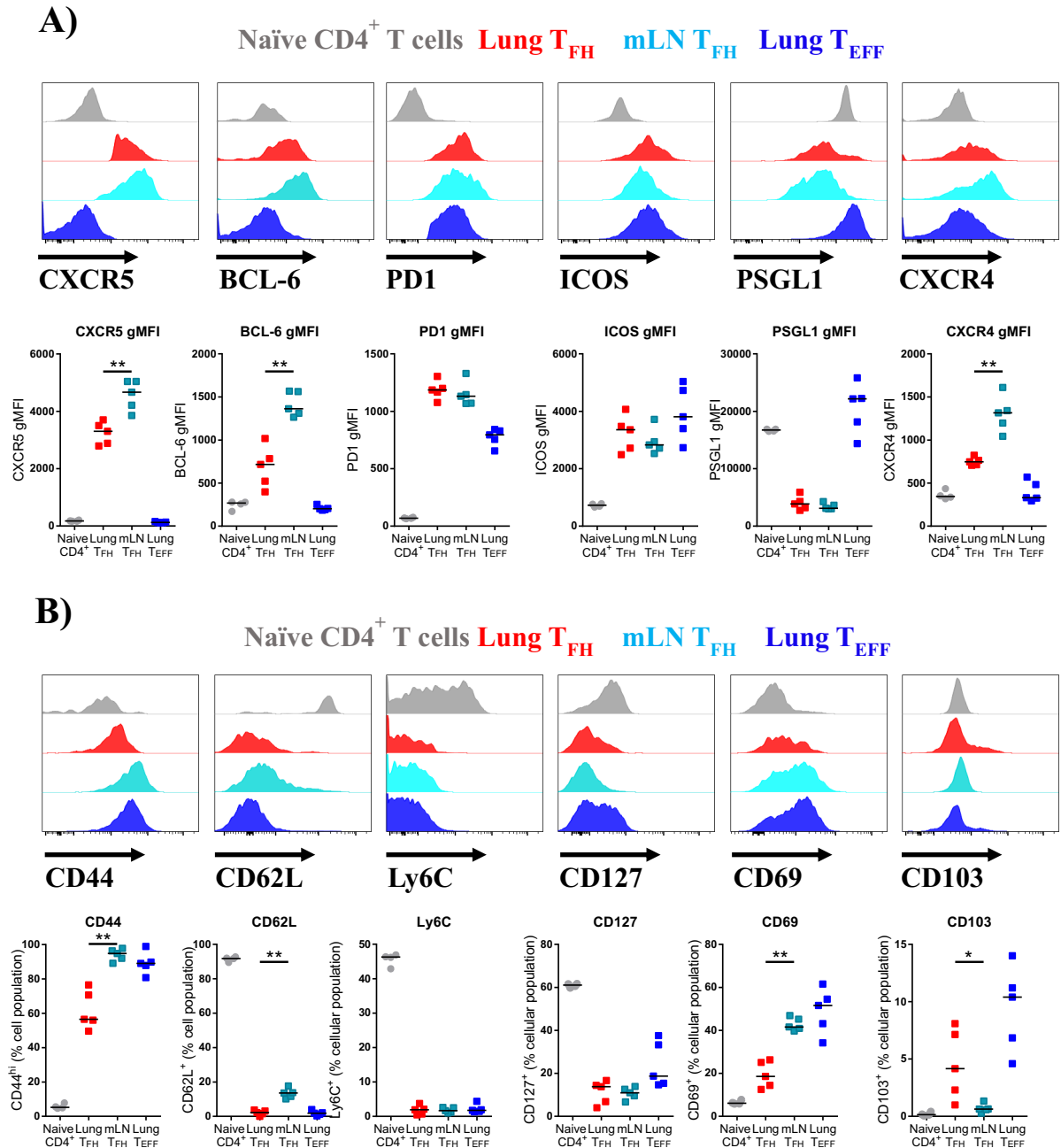


Figure 5.13: Lung T_{FH} express distinctive T_{FH} markers

Adult female C57BL/6 mice were exposed (i.n) to PBS and HDM 5 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v) with FITC-labelled CD45 antibody, 3-5 mins before culling. Tissue resident cells were defined as CD45⁻ cells. **A)** Histograms of geometric mean fluorescent intensity (gMFI) of T_{FH} related cellular markers and gMFI quantification on mLN naïve CD4⁺ T cells (CD45⁻CD4⁺Foxp3⁻) from PBS treated mice and lung T_{FH} (CXCR5⁺PD1⁺Foxp3⁻CD4⁺), mLN T_{FH} and lung T_{EFF} (CXCR5⁺PD1⁺Foxp3⁻CD4⁺) from HDM exposed animals **B)** Histograms of mean fluorescent intensity and quantification of the proportion of T cell marker positive cells in lung T_{FH}, mLN T_{FH} and Lung T_{EFF} cells. Statistical significance between the two T_{FH} groups was determined using a Mann Whitney U test. n=4-5 per group. Data is representative of 2 independent experiments.

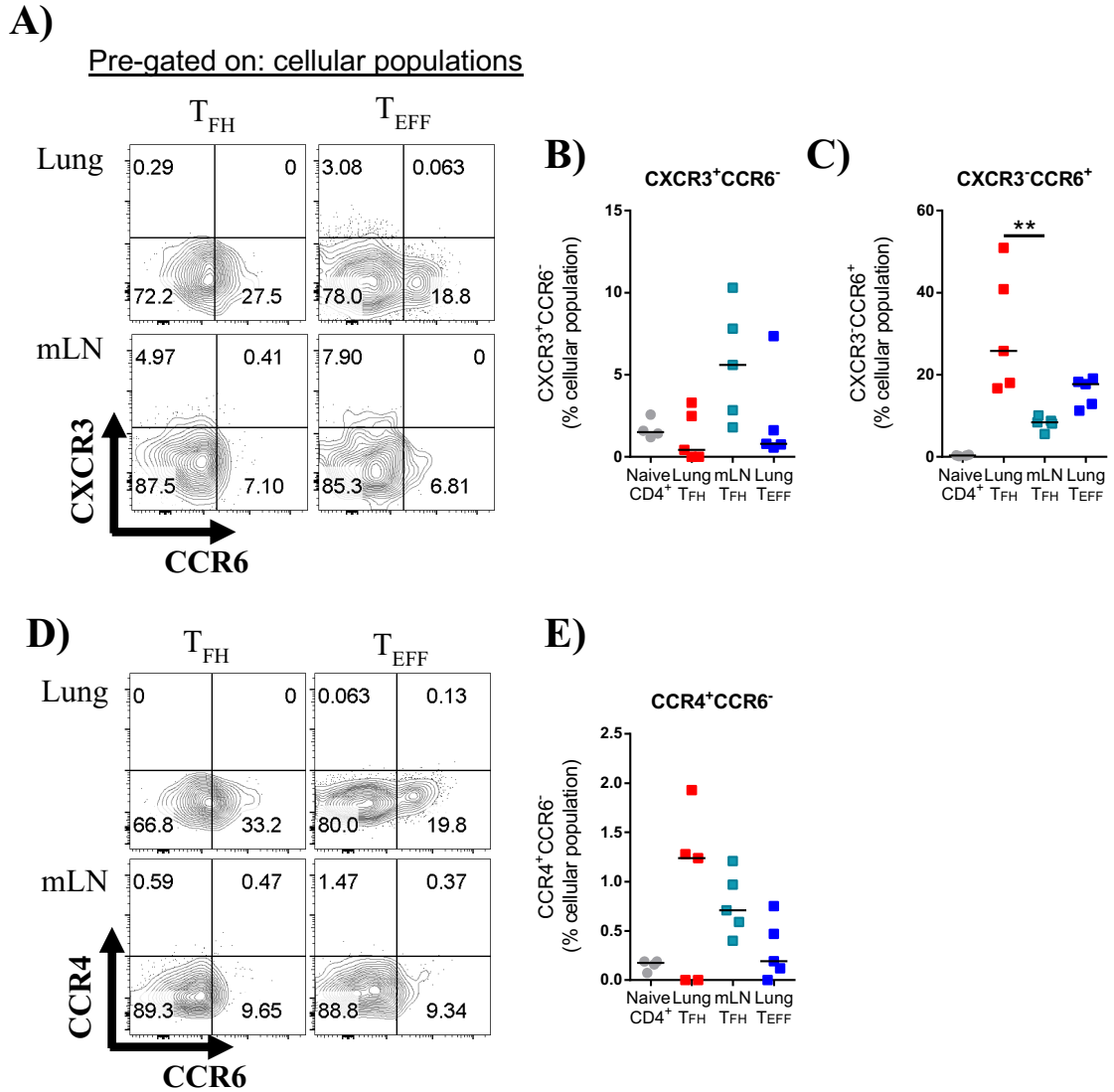


Figure 5.14: Lung T_{FH} express T_{FH17} associated markers

Adult female C57BL/6 mice were exposed (i.n) to PBS and HDM 5 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v) with FITC-labelled CD45 antibody, 3-5 mins before culling. Tissue resident cells were defined as CD45⁻ cells. Data from HDM treated animals is displayed. Lung and mLN T_{FH} and T_{EFF} cells from HDM treated animals were compared to mLN naïve CD4⁺ T cells from PBS treated animals. **A)** Representative flow plots of CXCR3 and CCR6 staining on lung and mLN T_{FH} and T_{EFF} cells, **B)** Proportions of CXCR3⁺CCR6⁻ T_{FH} , T_{EFF} cells and naïve CD4⁺ T cells. **C)** Proportions of CXCR3⁺CCR6⁺ T_{FH} , T_{EFF} cells and naïve CD4⁺ T cells, **D)** Representative flow plots of CCR4 and CCR6 staining on lung and mLN T_{FH} and T_{EFF} cells, **E)** Proportions of CCR4⁺CCR6⁻ T_{FH} , T_{EFF} cells and naïve CD4⁺ T cells. Statistical significance between the two T_{FH} groups was determined using a Mann Whitney U test. n=4-5 per group. Data is representative of 2 independent experiments.

5.5.8. Lung T_{FH} contain a subpopulation of IL-17A producing cells

Given that the lung T_{FH} population appeared to have a more T_{FH17} phenotype than their mLN counterparts, IL-17A producing capacity by lung T_{FH} was further examined. To understand this adult IL-17A fate reporter mice (*il-17a^{cre}rosa-26rfp^{fl/fl}*) (Hirota et al., 2011) were exposed to HDM or PBS, 5 times a week for 5 weeks. These mice permanently report all cells that have ever produced IL-17A as red florescent protein (RFP) positive cells. Data was compared to littermate controls, *rosa-26rfp^{fl/fl}* (WT) (**Figure 5.15A**).

Upon examination of the total IL-17A⁺RFP⁺ lymphocyte population, it was clear that after allergen exposure, IL-17A-RFP⁺ cells were mostly restricted to the lung tissue (**Figure 5.15B**). Very few IL-17A-RFP⁺ cells were identified in the mLN or lungs of PBS controls (**Figure 5.15B**). The predominate source of IL-17A within the lungs were the T_{EFF} cells, presumably Th17 cells (**Figure 5.15C**). However, when the lung T_{EFF} were excluded, lung $\gamma\delta$ T cells were the next largest producer (**Figure 5.15D**). IL-17A was also produced by lung T_{FH} and NKT cells after allergen treatment (**Figure 5.15D**).

Using the reporter mice, whether cells had ever produced IL-17A could be determined without the need for ex vivo stimulation. No RFP signal was observed in any of the analysed T cells in the mLN and lungs of WT mice. Again, mLN T_{FH} expressed very little RFP, while approximately 20% of lung T_{FH} cells were IL-17A-RFP⁺ (**Figure 5.16A, B**). IL-17A-RFP was also strongly expressed by lung T_{EFF} cells, $\gamma\delta$ T cells and natural killer T (NKT) cells (**Figure 5.16A-E**), with the proportion of IL-17A-RFP⁺ T_{EFF} and $\gamma\delta$ T cells greater than observed by the lung T_{FH} (**Figure 5.16A, C-D**). Thus the use of a reporter mouse confirmed that the lung was the major site of IL-17A production during chronic AAD, and that a proportion of lung, but not mLN, T_{FH} had previously, or were currently, IL-17A producing cells.

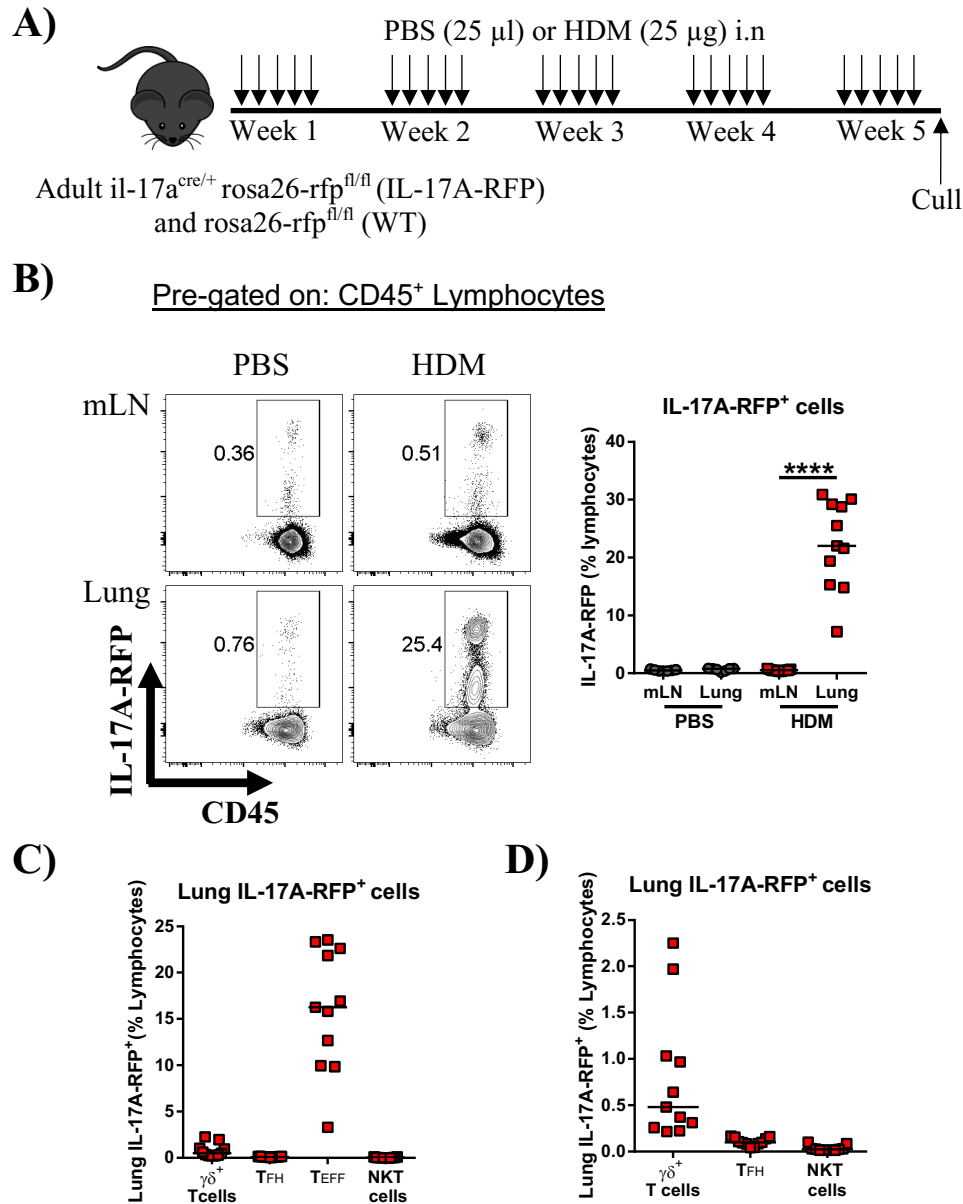


Figure 5.15: IL-17A is predominately produced in the lungs by T_{EFF} cells

Adult *il-17a^{cre/+} rosa26-rfp^{fl/fl}* (IL-17A-RFP) and *rosa26-rfp^{fl/fl}* (WT) mice were exposed (i.n) to PBS and HDM, 5 times a week for 5 weeks. **A)** Schematic of experimental design, **B)** Flow cytometry was used to determine the frequency of IL-17A-RFP⁺ cells in the mLN and lungs of PBS and HDM treated animals, **C)** Frequency of IL-17A-RFP⁺ cells in different cellular populations. **D)** Frequency of IL-17A-RFP⁺ cells in different cellular populations when the T_{EFF} cells are removed. Statistical significance between mLN and lung cells was determined using a Mann Whitney U test. Median data is displayed. n=7-10. Data is representative of 2 independent experiments.

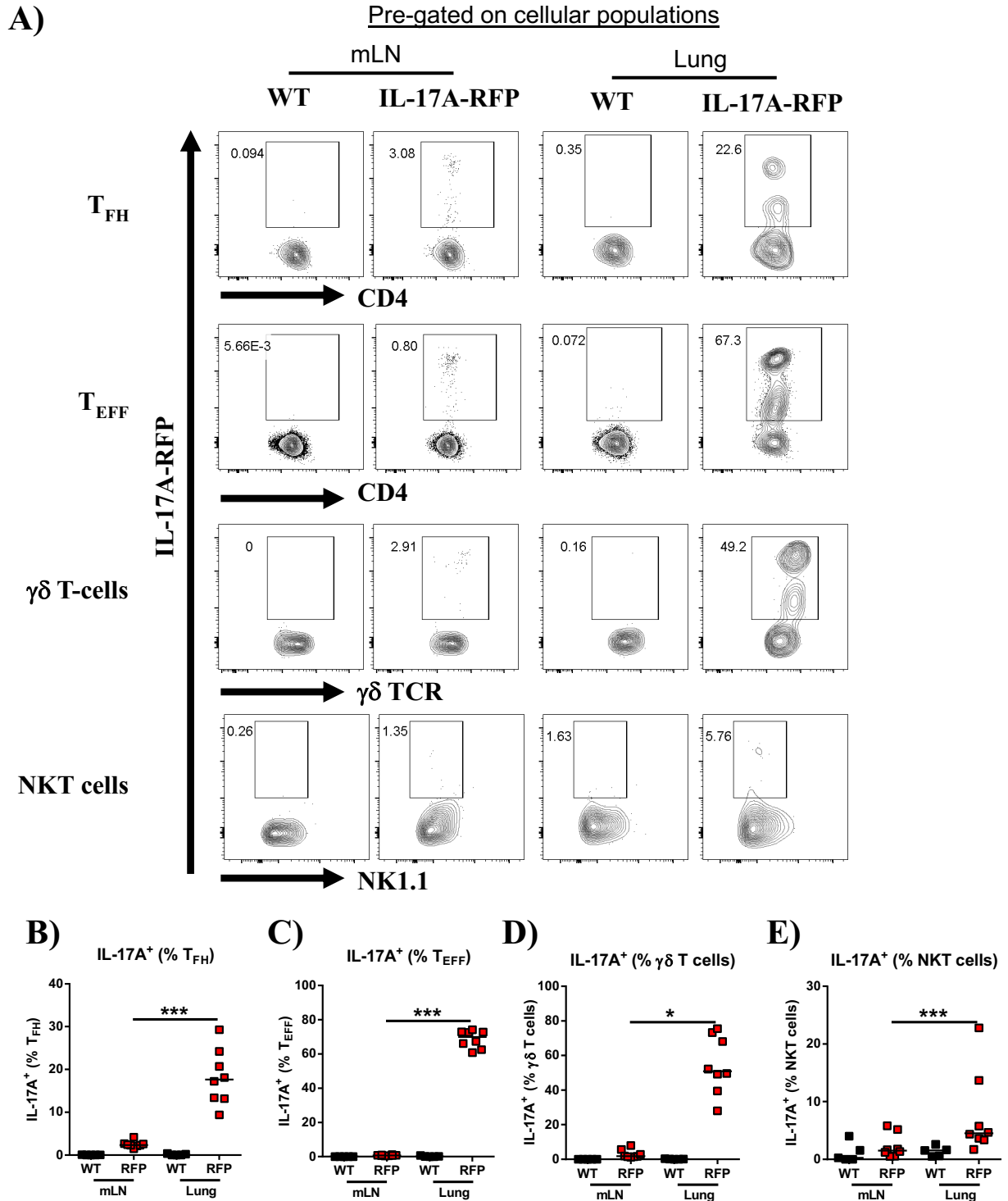


Figure 5.16: IL-17RFP⁺ cells reside within the lung tissue

Adult *il-17a^{cre/+}rosa-26-rfp^{fl/fl}* (IL-17A-RFP) and *rosa-26-rfp^{fl/fl}* (WT) mice were exposed (i.n) to PBS and HDM, 5 times a week for 5 weeks. Flow cytometry was used to determine the frequency of IL-17A-RFP⁺ T cell populations in the mLN and lungs of WT and RFP HDM treated animals, **A)** Representative flow plots of IL-17A-RFP⁺ cells in different cellular populations. Frequency of IL-17A-RFP⁺ T cells within different cellular populations was quantified, **B)** T_{FH} , **C)** T_{EFF} cells, **D)** $\gamma\delta$ T cells, **E)** Natural killer T (NKT) cells. Statistical significance between allergen treated mLN and lung cells was determined using a Mann Whitney U test. Median data is displayed. n=7-10. Data is representative of 2 independent experiments.

5.5.9. T_{FH} gene expression is distinct from other CD4⁺ T cells

To further phenotype and examine differences between the lung and mLN T_{FH}, gene expression was examined and compared to T_{EFF} cells and splenic naïve CD4⁺ T cells (CD4⁺CD44⁻CD62L⁺). Briefly mice were treated with PBS or HDM 5 times a week for 5 weeks. Tissue resident cells were defined using intravenously FITC-labelled CD45 staining. T cell populations of interest were then sorted and gene expression was assessed by RT-qPCR.

Cxcr5 and *Bcl6* gene expression was restricted to the mLN and lung T_{FH} (**Figure 5.17A, B**), with the lung T_{FH} expressing less *Cxcr5* transcript than their mLN counterparts (**Figure 5.17A**). *Gata3* was not expressed by either T_{FH} population (**Figure 5.17C**), while these cells expressed very low levels of the other lineage defining transcription factors, *Tbx21* and *Rorc* (**Figure 5.17D, E**). However, the lung T_{FH} expressed slightly more *Rorc* transcript than their mLN counterparts (**Figure 5.17E**). Expression of *Gata3*, *Tbx21* and *Rorc* was strongly expressed by T_{EFF} cells in the mLN and lungs, indicating the presence of multiple T_{EFF} cell populations (**Figure 5.17C-E**). *Prdm1* encodes for the antagonist of Bcl-6, Blimp-1, which is not normally expressed in T_{FH} populations (Johnston et al., 2009). In AAD T_{EFF} cells which expressed low *Bcl6*, instead expressing higher levels of *Prdm1*, with the greatest expression observed in the lung T_{EFF} cells (**Figure 5.17F**). The lowest *Prdm1* expression was found on the mLN T_{FH} consistent with high *Bcl6*, but the lung T_{FH} despite expressing h*Bcl6* expressed *Prdm1* to a level comparable to mLN T_{EFF} cells (**Figure 5.17F**). Taken together this data shows that when observing transcription factor gene expression, lung T_{FH} express transcripts associated with T_{FH}.

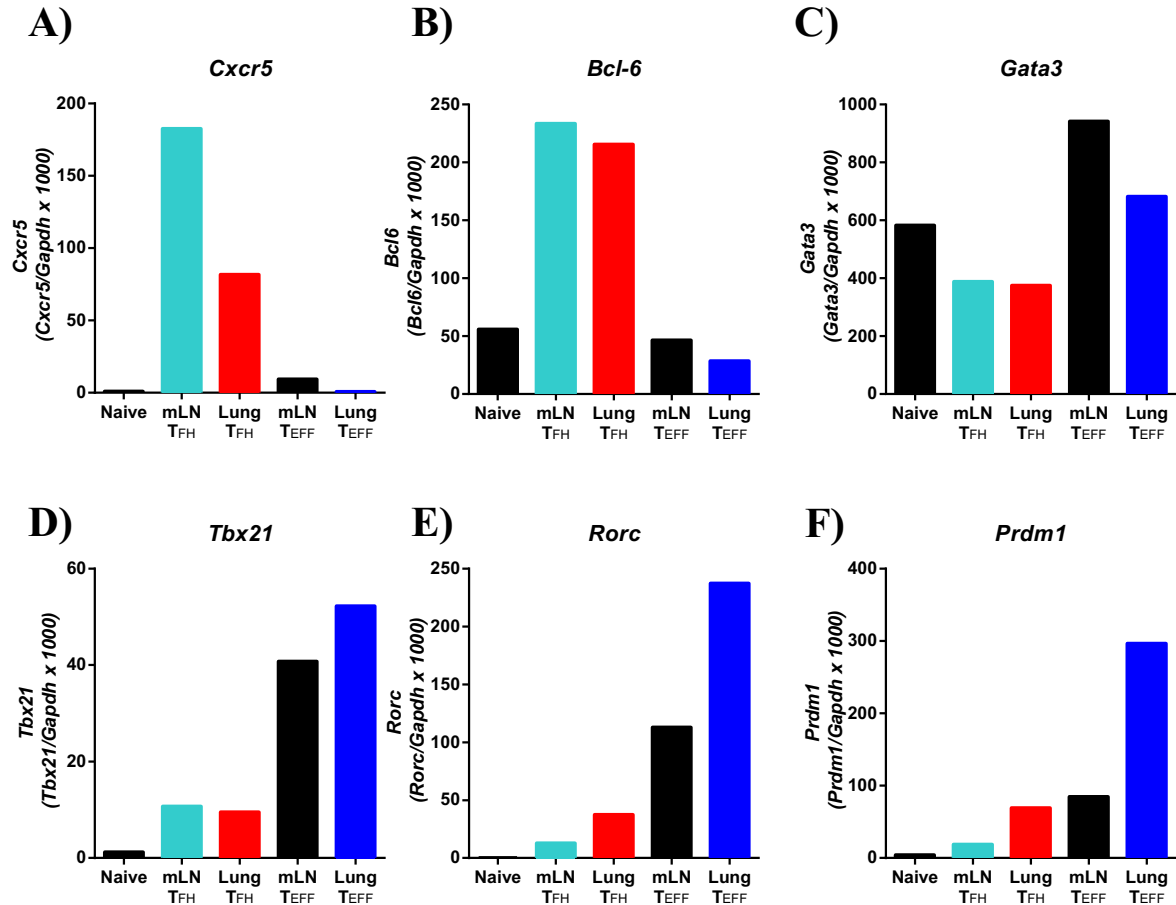


Figure 5.17: Lung T_{FH} express T_{FH} associated transcripts

Adult female C57BL/6 mice were exposed (i.n) to PBS and HDM 5 times a week for 5 weeks. Intravascular cells were stained by injecting (i.v) with FITC-labelled CD45 antibody, 3-5 mins before culling. Tissue resident cells were defined as CD45⁺ cells. T_{FH} and T_{EFF} were sorted from the lungs and mLN and compared to naïve splenic CD4⁺ T cells (CD4⁺CD44^{lo}CD62L⁺). Gene expression was determined by qPCR and the data was presented relative to *Gapdh*. **A) *Cxcr5*, B) *Bcl-6*, C) *Gata3*, D) *Tbx21*, E) *Rorc*, F) *Prdm1*.** Data is pooled from 5 individual mice. Experiment, n=1.

Next, cytokine gene expression was examined in the sorted T cell populations (**Figure 5.18**).

Il4 and *Il21* transcript was expressed by all 4 of the activated T cell populations analysed, with greater expression being found in the mLN T_{FH} than the lung T_{FH} group (**Figure 5.18A, B**).

Thus, both T_{FH} populations expressed the signature T_{FH} associated cytokines. The type 2 cytokines, *Il5* and *Il13* transcripts were poorly detected in lung T_{FH}, but were detected at higher levels in the lung T_{FH} than the mLN T_{FH} population (**Figure 5.18C, D**). Importantly however T_{EFF} cells expressed higher levels of *Il5* and *Il13*, with the highest expression in the lung T_{EFF}

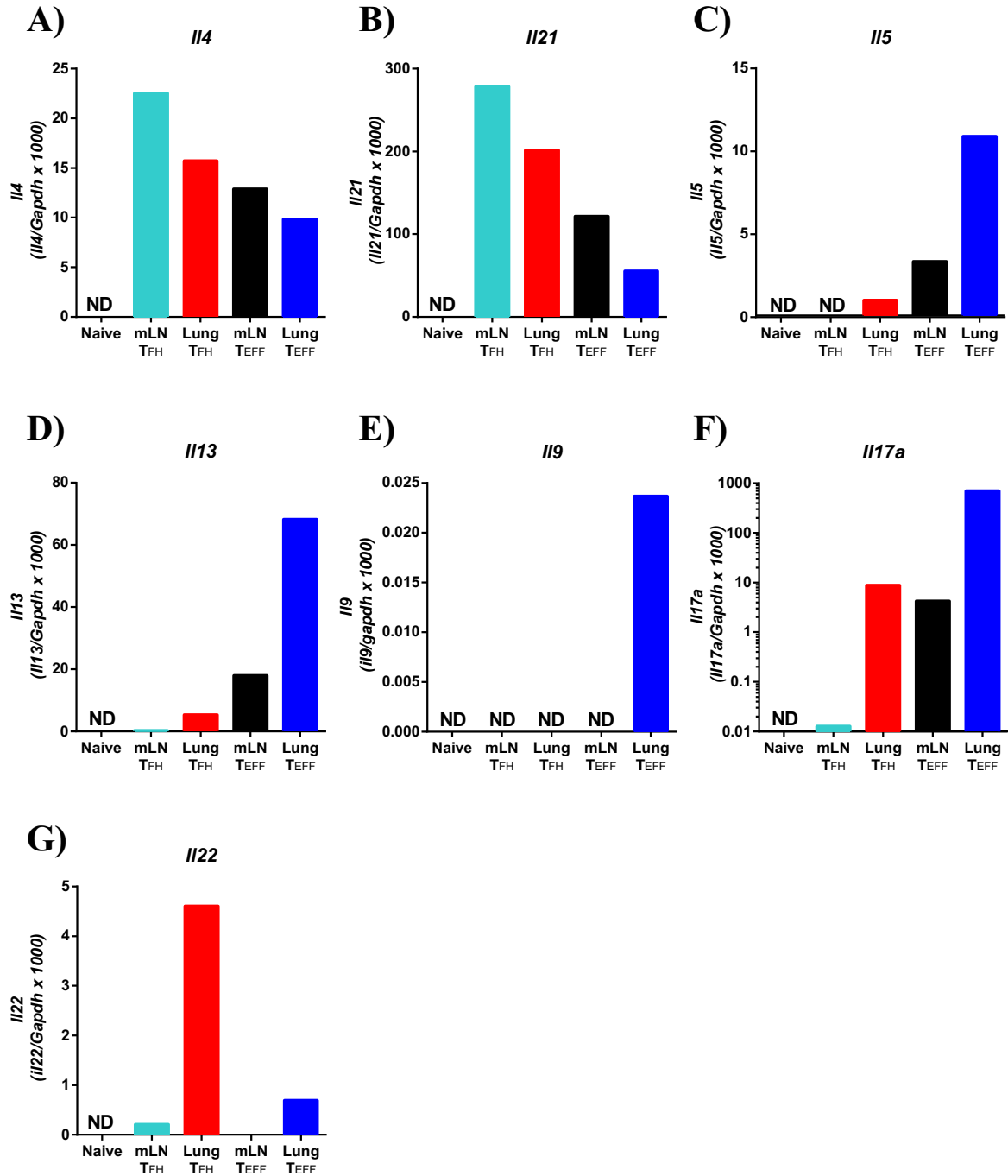


Figure 5.18: Lung T_{FH} express IL-4, IL-21 and IL-17A

Adult female C57BL/6 mice were exposed (i.n) to PBS and HDM 5 times a week for 5 weeks. Intravascular cells were stained by injecting (i.v) with FITC-labelled CD45 antibody, 3-5 mins before culling. Tissue resident cells were defined as CD45⁺ cells. T_{FH} and T_{EFF} were sorted from the lungs and mLN and compared to naïve splenic CD4⁺ T cells. Gene expression was determined by qPCR and the data was presented relative to *Gapdh*. **A) IL4, B) IL21, C) IL5, D) IL13, E) IL9, F) IL17a, G) IL22**. Data is pooled from 5 individual mice. Experiment, n=1.

cells (**Figure 5.18C, D**). *IL9*, which has also been highlighted as a cytokine of interest in allergic asthma (Kearley et al., 2011, Cheng et al., 2002, Soroosh and Doherty, 2009), was only detected in the lung T_{EFF} cells, but at what appeared to be very low transcript levels (**Figure 5.18E**). Given both Th2 cell associated cytokines, and the Th2 master transcriptional regulator, were predominantly found in lung T_{EFF} cells, but not elsewhere, this fits with the presence of lung Th2 cells within in this population.

Lastly, the Th17 associated cytokines, *IL17a* and *IL22* were assessed. *IL17a* transcript was detected at a greater level in the lung T_{FH} population than the mLN T_{FH} population (**Figure 5.18F**). The level of expression was comparable to that of mLN T_{EFF} cells and the highest level was detected in the lung T_{EFF} cells (**Figure 5.18F**). In contrast *IL22* expression was at its highest level in the lung T_{FH} overall (**Figure 5.18G**). Taken together, this expression shows that lung T_{FH}, like their mLN T_{FH} counterparts expressed T_{FH} associated cytokines but had a wider cytokine repertoire, in particular, with respect to *IL17a* and *IL22*.

5.5.11. Summary of T_{FH} phenotyping data

Table 7 gives a summary of the phenotyping data comparing mLN and lung T_{FH} and lung T_{EFF} cells. Overall, lung T_{FH} were closely related to mLN T_{FH} but also produced higher levels of IL-17A and expressed *IL22* transcript.

A)

	mLN T _{FH}	Lung T _{FH}	Lung T _{EFF} cells
CXCR5	++	+	-
PD1	++	++	++
BCL-6	++	+	-
GATA3	-	-	++
PSGL1	-	-	++
CXCR4	++	+	-
CCR6	+	++	++
IL-4	+	+	+
IL-21	+	+	+
IL-5	-	-	+
IL-13	-	-	+
IL-17A	+	++	++
IL-22	-	++	+

Table 7: Summary of T_{FH} phenotyping in mLN T_{FH}, lung T_{FH} and lung T_{EFF} cells.
High expression (++), expression (+) and no/low expression (-).

5.4. Discussion

The aim of this chapter was to understand the phenotype of the CXCR5⁺PD1⁺CD4⁺ T cells induced in the lungs and the mLN during chronic AAD. The data from this chapter clearly shows that CXCR5⁺PD1⁺CD4⁺ T cells are induced at both sites display key markers of the T_{FH} lineage while being devoid of markers associated with Th2 cells. Interestingly, the data also suggests the cytokine repertoire to differ between these bona fide mLN and lung T_{FH} populations, with the lung T_{FH} displaying a T_{FH17} signature.

5.4.1. CXCR5⁺PD1⁺CD4⁺ T cells induced during chronic AAD are bona fide T_{FH}

The primary role of T_{FH} is to interact with B cells and drive their proliferation and differentiation into memory B cells and plasma cells which generate high affinity, isotype switched antibody (Vinuesa et al., 2016). The data presented here shows T_{FH} induced in the mLN and locally within the lungs of allergen exposed animals to be tissue resident cells. cT_{FH} populations have been shown to exist in the blood of mice during several infectious models and during human disease, including allergic inflammation (Hale et al., 2013, Simpson et al., 2010, Gong et al., 2018, Schulten et al., 2017, Kim et al., 2016). Therefore, it was particularly important for the highly vascularised lung to determine whether identified T_{FH} populations were in the blood or the tissue. Given that lung T_{FH} populations were tissue resident rather than circulating, the data are consistent with information from chapter 3 showing a lack of cT_{FH} within the blood of allergen treated animals.

Induced T_{FH} populations were examined using a combination of flow cytometry and gene expression analysis and were found to possess a prototypical T_{FH} phenotype. T_{FH} at both sites expressed high levels of CXCR5, PD1, Bcl-6 and ICOS, while expressing low levels of PSGL1. Bcl-6 is the master regulator of T_{FH} and is known to be necessary and sufficient for naïve CD4⁺ T cells to commit to the T_{FH} lineage (Johnston et al., 2009, Nurieva et al., 2009, Yu et al., 2009). Bcl-6 was found to be expressed at a lower level on the lung T_{FH} than their mLN counterparts. This is consistent with data from other groups showing T_{FH} populations that

reside outside of SLOs tending to have reduced Bcl-6 expression compared to their SLO counterparts or no expression at all (He et al., 2013b, Zhang et al., 2016, Bentebibel et al., 2013). It has been proposed to be a result of these cells not residing within a GC and therefore no longer needing to retain high levels of this marker. However, the lung T_{FH} induced during allergic disease probably still exist in some sort of a B cell structure and follicle T_{FH} still express Bcl-6 but not to the level observed in GC (Kitano et al., 2011). Therefore, the smaller GC induced in the lung during this model could also explain the lower Bcl-6 expression on these lung T_{FH} compared to their mLN counterparts. Despite this, T_{FH} outside the GC are still dependent on Bcl-6 for their generation (He et al., 2013b) and the same is likely true for the lung T_{FH} population.

The migration of T_{FH} is tightly controlled by the balance of the chemokine receptors, CXCR5 and CCR7 (Haynes et al., 2007). This is critical for allowing T_{FH} to be in close proximity to B cells by migrating along CXCL13 gradients. Consistent with this both lung and mLN T_{FH} express high levels of CXCR5. While CCR7 itself was not directly measured, the CCL19 and CCL21 binding protein, PSGL1 was poorly expressed on both T_{FH} populations. PSGL1 is a direct target of the Bcl-6 repression and its downregulation is important for T_{FH} localisation (Poholek et al., 2010). CXCR4, the chemokine receptor for CXCL12 was found to be expressed on both T_{FH} but not on the T_{EFF} cells, consistent with data showing Bcl-6 overexpression to drive CXCR4 expression in T_{FH} (Yu et al., 2009). Taken together this data shows that the lung and mLN T_{FH} populations were capable of migrating towards areas that B cells reside as long as they receive the appropriate chemokine signalling.

CD127 (IL-7R α) expression was considerably lower on both T_{FH} populations and the lung T_{EFF} cells compared to naïve CD4⁺ T cells. In T_{EFF} cells this downregulation is potentially mediated by IL-2 (Xue et al., 2002). However, IL-2 signalling actually inhibits the T_{FH} differentiation program (Ballesteros-Tato and Randall, 2014) and Bcl-6 directly targets *il7r* and thereby antagonises IL-7R/STAT5 signalling which is inhibitory to T_{FH} differentiation program (Liu et al., 2016). Both lung and the mLN T_{FH} populations contained antigen experienced cells expressing high CD44 and low CD62L, and both populations had a subpopulation of cells

expressing the activation marker CD69. While, the residency marker, CD103 was not expressed by the majority of mLN and lung T_{FH}, it was expressed by a small population of lung T_{FH}.

From a functional point of view both the mLN and lung T_{FH} populations were capable of producing IL-4 and IL-21 and therefore possess the ability to help B cells to proliferate and differentiate. Both mLN and lung T_{FH} expressed *Il4* and *Il21* transcript, but both were expressed at a lower level in the lung population. By flow cytometry a similar proportion of T_{FH} at both sites were IL-4⁺ but a lower proportion of T_{FH} within the lung were IL-21⁺. Recently, it has been suggested that IL-4 and IL-21 can be used to define 3 distinct subsets of T_{FH} (IL-4⁺IL-21⁻, IL-4⁺IL-21⁺ and IL-4⁻IL-21⁺). This study suggested the double positive IL-4⁺IL-21⁺ T_{FH} to most efficiently drive GC formation, class switch recombination and plasma cell generation (Weinstein et al., 2016). Thus, the presence of heightened IL-21 in combination with IL-4 may suggest mLN T_{FH} represent a more functional T_{FH} population than their lung counterparts in regards to generating a humoral response.

In none of the studies carried out did T_{FH} express significant *Gata3* transcript or GATA3 protein. GATA3 expression was limited to Th2 cells, suggesting there was not a transcriptional overlap between these cells. This observation in AAD is distinct from observations in mice after *Schistosoma masoni* infection, where T_{FH} and Th2 cells express comparable levels of *Gata3* transcript (Zaretsky et al., 2009), and suggests the relationship between Th2 and T_{FH} cells during type 2 immune responses may depend on the site and type of inflammation induced. However, this does fit with differential transcriptional control at the *Il4* locus. In T_{FH} *Il4* transcription is driven by the cooperation of c-maf and BATF/IRF4 acting at the hypersensitivity region V (CSN2) region of the *Il4* locus. While in Th2 cells, this transcription is driven by GATA3 acting at the *Il4* promoter (Sahoo et al., 2015). Indeed IL-4 production by T_{FH}, but not Th2 cells, can be eliminated by selectively removing CSN2 region of the *Il4* promoter (Vijayanand et al., 2012). Thus, as is the case after vaccination and infection T_{FH} induced during chronic AAD do not appear to require GATA3 expression to function, nor to produce IL-13, and consistent with this do not produce other type 2 cytokines, including IL-5

and IL-9. This aligns with a study by Kobayashi and colleagues which show that transfer of Th2 cells but not T_{FH} into T cell and ILC deficient animals could drive BAL IL-5 and IL-13 secretion (Kobayashi et al., 2017). While the data presented here shows T_{FH} and Th2 cells to represent 2 distinct cellular populations, it cannot rule out the possibility that T_{FH} can transition into Th2 cells as was suggested by cell transfer experiments by the lab of Beatriz Leon (Ballesteros-Tato et al., 2016). Likewise, it also remains, possible that Th2 cells themselves might acquire T_{FH} like capacities (Zaretsky et al., 2009). However, it is worth noting the work of Locksley and colleagues which describe using a series of reporter mice that IL-4 and IL-13 derive from separate CD4⁺ T cell populations during helminth infection, consistent with separation between IL-4⁺ T_{FH} and IL-13⁺ Th2 cells (Liang et al., 2011).

5.4.2. Lung T_{FH} have a T_{FH17} phenotype

Interestingly, during chronic AAD, which is traditionally considered a Th2 mediated disease, T_{FH} in the lungs were identified that possessed a T_{FH17}-like phenotype. When comparing the lung and the mLN T_{FH} it was observed that a greater proportion of the lung T_{FH} were IL-17A⁺ or CCR6⁺. Consistent with this, *Il17a* and *Rorc* transcript were higher in the lung T_{FH} compared to their mLN counterparts. Lung T_{FH} also expressed high levels of *Il22* transcript, a cytokine closely associated with Th17 cells compared to other analysed cellular populations. Thus, this data showed that the lung T_{FH}, or a subset of them, were far more likely to have a T_{FH17} phenotype than their mLN counterparts. Overall the mLN appear to have few IL-17A⁺ cells upon allergen challenge while a substantial frequency of IL-17A⁺ cells were observed in the lungs during chronic AAD. Interestingly, Bcl-6 is a transcriptional repressor and is known to bind to promoters and enhancers associated with the differentiation of other CD4⁺ T cell lineages (Hatzi et al., 2015, Nurieva et al., 2009, Yu et al., 2009). Bcl-6 has been suggested to limit Th17 differentiation by specifically binding the *Rorc* promoter (Yu et al., 2009), while another group have suggested that Bcl-6 does not bind the *Rorc* promoter but instead targets other Th17 associated sites, including *Rora* and an IL-17A/F enhancer (Hatzi et al., 2015).

Regardless, it would be interesting to understand whether the mild co-expression of *Bcl6* and *Prdm1* in the lung T_{FH} is sufficient to allow more flexibility at the *Rorc* locus in lung T_{FH}.

IL-17A is produced by multiple cell types during allergic disease and in this study the predominant source of IL-17A are Th17 cells within the T_{EFF} cellular population. IL-17A has been found in the lungs of asthmatic patients and has been found to correlate with disease severity, with highest concentrations found in moderate to severe asthmatics compared to mild patients (Chakir et al., 2003). Despite, IL-17A being linked to more severe asthma it has been suggested that the cellular source of IL-17A is critical for function during allergic disease. IL-17A producing Th17 cells have been shown to drive steroid insensitive mucus secretion and AHR, while IL-17A from $\gamma\delta$ T cells has been shown to be important for the resolution of AAD (Murdoch and Lloyd, 2010). Furthermore, IL-17A from NKT cells has been shown to drive neutrophilia (Michel et al., 2007) and is closely associated with ozone induced asthma (Pichavant et al., 2008). However, the role of IL-17A from T_{FH} during chronic AAD has not been fully explored.

IL-17A has also been linked to humoral immunity through the formation of TLS within the lung called iBALT. IL-17A produced by T cells has been shown to act on FDCs to produce the chemokines CXCL13 within the lungs (Rangel-Moreno et al., 2011a) or to drive CXCL12 production by fibroblastic reticular cells (Fleige et al., 2014). These chemokines are not normally found within the lungs and have been shown to drive the formation of organised lymphocyte aggregates (Rangel-Moreno et al., 2011a, Fleige et al., 2014). Interestingly, one group showed *in vitro* generated T_{FH17} to be superior to Th17 cells in driving the formation of these iBALT structures (Rangel-Moreno et al., 2011a). In chapter 3 it was shown that both HDM and ALT exposure can trigger the formation of lung GC B cells and that lung T_{FH} strongly positively correlate with lung GC B cell proportions and numbers. Given that these lung T_{FH} cells also have a T_{FH17}-like phenotype it maybe that these cells play a role in the formation of TLS in the lung that support these GC B cells. This concept will be explored in more depth in chapter 6.

5.4.3. Concluding remarks

This chapter demonstrates that during chronic AAD T_{FH} generated in the mLN and the lung tissue are phenotypical T_{FH}. They both express key T_{FH} markers, including CXCR5, PD1, Bcl-6, ICOS, CXCR4, while expressing low levels of PSGL1 and Ly6C. T_{FH} induced at both sites can produce the signature T_{FH} cytokines, IL-4 and IL-21 and during chronic AAD did not take on phenotypical features associated with Th2 cells. T_{FH} did not express GATA3 nor did they produce the type 2 cytokine IL-5, -9 or IL-13. In many regards, lung T_{FH} resembled their mLN counterparts, however, the lung T_{FH} appeared skewed to a T_{FH17} phenotype. In the next chapter the role of T_{FH}, IL-17A and lung GC during chronic AAD will be examined.

Chapter 6: T_{FH} are critical regulators of chronic allergic airway disease

6.1. Introduction

In the previous chapter, it was shown that T_{FH} induced during allergic airway disease both in the mLN and the lungs appeared to share characteristics with stereotypical “bona fide” T_{FH} . They expressed many of the phenotypical T_{FH} markers, including CXCR5, PD1, BCL-6 and ICOS, while also producing the major T_{FH} associated cytokines IL-4 and IL-21. Interestingly however the lung T_{FH} appeared to be more skewed towards a T_{FH17} phenotype being IL-17A⁺ and expressing the transcription factor ROR γ t. Given that IL-17A has been linked to iBALT formation during pulmonary infection by causing ectopic CXCL13 secretion by FDCs and that the transfer of T_{FH17} into T cell deficient mice strongly drives iBALT formation in response to antigenic stimulation (Rangel-Moreno et al., 2011a) it could be hypothesized that these lung T_{FH17} cells that develop during chronic AAD drive the formation of TLS.

Moreover, throughout the previous work the observation of local T_{FH} and GC B cells has been suggestive of the existence of TLS formation in the lungs of chronic allergen exposed animals. iBALT structures have been identified in the lungs of patients with a number of chronic disorders including COPD (Hogg et al., 2004), RA (Rangel-Moreno et al., 2006) and cancer (Dieu-Nosjean et al., 2008), and suggestive lymphoid aggregates have been identified in the lungs of asthmatic patients (Elliot et al., 2004) and chronic allergen exposed mice (Johnson et al., 2004). The contribution of iBALT to pathology appears to vary extensively depending on the disease context. In non-small cell lung cancer iBALT have been linked to better disease prognosis (Dieu-Nosjean et al., 2008) and during TB infection iBALT formation around granulomas in human and mice and are thought to help control infection (Ulrichs et al., 2004, Khader et al., 2009) even in the absence of SLOs (Day et al., 2010). iBALT can also have pathogenic roles during autoimmune diseases when pulmonary complications are present such as Sjögren’s syndrome and RA by contributing a local source of autoantibodies helping to drive pathology (Rangel-Moreno et al., 2006). In COPD iBALT are generally found in the lungs of patients with the most severe disease (Hogg et al., 2004) but whether these structures contribute to pathology or exist as part of a protective response to infections that

settle in at later stages of the disorder is not truly understood. Taken together, it is clear these structures have many purposes and thus it is critical to understand whether T_{FH} and GC B cells in this model of chronic AAD form structures suggestive of iBALT. Furthermore, if TLSs form what role does IL-17A play to this process and what contribution do these structures or local T_{FH} and GC B cell make to pathogenesis.

In chapter 4, intervening with T_{FH} responses during chronic AAD via α -ICOS-L blockade improved established chronic allergic disease. However, this method resulted in only transient depletion of local and systemic T_{FH}, after disease was established, and was not specific enough to examine the precise contribution of T_{FH} to disease pathogenesis without taking into account any potential side effects on other cellular populations. Therefore, in this chapter T_{FH} responses were examined using the much more specific *cd4^{cre}bcl-6^{fl/fl}* mouse line in which T cells are incapable of expressing Bcl-6 and thus CD4⁺ T cells cannot differentiate into T_{FH} (Hollister et al., 2013). Initially these *cd4^{cre}bcl-6^{fl/fl}* mice were shown in a 10 day sheep red blood cell immunisation model to be deficient in T_{FH} and GC B cells (Hollister et al., 2013). But have also subsequently been used in an acute OVA and IL-33 sensitisation and challenge model of allergic disease and show T_{FH} to be critical for the generation of allergen specific IgE (Kobayashi et al., 2017). However, the impact of this T_{FH} deficiency on other hallmark features of allergic disease such as AHR has not been assessed or examined in the context of a more clinically relevant chronic AAD models. In particular it would be interesting to try and understand the contribution of lung T_{FH} versus SLO T_{FH}.

Overall, the aim of this current chapter was to understand the role of that T_{FH}, IL-17A and lung resident GC responses play during chronic AAD.

6.1.1. Hypothesis

T_{FH} and GC B cells induced in the lungs are organised into lymphoid aggregates and T_{FH} are critical for the development of chronic allergic airway disease

6.1.2. Aims

- 1) To determine if lung T_{FH} and GC B cells are found in lung resident TLS
- 2) To determine the role of local versus systemic T_{FH} and GC B cell responses to chronic AAD
- 3) To understand the role of T_{FH} during allergic disease pathogenesis

6.2. Methods

6.2.1. Intravascular discrimination of cellular populations

Adult female C57BL/6 mice were exposed (i.n) to HDM (25 µg) or PBS (25 µl), 5 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v) mice via the tail vein with FITC-labelled CD45 antibody, 3-5 mins before culling. Tissue resident cells were defined as CD45⁻ cells. Cellular populations were assessed by flow cytometry (**Figure 6.1A**).

6.2.2. IL-17A knockout mouse studies

Adult female and male *rosa26-rfp^{fl/fl}* (WT), *il-17a^{cre/+}rosa26-rfp^{fl/fl}* (HET) and *il-17a^{cre/cre}rosa26-rfp^{fl/fl}* (17A-KO) mice were exposed (i.n) to HDM (25 µg) or PBS (25 µl) 5 times a week for 5 weeks. To examine the impact of loss of IL-17A production during chronic allergic airway disease mice were culled at the end of week 5 and the lungs and mLN were harvested. Cellular populations were determined by flow cytometry, while lung, BAL, NAL and serum antibody were assessed by ELISA. PCLS were imaged by wide-field microscopy (**Figure 6.1B**).

6.2.3. *cd4^{cre}bcl-6^{fl/fl}* mouse and chronic allergic disease studies

Adult female and male *bcl-6^{fl/fl}* (WT) or *cd4^{cre}bcl-6^{fl/fl}* (T-Bcl6^Δ) were exposed (i.n) to HDM (25 µg), ALT (10 µg) or PBS (25 µl) 5 times a week for 5 weeks. To assess the impact of loss of Bcl-6 expressing CD4⁺ T cells on chronic allergic airway disease, mice were culled at the end of week 5. Mice were sacrificed 18 hours after the final dose for each time-point. Lung function was assessed using the flexiVent system. Blood and BAL were collected, and the lungs and mLN were harvested. Cellular populations were determined by flow cytometry and lung, serum and BAL antibody levels were analysed by ELISA. PCLS were imaged by confocal microscopy (**Figure 6.2A**).

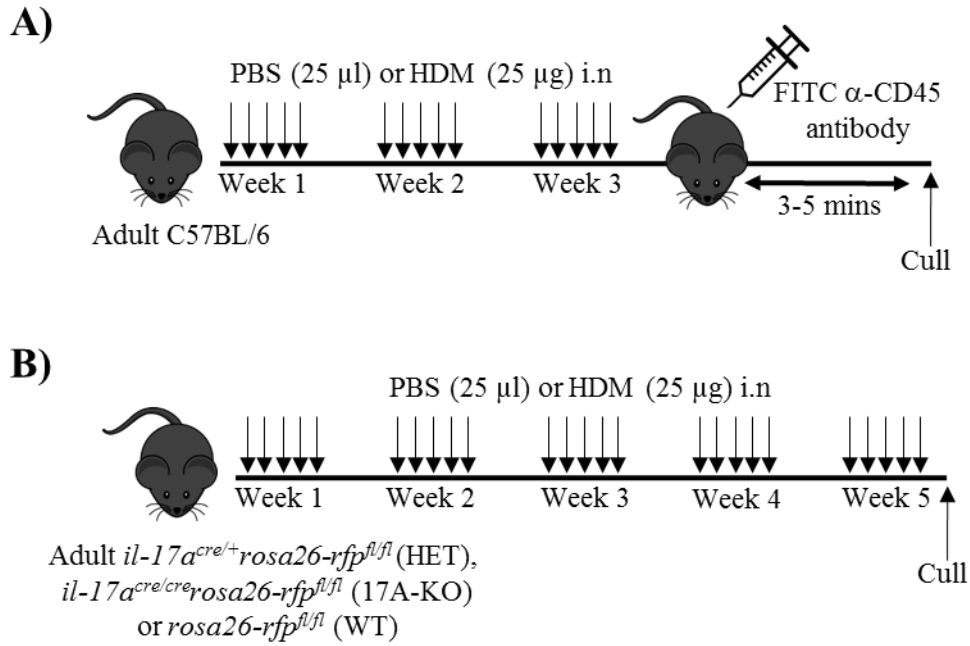


Figure 6.1: Methods 1

A) Adult female C57BL/6 mice were exposed (i.n) to 25 µg HDM or 25 µl PBS 5 times a week for 3 weeks. At the end of week 3 mice were intravenously injected (i.v) via the tail vein with 3 µg of FITC labelled anti-CD45 antibody. Mice were culled 3-5 mins later. **B)** Adult *il-17a^{cre/+}rosa26-rfp^{fl/fl}* (HET), *il-17a^{cre/cre}rosa26-rfp^{fl/fl}* (17A-KO) or littermate controls *rosa26-rfp^{fl/fl}* (WT) mice were exposed (i.n) to HDM 5 times a week for 5 weeks. Mice were culled at the end of week 5. All mice, regardless of study design were culled 18 hours after the final dose.

6.2.4. *cd4^{cre}bcl6^{fl/fl}* bone marrow chimera studies

Adult female *cd4^{cre}bcl6^{fl/fl}* CD45.2 (T-Bcl6^Δ) or *bcl6^{fl/fl}* CD45.2 (WT) bone marrow were mixed at a 1:1 ratio with WT CD45.1 bone marrow. 5 x 10⁶ mixed bone marrow cells were transferred into irradiated female WT C57BL/6 mice by intravenous injection. After 9 weeks of rest to reconstitute the bone marrow mice were then exposed to HDM (25 µg) or PBS (25 µl) 5 times a week for 3 weeks. BAL was collected and the lungs and mLN were harvested. Lung function was assessed using the flexiVent system and cellular populations were determined by flow cytometry (**Figure 6.2B**).

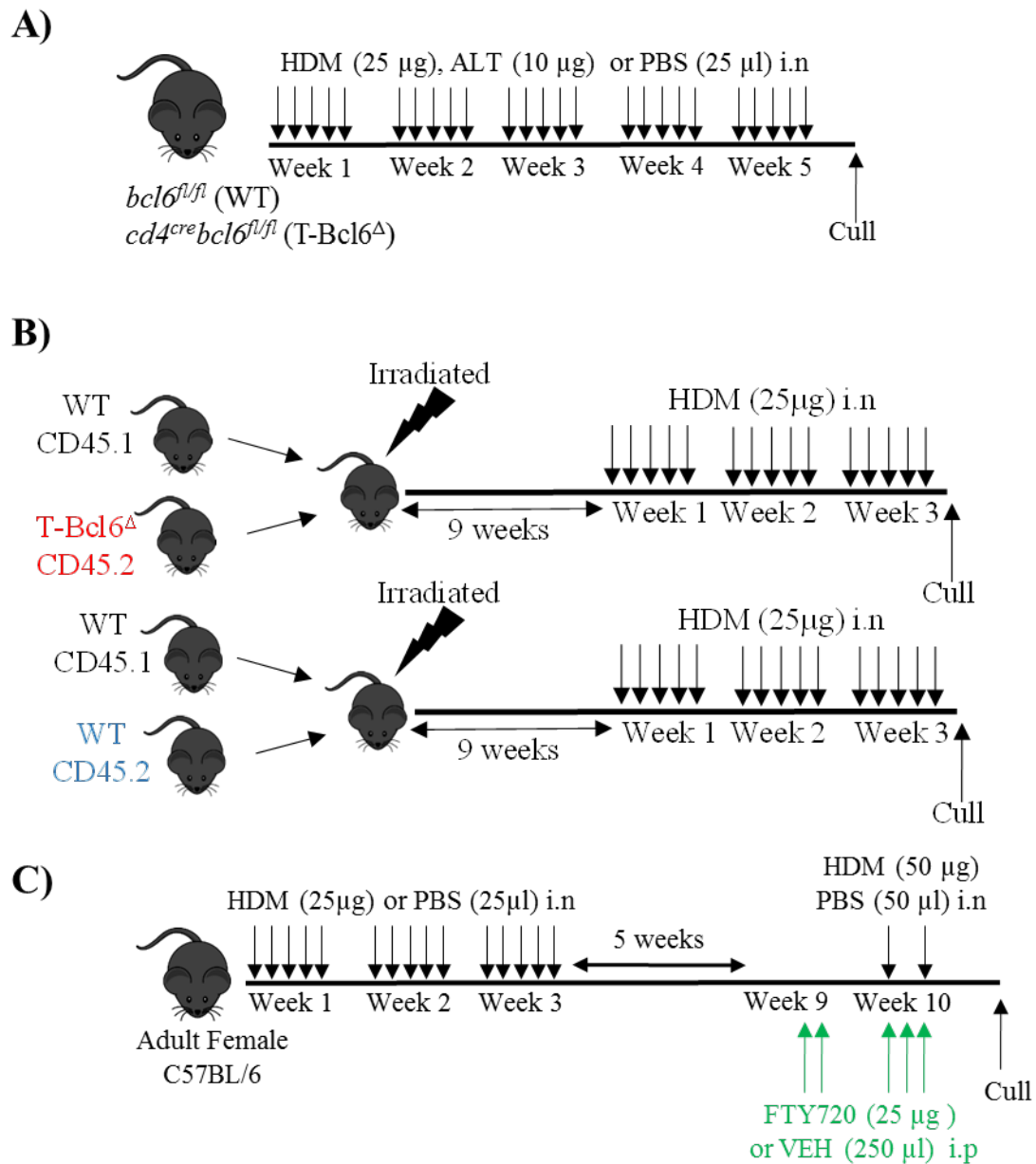


Figure 6.2: Methods 2

Adult *cd4^{cre}bcl6^{fl/fl}* (T-Bcl6^Δ) or *bcl6^{fl/fl}* (WT) mice were exposed (i.n) to 25 µg HDM, 10 µg ALT or 25 µl PBS 5 times a week for 5 weeks. **B)** Mixed bone marrow chimeras were created by transferring (i.v) 5×10^6 *cd4^{cre}bcl6^{fl/fl}* CD45.2 (T-Bcl6^Δ): CD45.1 (WT) or *bcl6^{fl/fl}* (WT): CD45.1 (WT) bone marrow into irradiated female C57BL/6 recipient mice. Mice were given 9 weeks to reconstitute their bone marrow then exposed (i.n) to 25 µg HDM or 25 µl PBS 5 times a week for 3 weeks. **C)** Adult C57BL/6 female mice were exposed (i.n) to 25 µg HDM 5 times a week for 3 weeks. Mice were then rested for 5 weeks before being administered 5 doses of 25 µg FTY720 or a 5% DMSO vehicle control (VEH) and being re-challenged with 2 doses of 50 µg HDM or 50 µl PBS. All mice, regardless of study design were culled 18 hours after the final dose.

6.2.5. FTY720 allergen re-challenge studies

Adult female C57BL/6 mice were exposed (i.n) to HDM (25 µg) or PBS (25 µl) 5 times a week for 3 weeks to establish chronic allergic disease. After a 5 week rest period mice were injected (i.p) with 5 daily doses of the drug FTY720 (25 µg) or a vehicle control (5 % DMSO) and re-challenged with either HDM (50 µg) or PBS (50 µl). Mice were culled 18 hours after the final re-challenge dose. BAL and serum were collected and the lungs and mLN were harvested. Lung function was assessed using the flexiVent system and cellular populations were determined by flow cytometry. Serum and BAL antibodies were assessed by ELISA and PCLS were imaged by wide-field microscopy (**Figure 6.2C**).

6.3. Results

6.3.1. Germinal centre and memory B cells are tissue resident cells

Chronic allergen exposure results in the movement of immune cells into the lungs, and their proliferation (Johnson et al., 2004, Gregory et al., 2009a). To examine the phenotype of B cells within the lungs intravascular staining was utilised (**Figure 6.3A**). Unsurprisingly, as the mLN is a poorly vascularised organ in both the PBS and HDM treated animals almost all the B cells were within the CD45⁻ tissue resident population (**Figure 6.3B**). While the majority of the B cells within the lungs of PBS treated animals were within the circulation with very few being CD45⁻ (**Figure 6.3B**). However, after allergen challenge most of the B cells were within the lung tissue itself (**Figure 6.3B**). Thereby, indicating that after allergen treatment there is a large infiltration, and potentially *in situ* proliferation, of B cells in the lung tissue.

Naïve B cells (IgD⁺IgM⁺) were identified in both the CD45⁻ and CD45⁺ compartments of both PBS and HDM treated animals (**Figure 6.3C**). However, in the mLN regardless of whether animals were PBS or HDM treated, the majority of naïve B cells were found within the CD45⁻ pool (**Figure 6.3D**). In contrast, in the lungs of PBS mice, except for one animal, naïve B cells were almost exclusively within the circulating CD45⁺ pool rather than within the lung tissue (**Figure 6.3D**). However, upon allergen exposure naïve B cells could be identified at similar numbers within both lung fractions (**Figure 6.3C, D**).

IgD⁻IgM⁻ B cells were only found within the CD45⁻ population of lung and mLN B cells (**Figure 6.3C**). Consistent with this GC B cells were also only identified within the CD45⁻ tissue resident population of the lung and mLN of allergen treated animals rather than within the circulation (**Figure 6.3E-G**). As expected GC B cells were not seen in either the CD45⁻ or CD45⁺ fraction of PBS treated animals (**Figure 6.3E-G**). Similarly, memory B cells were predominately identified within the tissue resident population in the lung and mLN of allergen treated animals (**Figure 6.4A-C**). Taken together this implies that following allergen treatment most B cells, comprising of naïve B cells, GC B cells and memory B cells are found within the lung tissue rather than within the circulation.

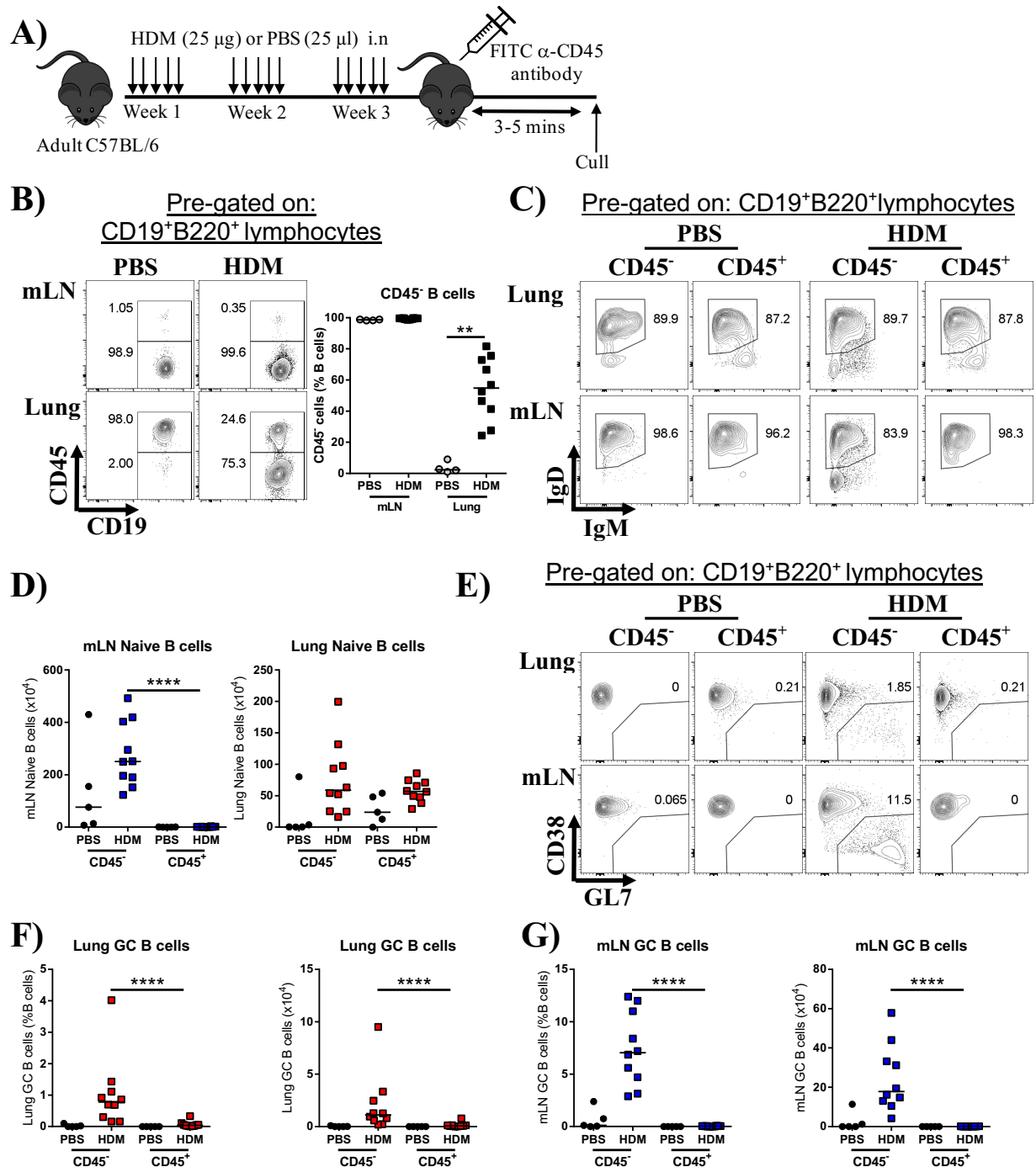


Figure 6.3: Chronic allergen exposure induces lung resident germinal centres

Adult female C57BL/6 mice were exposed (i.n.) to PBS and HDM 3 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v.) with FITC-labelled anti-CD45 antibody, 3-5 mins before culling animals. Tissue resident cells were defined as CD45⁻ cells. **A)** Schematic of experimental design, **B)** Flow cytometry was used to determine the proportion of tissue resident (CD45⁻) and circulating (CD45⁺) B cells, pre-gated on CD19⁺B220⁺ lymphocytes. Representative plots and quantified data is displayed. **C)** Representative flow plots of naive B cells (IgD⁺IgM⁺) B cells within the CD45⁺ and CD45⁻ compartments, pre-gated on CD19⁺B220⁺ lymphocytes, **D)** Numbers of CD45⁻ and CD45⁺ naive B cells in the mLN and lungs, **E)** Representative flow plots of mLN and lung tissue resident and circulating GC B cells, pre-gated on CD19⁺B220⁺ lymphocytes. **F)** Frequency and number of lung GC B cells. **G)** Frequency and number of mLN GC B cells. Statistical significance was determined using Mann Whitney U analysis. Data is pooled from 2 independent experiments. n=4-10.

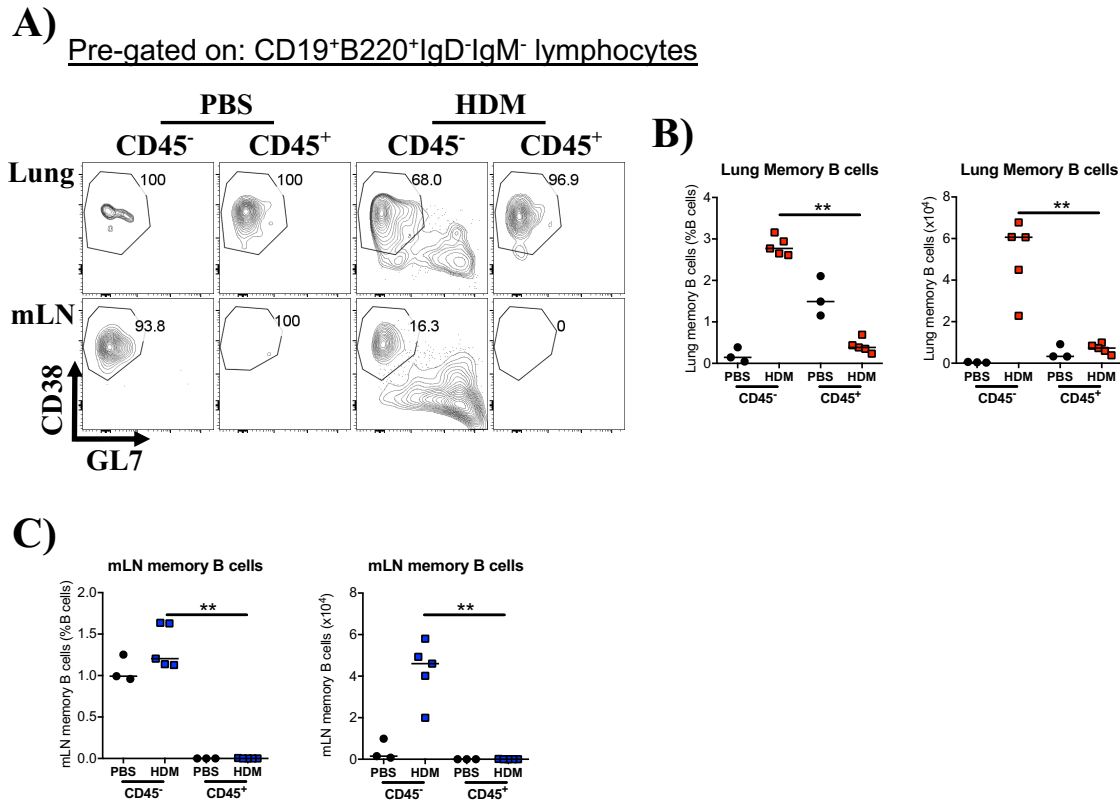


Figure 6.4: Chronic allergen exposure generates tissue resident lung memory B cells
Adult female C57BL/6 mice were exposed (i.n) to PBS and HDM 3 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v) with FITC-labelled anti-CD45 antibody, 3-5 mins before culling animals. Tissue resident cells were defined as CD45⁻ cells. **A)** Representative flow plots of memory B cells in the mLN and lungs of PBS treated animals in the CD45⁺ and CD45⁻ population. **B)** Frequency and number of lung memory B cells, **C)** Frequency and number of mLN memory B cells. Mann Whitney U analysis used to compare between groups. n=5 per group.

6.3.2. Tertiary Lymphoid Structures are found in the lung tissue

To examine B cell organisation within the lungs, PCLS from wild type C57BL/6 mice treated with either PBS or HDM for 3 weeks were imaged (**Figure 6.5A**). In the lungs of PBS treated animals B220⁺ B cells and CD4⁺ T cells could be easily identified and were scattered sporadically around the tissue (**Figure 6.5B**). While, in the lungs of allergen treated animals, B220⁺ B cells were organised into large clusters around the airways with CD4⁺ T cells being found within or around the clusters (**Figure 6.5B**). Furthermore, the B cell clusters contained Ki67⁺ and GL7⁺ B cells indicative of GC B cells (**Figure 6.5B**). While, CD21/35⁺ B220⁻ cells with morphology suggestive of FDCs (Allen et al., 2004) were found within these B cell

aggregates (**Figure 6.5C**), indicating that these structures contained the major components of GCs. Consistent with the presence of these organised lymphoid structures, the homeostatic chemokine CXCL13 which is expressed in the lungs during TLS formation was detected in the lungs of HDM treated animals at a significantly higher concentration than in PBS control animals (**Figure 6.5D**). Taken together these data suggest that allergen challenge results in the proliferation and infiltration of B cells into the lungs, forming TLS located close to airways.

6.3.3. The lungs are a site of active germinal centre switch and IgA memory

To investigate whether these lung resident TLS represent active GCs, tissue resident B cells were sorted from the lungs of 3-week allergen treated animals. CSR is an antibody diversification event that generally occurs within GCs and germline transcripts (GLT) and switch circle transcripts (SCT) should only be found at sites which are actively or have undergone CSR (Kinoshita et al., 2001) (**Figure 1.4**). Therefore, qPCR was performed on RNA extracted from lung resident B cells. For comparison, tissue resident B cells were also sorted from the mLN of allergen treated animals, a site where CSR is known to occur and splenic resident B cells were also sorted from PBS treated animals (**Figure 6.6**). As expected *IgE* GLTs, *IgG1* SCT and *IgA* SCT could not be detected in the splenic B cells and only minimal *IgG1* GLT expression was observed at this site (**Figure 6.6A**). *IgE* GLT could be detected in the lungs of allergen exposed animals, but the transcript was expressed at a much higher level in the mLN. In contrast *IgG1* GLT was expressed in the lungs, at a higher level than in mLN B cells, where it was very poorly detected in some animals. *IgG1* SCTs were similarly detected in the lungs and mLN, while *IgA* SCTs were only detectable in the lungs of allergen exposed animals (**Figure 6.6A**). Overall, this data implies that CSR is occurring in the lungs and mLNs to IgE, IgG1 and suggests the lung to represent a selective site for IgA switch during AAD.

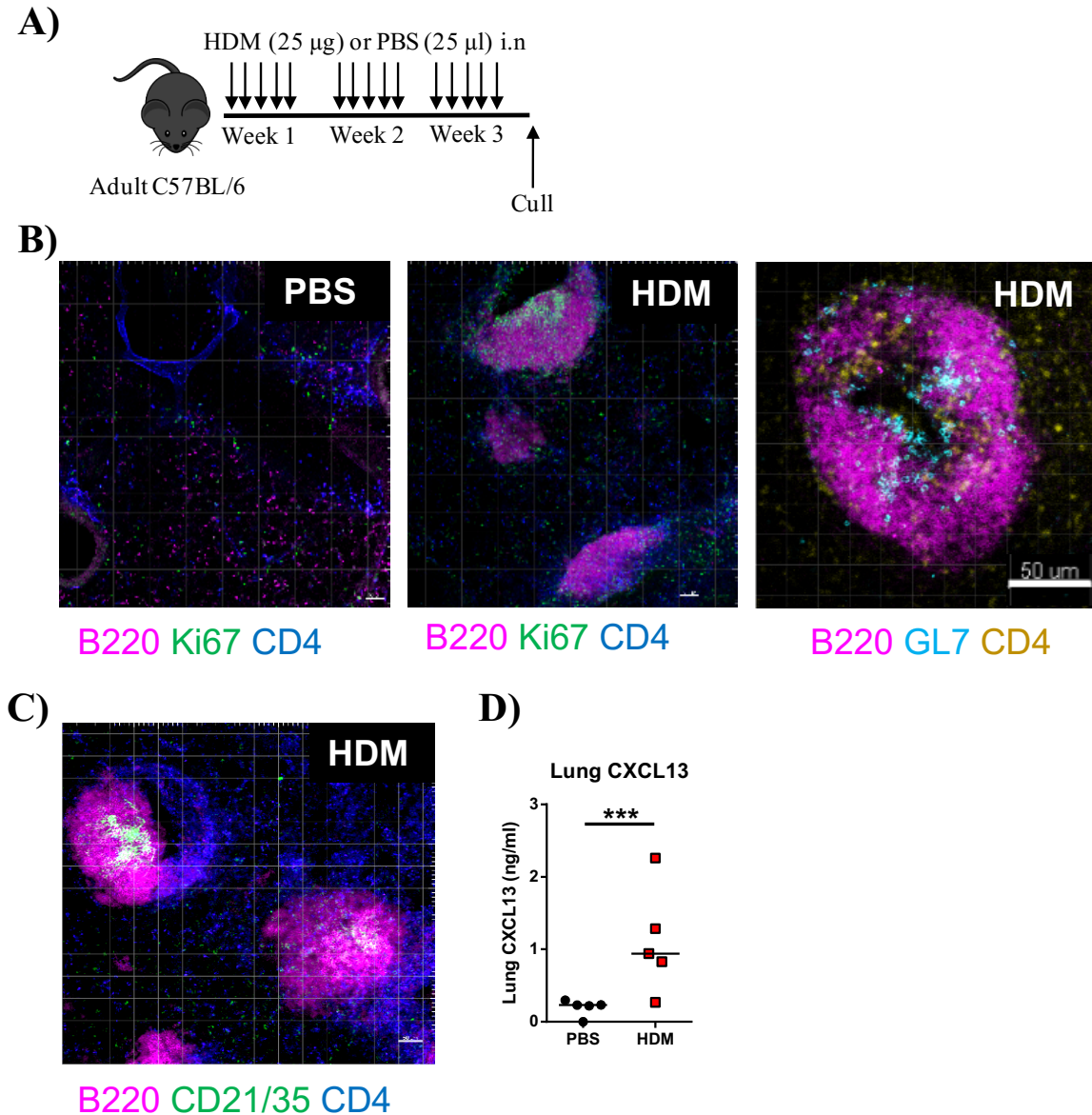


Figure 6.5: Tertiary lymphoid structures form in the lungs of chronic allergen treated animals

Adult female C57BL/6 mice were exposed (i.n) to 25 µg HDM or 25 µl PBS, 5 times a week for 3 weeks. **A)** Schematic of the experimental design, **B-D)** Representative confocal imaging of precision cut lung slices from PBS and HDM treated animals. B220 (B cells), CD4 (T cells), Ki67 (Proliferating cells-GC), GL7 (GC B cells), CD21/35 (Follicular dendritic cells). **B)** Lungs from PBS and HDM treated animals were stained with anti-B220, CD4 and Ki67 antibodies, while lungs from HDM treated animals were also stained with anti-B220, GL7 and CD4 antibodies, **C)** Lungs from HDM treated animals stained with anti-B220, CD21/35 and CD4 antibodies. **D)** CXCL13 was determined by ELISA in mice exposed to PBS or HDM 5 times a week for 3 weeks, n=5 mice per group. Mann Whitney U analysis used to compare between groups.

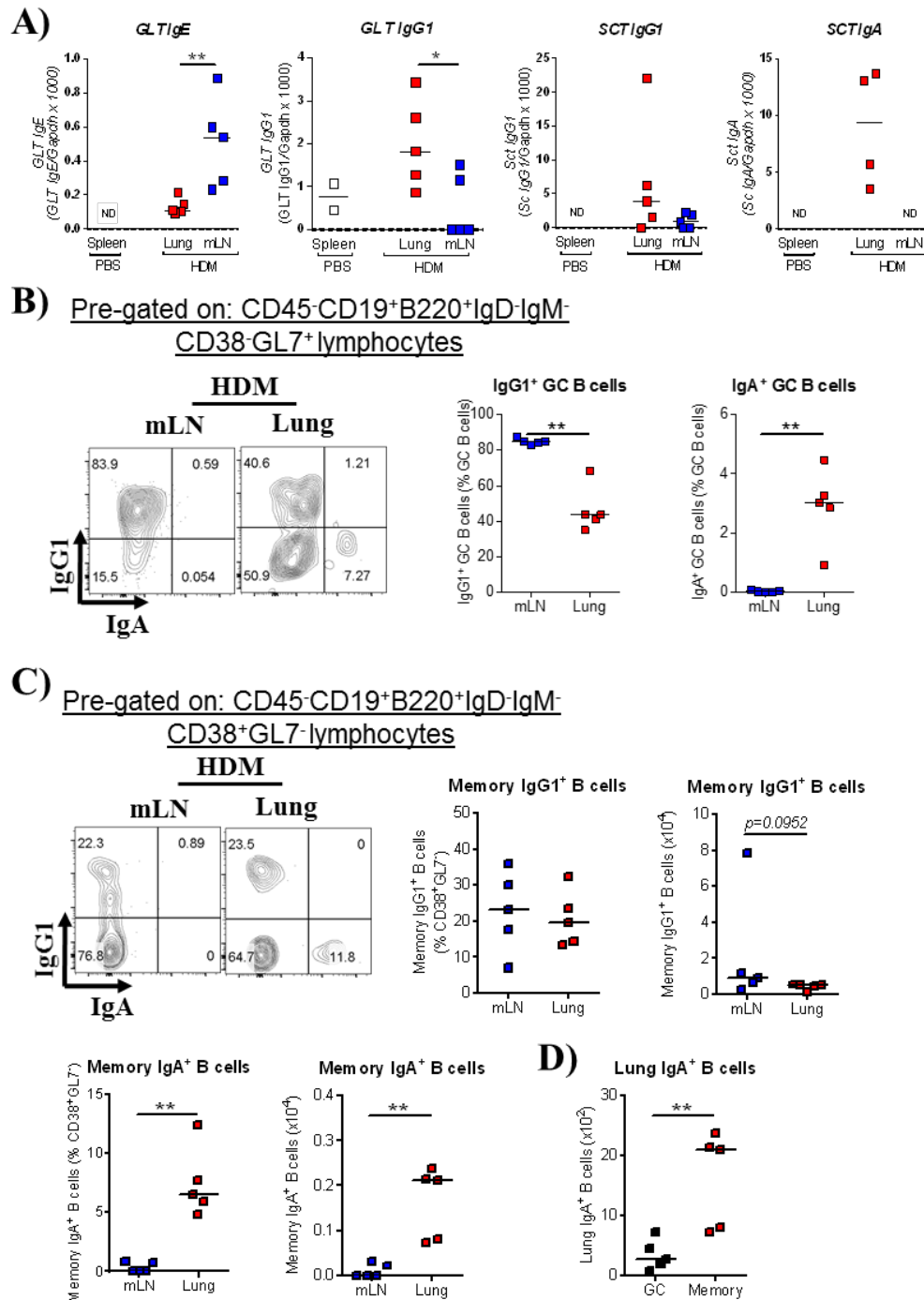


Figure 6.6: Lung GCs are sites of active class switch recombination

A-B) Adult female C57BL/6 mice were exposed (i.n) to PBS and HDM 3 times a week for 3 weeks. Intravascular cells were stained by injecting (i.v) with FITC-labelled anti-CD45 antibody, 3-5 mins before culling animals. Tissue resident cells were defined as CD45⁻ cells. **A)** Gene expression of class switch recombination transcripts was determined by real time qPCR. Switch circle transcripts (SCTs), germline transcripts (GLTs). Data is presented relative to *Gapdh*. **B-C)** Flow cytometry was used to analyse tissue resident GC and memory B cell populations. **B)** Representative images and quantification of IgG1⁺ and IgA⁺ GC B cells in the mLN and lungs of allergen treated animals, **C)** Representative images and quantification of IgG1⁺ and IgA⁺ memory B cells in the mLN and lungs of allergen treated animals. **D)** Lung IgA⁺ GC and memory B cell numbers. Mann Whitney U analysis used to compare between groups. n=5 per group.

As IgA transcript had been identified in sorted lung B cells whether IgA⁺ B cells could be identified in the lungs of allergen treated animals was next analysed. To understand this tissue resident cells were examined using the FITC-CD45 intravascular discrimination technique. Consistent with data represented in chapter 3, IgG1⁺ GC B cells were identified in the mLN and the lungs of allergen exposed animals, being found at a higher frequency in the mLN (**Figure 6.6B**). However, IgA⁺ GC B cells could only be identified in the lungs but not the mLN of allergen exposed animals (**Figure 6.6B**). Furthermore, upon examination of the memory B cells populations it was clear that while a similar proportion and number of memory B cells were IgG1⁺ in the mLN and the lungs (**Figure 6.6C**), a higher proportion and number of lung memory B cells were IgA⁺ and this population was virtually absent in the mLN (**Figure 6.6C**). Interestingly, the largest IgA⁺ cell pool was found in the memory population rather than the GC pool (**Figure 6.6D**), indicating that while IgA switch is occurring in the lungs much of these lung IgA⁺ B cells have a memory phenotype.

6.3.4. FTY720 successfully limits lymphocyte egress

In order to understand the role that lung resident TLS play in the development of allergic disease, a re-challenge study was performed to try and examine disease when cells are incapable of leaving lymphoid organs or entering into the lungs (Teijaro et al., 2011, Matloubian et al., 2004). It is widely known that lymphocytes and mature thymocytes egress from SLOs and the thymus, is strongly dependent on the interaction of S1PR with the lysophospholipid, sphingosine 1 phosphate (S1P) at efferent lymphatic vessels or blood vessels (Matloubian et al., 2004). Therefore, lymphocyte egress can be prevented using a drug called FTY720 which has similar homology to S1P. FTY720 becomes phosphorylated *in vivo* and functions as a high affinity agonist for S1PR, causing receptor internalisation, and thereby prevents lymphocyte circulation, sequestering the cells within SLOs and the thymus (Hideki et al., 2000, Graler and Goetzl, 2004). To examine the impact of lung resident cells on allergic disease, adult female mice were exposed to HDM for 3 weeks to establish AAD. Mice

were then rested for 5 weeks before being re-exposed to 2 doses of HDM (i.n) in the presence of the FTY720 or a vehicle (VEH) control before being culled (**Figure 6.7A**). The different experimental groups and their purpose are summarised in **Figure 6.7B**.

As expected all mice treated with FTY720 had reduced B cell, CD8⁺ and CD4⁺ T populations in the blood compared to their VEH treated control groups, consistent with FTY720 preventing lymphocyte egress and thus reducing circulating lymphocyte populations (**Figure 6.7B-F**). In the FTY720 treated mice the remaining blood CD4⁺ T cells were CD44^{hi}CD62L⁻, implying that they were antigen experienced (**Figure 6.7G**). Furthermore, the number of lymphocytes in the lung was also reduced in the FTY720 treated animals compared to their VEH treated controls, with the exception for lung CD4⁺ T cells in allergen rechallenged animals (**Figure 6.8A-C**). Similarly, to the blood most of the CD4⁺ T cells within the lungs were antigen experienced following FTY720 exposure (**Figure 6.8D**). This trend of reduced lymphocyte populations was not observed for mLN lymphocyte populations where no major differences were observed between groups in terms of CD19⁺ B cells (**Figure 6.8E**), CD8⁺ T cells (**Figure 6.8F**), CD4⁺ T cells (**Figure 6.8G**) apart from both T cells population showing a slight reduction between HDM/VEH/PBS mice compared to HDM/720/PBS mice (**Figure 6.8F, G**). Once again there was a trend of increased antigen experienced cells in FTY720 treated mice in the mLN (**Figure 6.8H**). Overall the data shows the FTY720 to be successful at limiting lymphocyte egress in all drug treated groups and suggested a loss of the naïve circulating CD4⁺ T cell pool.

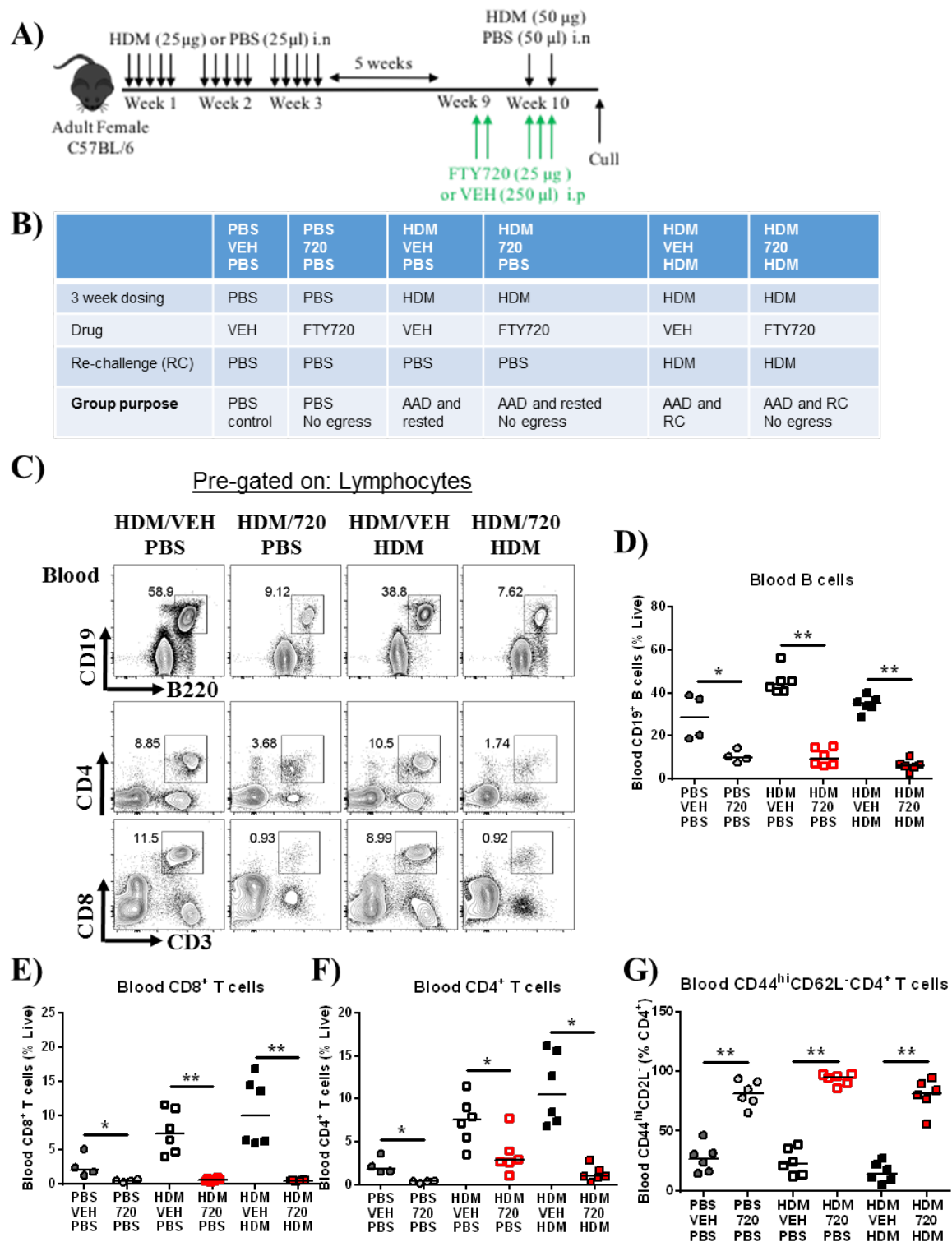


Figure 6.7: FTY720 treatment reduces lymphocyte circulation in the blood

Adult wild type female C57BL/6 mice were exposed (i.n.) to 25 µg HDM or 25 µl PBS, 5 times a week for 3 weeks. After 5 weeks of rest mice were then injected (i.p.) with 5 doses of 25 µg FTY720 or a vehicle (VEH) control and re-challenged with 2 doses (i.n.) of 50 µg HDM or PBS. **A)** Schematic of the dosing schedule, **B)** Table of the 6 experimental groups and their purpose, **C-G)** Flow cytometry was used to examine lymphocyte populations in the blood, **C)** Representative images of HDM treated animals, **D)** CD19⁺ B cells, **E)** CD8⁺ T cells, **F)** CD4⁺ T cells, **G)** CD44^{hi}CD62L⁻CD4⁺ T cells. n=4-6. Mann Whitney U analysis was used to determine statistical significance between groups. The experiment was performed once.

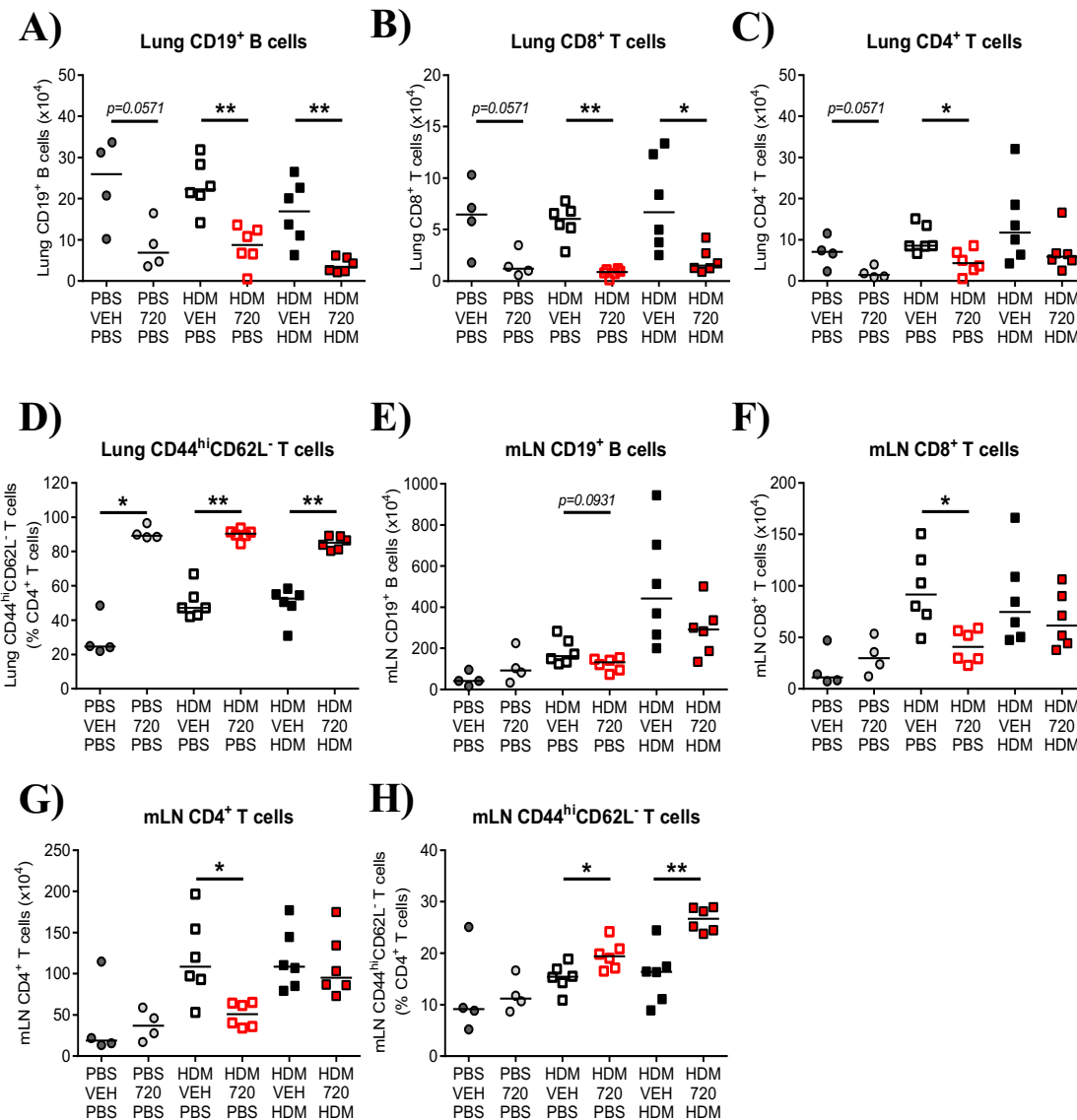


Figure 6.8: FTY720 treatment reduces lymphocyte populations in the lung

Adult wild type female C57BL/6 mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 5 times a week for 3 weeks. After 5 weeks of rest mice were then injected (i.p) with 5 doses of 25 μ g FTY720 or a vehicle (VEH) control and re-challenged with 2 doses (i.n) of 50 μ g HDM or PBS. **A-D)** Numbers of lung lymphocyte populations, **A)** CD19⁺ B cells, **B)** CD8⁺ T cells, **C)** CD4⁺ T cells, **D)** CD44^{hi}CD62L⁻CD4⁺ T cells, **E-H)** Numbers of mLN lymphocyte populations, **E)** CD19⁺ B cells, **F)** CD8⁺ T cells, **G)** CD4⁺ T cells, **H)** CD44^{hi}CD62L⁻CD4⁺ T cells. $n=4-6$. Mann Whitney U analysis was used to determine statistical significance between groups. The experiment was performed once.

6.3.5. Allergen disease following re-challenge was similar regardless of the ability for lymphocyte egress or not

Given that lymphocyte egress was limited in FTY720 treated animals compared to mice given the VEH control, the impact on allergen re-challenge was next examined (**Figure 6.9**). Mice initially exposed to HDM and re-challenged to PBS had similar numbers of lung, BAL and mLN cells compared to PBS only treated animals (**Figure 6.9A-C**). However, following HDM re-challenge the number of lung, BAL and mLN cells was increased compared to the other groups and these numbers were comparable between the VEH and FTY720 exposed mice (**Figure 6.9A-C**). ILN and splenic cell numbers were similar in all analysed groups (**Figure 6.9D, E**). Consistent with the increase in cell number at pulmonary sites following allergen re-exposure, BAL and lung neutrophil numbers were also increased and no difference was observed between the VEH or FTY720 treated mice (**Figure 6.9F, G**). Interestingly, BAL eosinophil numbers trended upwards when lymphocyte egress was limited during allergen re-challenge suggesting worsened eosinophilia (**Figure 6.9H**), while this increase was not observed in the lungs (**Figure 6.9I**).

Allergen re-challenge was not sufficient to significantly exacerbate airway resistance, elastance or compliance compared to mice initially exposed to HDM and re-challenged with PBS (**Figure 6.9J**). However, both resistance and elastance were slightly increased at 300mg/ml in the 2 HDM re-challenged groups. Furthermore, compliance was lower in the FTY720 and allergen treated groups compared to their respective control animals suggesting this to be an effect of the drug rather than the re-challenge status (**Figure 6.9J**).

Taken together, this data shows the re-challenge mice to undergo disease exacerbation, complete with increased granulocyte infiltration but not AHR. Overall disease exacerbation occurred at similar level even when lymphocyte egress from SLOs was limited. FTY720 appeared to directly affect airway compliance in initially allergen exposed groups irrespective of rechallenge status.

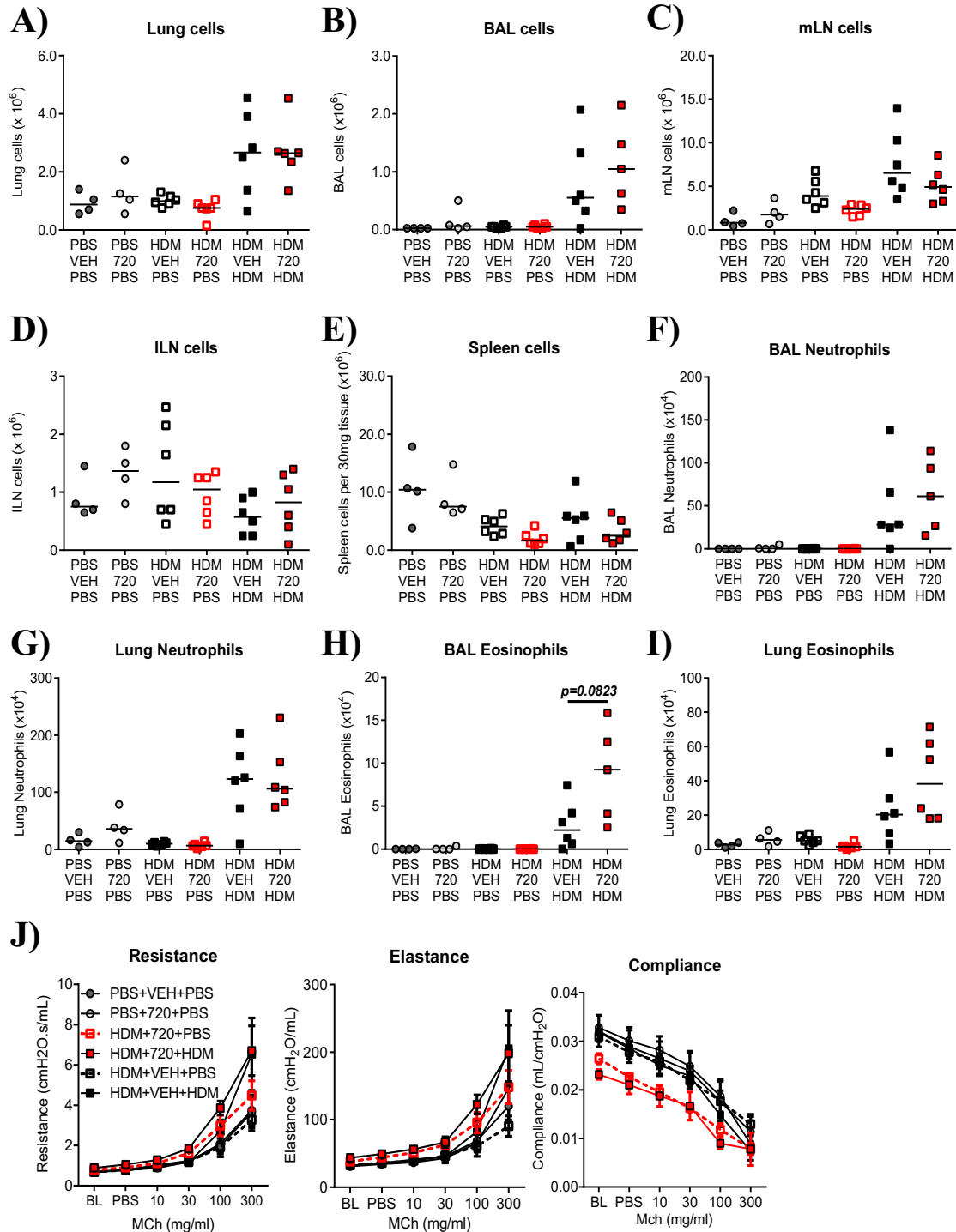


Figure 6.9: Lack of lymphocyte egress does not limit allergic disease rechallenge

Adult wild type female C57BL/6 mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 5 times a week for 3 weeks. After 5 weeks of rest mice were then injected (i.p) with 5 doses of 25 μ g FTY720 or a vehicle control and re-challenged with 2 doses (i.n) of 50 μ g HDM or PBS. **A-D)** Cell numbers were quantified in the, **A)** Lung, **B)** BAL, **C)** mLN, **D)** ILN, **E)** Spleen. **F-I)** Flow cytometry was used to determine the number of neutrophils and eosinophils, **F)** BAL neutrophils, **G)** Lung neutrophils, **H)** BAL eosinophils, **I)** Lung eosinophils, **J)** Airway hyperresponsiveness was measured by exposing mice to 10-300 mg/ml methacholine (MCh) using the flexiVent system. Airway resistance, elastance and compliance are displayed. n=4-6. Mann Whitney U analysis was used to determine statistical significance between groups. The experiment was performed once.

6.3.6. Limiting lymphocyte egress reduces B cell aggregation but does not dramatically impact humoral immunity

As a reduction in circulating lymphocyte populations was observed when lymphocyte egress was blocked, the impact of this on lung B cell aggregation was examined by imaging PCLS using wide-field microscopy (**Figure 6.10**). Mice initially exposed to HDM and re-challenged with PBS lacked B220⁺ B cell aggregates that could be visualised by wide-field microscopy and aggregates were absent regardless of whether mice were exposed to VEH or FTY720 (**Figure 6.10A-C**). When animals were re-exposed to HDM in the presence of VEH or FTY720, B cell aggregates could be easily visualised by wide-field imaging (**Figure 6.10A**). The number of aggregates appeared to be trending down in mice re-challenged with HDM when egress was prevented compared to those where egress was possible (**Figure 6.10B, C**). In mice re-exposed to HDM with FTY720 the size of individual aggregates was smaller than that of VEH treated controls (**Figure 6.10D**) and the overall B cell aggregate area over 1000 μm^2 of lung tissue was also decreased (**Figure 6.10E**). This reduced aggregation was not due to reduced levels of the B cell chemokine, CXCL13 which was similar in both VEH and FTY720 treated, and HDM re-challenged mice (**Figure 6.10F**). Therefore, in the absence of lymphocyte egress, aggregates still form in the lungs, but the size of individual aggregates and the total aggregate area is reduced.

Given the reduction in B cell aggregation in allergen re-challenged mice when lymphocytes egress was limited, the effect on GC B cell populations was investigated. As expected GC B cells were not identified in mLN or the lungs of VEH or FTY720 treated PBS exposed animals (**Figure 6.11A, B**). Following the rest period GC B cells could be easily identified in the mLN of both VEH and FTY720 mice previously exposed to allergen and re-challenged with PBS (**Figure 6.11A**). But this was at a much lower frequency than is usually observed during peak disease. GC B cells in the mLN were not altered by allergen re-challenge and no difference was observed in the number of GC B cells between the VEH or FTY720 treated animals (**Figure 6.11A**). However, fewer GC B cells were observed in the mLN of FTY720 mice

previously exposed to HDM and rechallenged with PBS compared to their VEH control group (**Figure 6.11A**). Very few GC B cells were observed in the lungs of previously allergen exposed and rested animals and this was unchanged by allergen re-challenge (**Figure 6.11B**). Hence, GC B cell populations were not altered during allergen rechallenge when lymphocyte egress was possible or impossible.

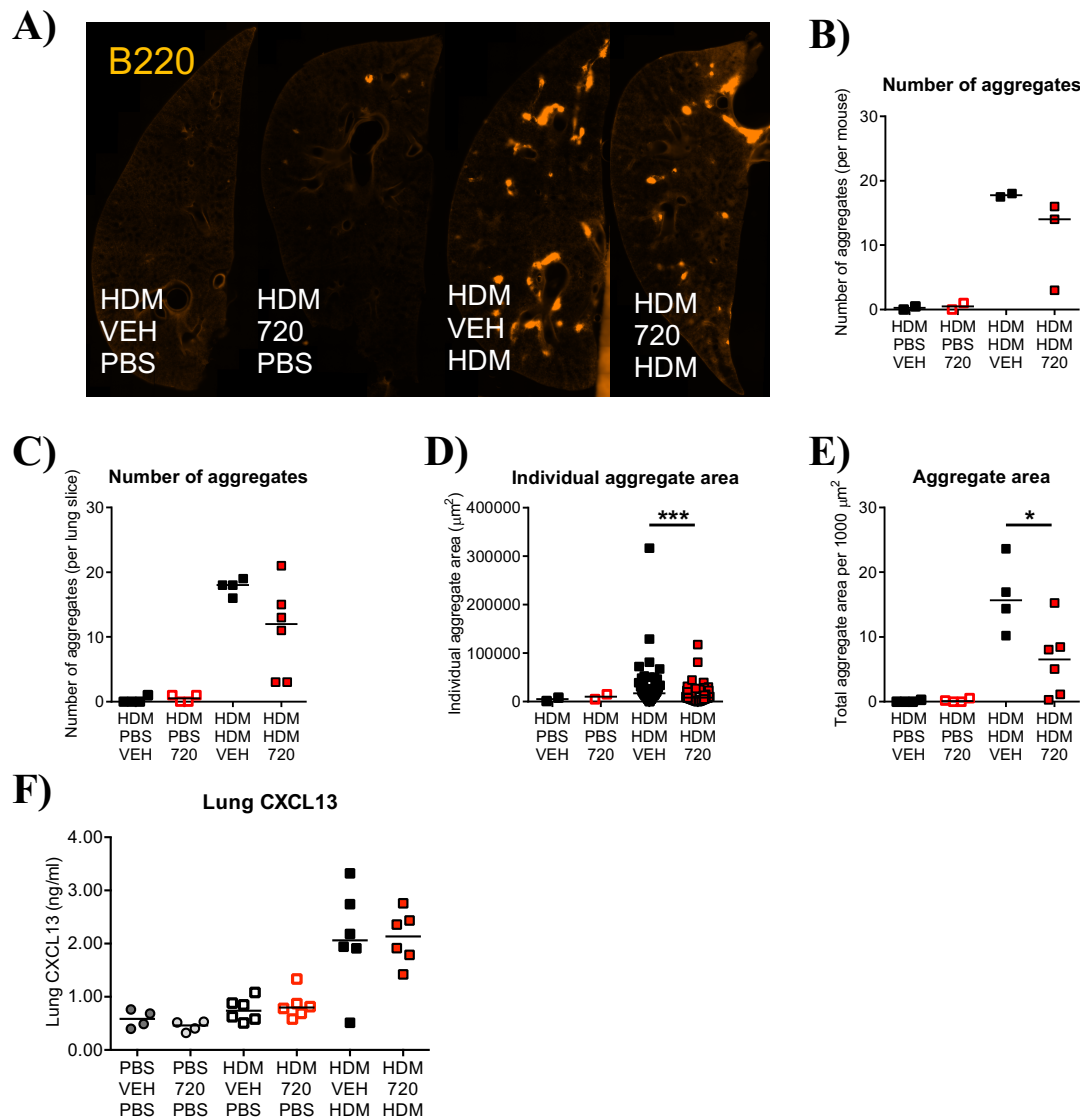
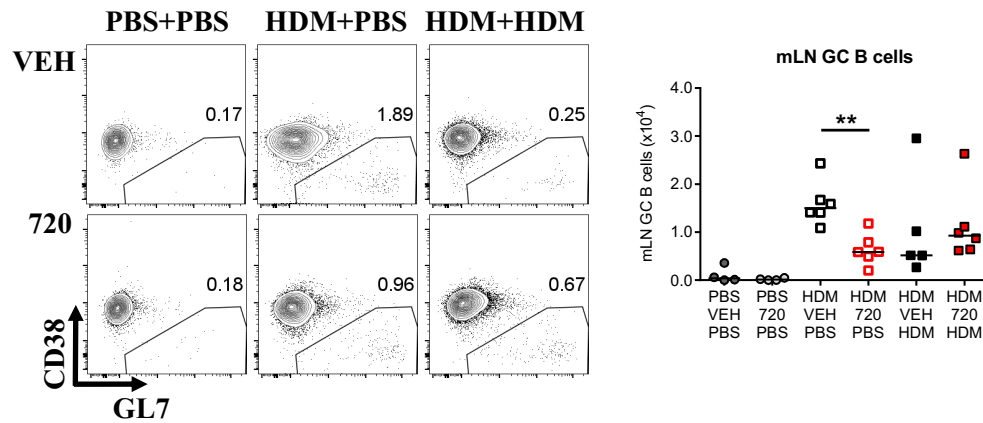


Figure 6.10: Re-challenged animals have increased pulmonary inflammation

Adult wild type female C57BL/6 mice were exposed (i.n) to 25 μg HDM or 25 μl PBS, 5 times a week for 3 weeks. After 5 weeks of rest mice were then injected (i.p) with 5 doses of 25 μg FTY720 or a vehicle control and re-challenged with 2 doses (i.n) of 50 μg HDM or PBS. **A-E**) Lung B cell clusters were imaged in PCLS from allergen treated animals using wide-field microscopy. 2 slices were imaged per mouse, **A**) Representative images of B220⁺ B cell clusters, **B**) Number of B cell aggregates per mouse –average over 2 slices, **C**) Number of aggregates per slice, **D**) Individual aggregate size, **E**) Aggregate area per 1000 μm² lung tissue. **F**) Lung CXCL13 was measured by ELISA. Mann Whitney U analysis was used to determine statistical significance between groups. The experiment was performed once.

A) Pre-gated on: CD19⁺B220⁺ lymphocytes



B) Pre-gated on: CD19⁺B220⁺ lymphocytes

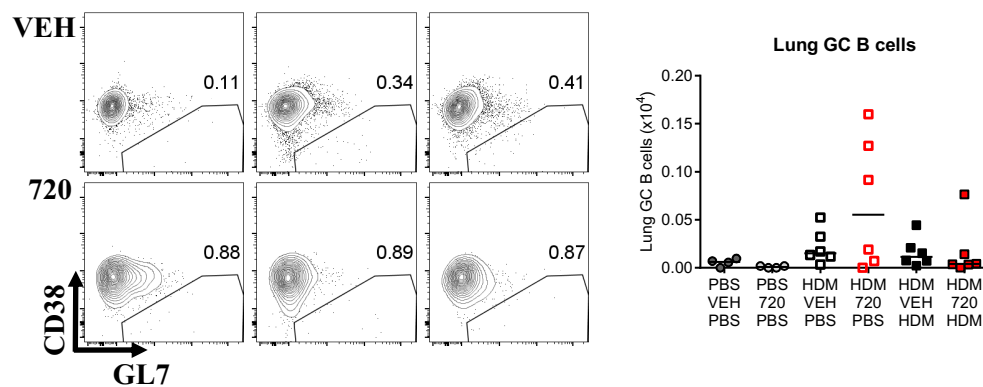


Figure 6.11: FTY720 does not alter GC B cell populations in the mLN or lungs upon allergen re-challenge

Adult wild type female C57BL/6 mice were exposed (i.n) to 25 µg HDM or 25 µl PBS, 5 times a week for 3 weeks. After 5 weeks of rest mice were then injected (i.p) with 5 doses of 25 µg FTY720 or a vehicle control and re-challenged with 2 doses (i.n) of 50 µg HDM or PBS. **A-B**) Flow cytometry was used to analyse GC B cells, **A**) mLN, **B**) Lung. n=4-6. Mann Whitney U analysis was used to determine statistical significance between groups. The experiment was performed once.

T_{FH} could still be identified in the mLN of mice initially exposed to HDM and rested for 5 weeks (**Figure 6.12A**). Upon HDM re-challenge the number and proportion of T_{FH} was increased compared to HDM and rested controls (**Figure 6.12A**). However, when lymphocyte egress was prevented by FTY720, both the proportion and number of mLN T_{FH} was increased compared to VEH HDM challenged mice (**Figure 6.12A**). No dramatic difference was

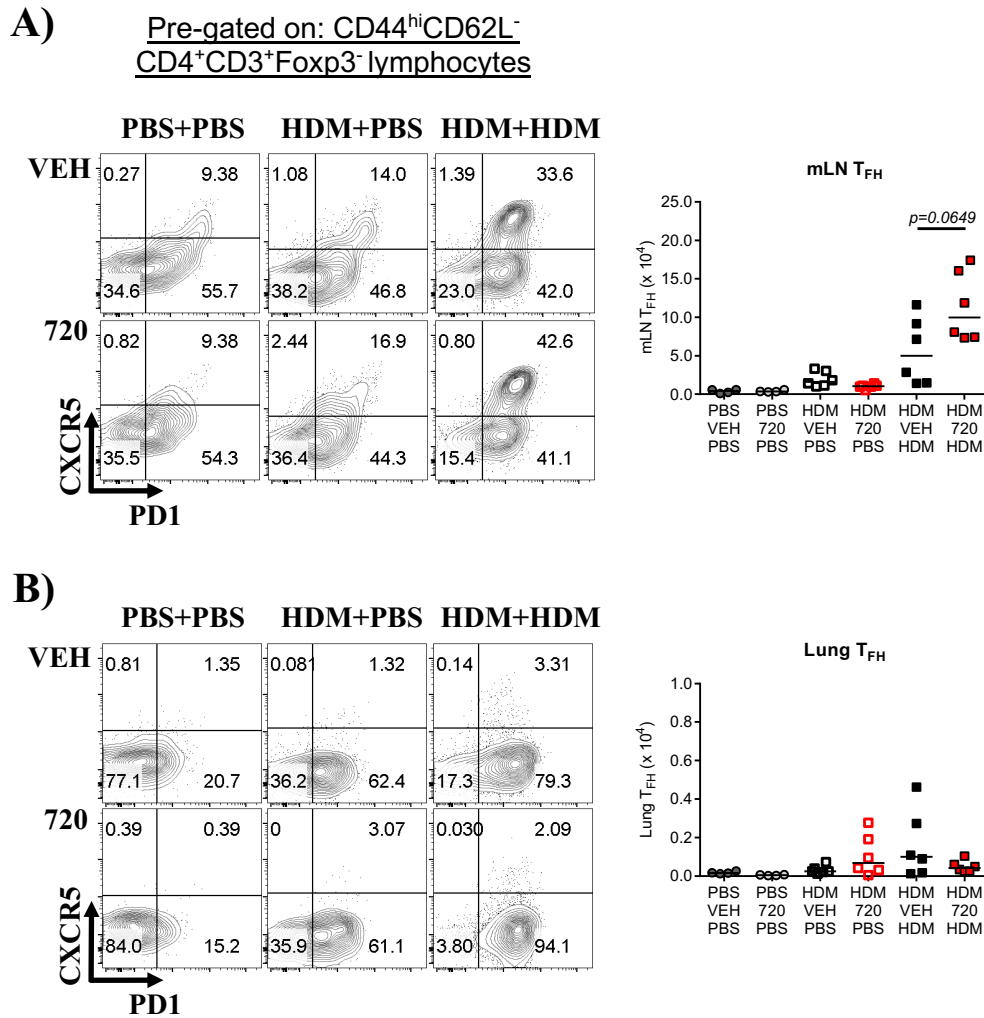


Figure 6.12: mLN T_{FH} increase upon allergen re-challenge when egress is prevented

Adult wild type female C57BL/6 mice were exposed (i.n) to 25 µg HDM or 25 µl PBS, 5 times a week for 3 weeks. After 5 weeks of rest mice were then injected (i.p) with 5 doses of 25 µg FTY720 or a vehicle control and re-challenged with 2 doses (i.n) of 50 µg HDM or PBS. **A-B)** Flow cytometry was used to analyse T_{FH} populations. Representative flow plots are displayed. **A)** mLN, **B)** Lung. n=4-6. Mann Whitney U analysis was used to determine statistical significance between groups. The experiment was performed once.

observed in lung T_{FH} populations in all analysed groups both after rest or re-challenge regardless of egress status (**Figure 6.12B**).

Lastly, antibody levels were measured to determine the effect of blocking lymphocyte egress during allergen re-exposure (**Figure 6.13**). HDM specific IgE was barely detected in the serum of any groups and was not increased upon allergen re-challenge and no difference was observed when egress was prevented (**Figure 6.13A**). HDM specific serum and lung IgG1

and lung and BAL IgA were elevated in all allergen treated animals compared to PBS treated controls and were found at a similar level in all allergen treated animals irrespective of PBS or HDM re-challenge or the ability to undergo lymphocyte egress or not (**Figure 6.13B-E**). Overall, this allergen re-challenge schedule did not exacerbate IgE as it did in the chapter 3 (**Figure 3.32E, F**).

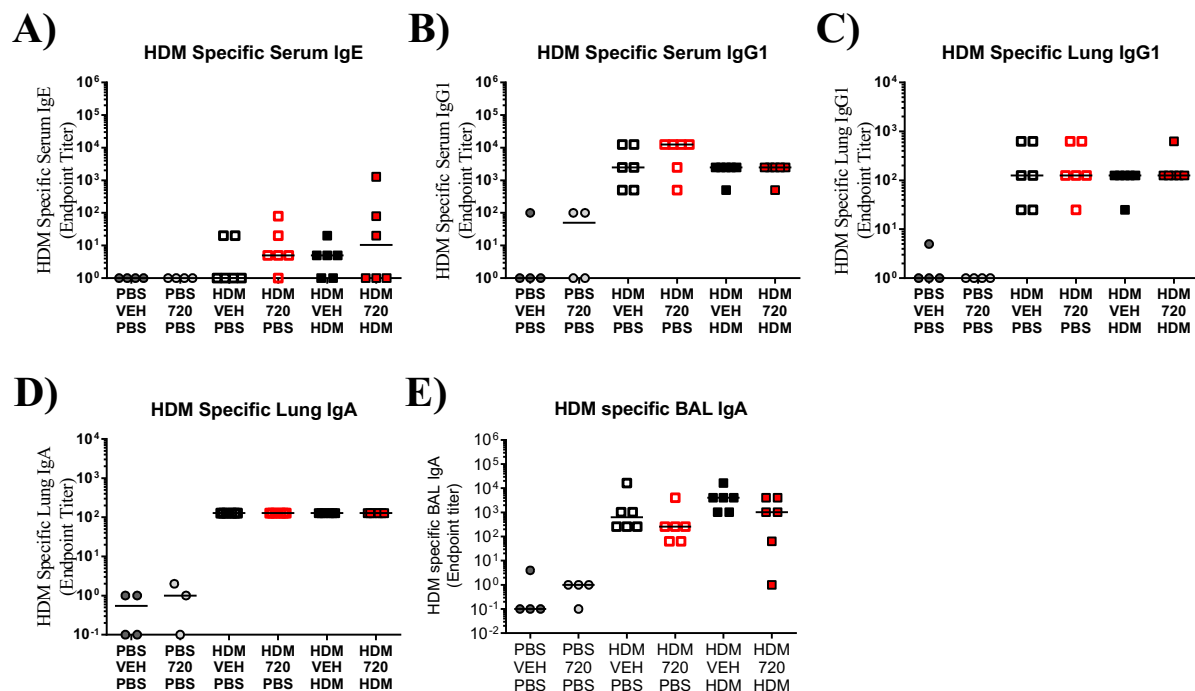


Figure 6.13: Allergen specific antibody is unchanged by re-challenge or lack of egress
Adult wild type female C57BL/6 mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 5 times a week for 3 weeks. After 5 weeks of rest mice were then injected (i.p) with 5 doses of 25 μ g FTY720 or a vehicle control and re-challenged with 2 doses (i.n) of 50 μ g HDM or PBS. Mice were culled 18 hours after the final allergen dose. HDM specific antibody was measured by ELISA and endpoint titer calculated, **A)** Serum IgE, **B)** Serum IgG1, **C)** Lung IgG1, **D)** Lung IgA, **E)** BAL IgA. n=4-6. Mann Whitney U analysis was used to determine statistical significance between groups. The experiment was performed once.

6.3.7. IL-17A does not drive lung GC formation

IL-17A has been implicated in the formation of lung TLS by driving the secretion of the chemokine CXCL13 from stromal cells (Rangel-Moreno et al., 2011b). Given that CD4⁺ T cells could be identified within the TLS in the lungs (**Figure 6.5**) and in chapter 5 lung T_{FH} were shown to produce IL-17A (**5.16A, B**), the importance of IL-17A to lung GC formation and

allergic disease was examined. To investigate this IL-17A KO (*il17^{cre/cre}rosa26-rfp^{fl/fl}*), IL-17A HET (*il17^{cre/+}rosa26-rfp^{fl/fl}*) and WT littermate control mice (*rosa26-rfp^{fl/fl}*) were exposed to HDM for 5 weeks (Figure 6.14A).

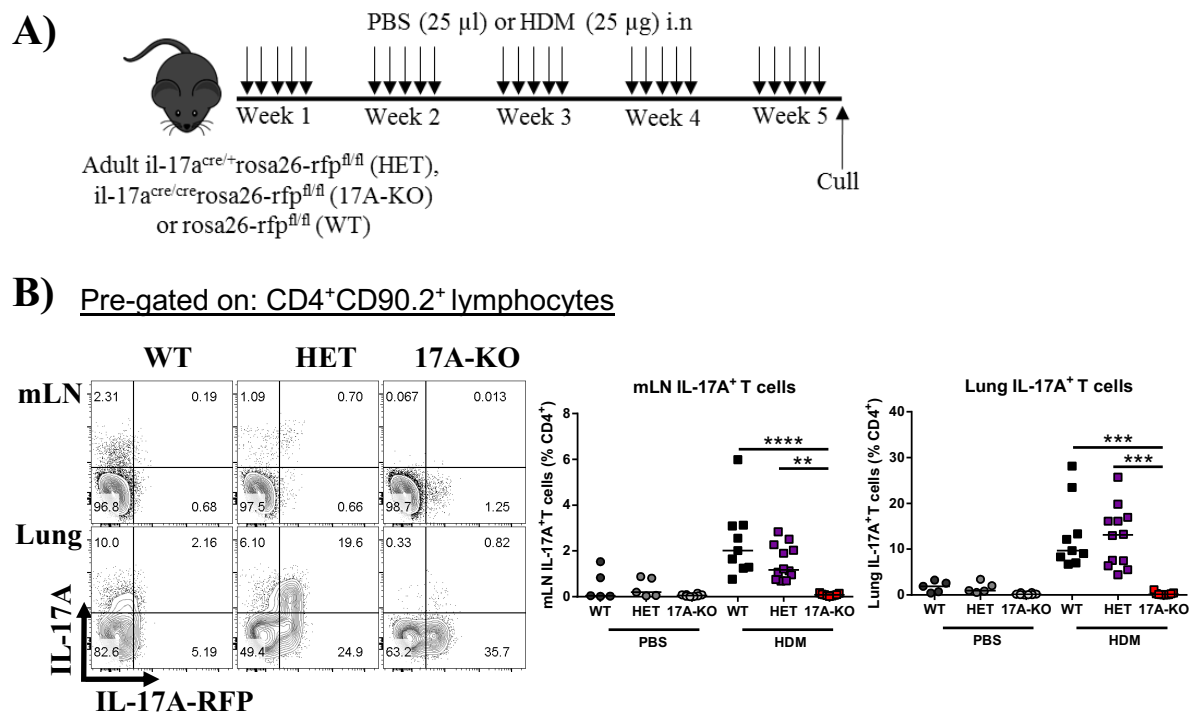


Figure 6.14: IL-17A knockout mice lack IL-17A⁺ cells

Adult *rosa26-rfp^{fl/fl}* (WT), *il17a^{cre/+}rosa26-rfp^{fl/fl}* (HET) and *il17a^{cre/cre}rosa26-rfp^{fl/fl}* (17A-KO) mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS 5 times a week for 5 weeks. **A)** Schematic of experimental design. Flow cytometry was used to analyse cellular populations. **B)** Representative flow plots of IL-17A⁺ CD4⁺ T cells in the mLN and lungs, IL-17A⁺ CD4⁺ T cells after PMA/ionomycin stimulation were quantified in the mLN and lungs. Data is pooled from 2 independent experiments. Statistical significance was determined using Kruskal Walis analysis with Dunn's multiple comparison test. n=5-12.

As expected in the mLN and the lungs of HDM exposed animals after PMA/ionomycin stimulation IL-17A⁺ CD4⁺ T cells were identified in both WT and HET mice but not in IL-17A KO animals (**Figure 6.14B**). The Rosa26 induced RFP reporter signal could be clearly identified in the mLN and lungs CD4⁺ T cells from HET and IL-17A KO mice (**Figure 6.14B**). T_{FH} are also producers of IL-17A during chronic allergic disease. T_{FH} were identified in the mLN and the lungs of HDM treated animals and no difference was observed in the proportion of T_{FH} indicating they were not reliant on IL-17A for their generation (**Figure 6.15A-C**). As expected HDM treated IL-17A KO mice lacked IL-17A production from lung T_{FH} populations after PMA/ionomycin stimulation (**Figure 6.15D, E**). Taken together this data shows that *il17a^{cre/cre}rosa26-rfp^{fl/fl}* lacked IL-17A⁺ CD4⁺ T cells.

Consistent with the absence of IL-17A⁺ cells in the lung and mLN after chronic AAD, IL-17A protein was completely absent in the IL-17A KO animals. However, IL-17A was strongly detected in the lungs of WT animals and to a lesser extent in those from HET animals (**Figure 6.16A**). Similar results were observed for *Il17a* gene expression (**Figure 6.16B**). Next, whether *Rorc* was upregulated in IL-17A deficient animals was examined (**Figure 6.16C**). *Rorc* appeared to increase the lungs of allergen treated animals compared to PBS controls, in particular the HET mice. Although, no significant differences were observed between the three allergen treated groups in terms of *Rorc* expression, the 17A-KO mice appeared to have the lowest *Rorc* expression (**Figure 6.16C**). Neither *Il17f* nor *Il22*, both major cytokine products of Th17 cells, were detectable in most animals and no difference was observed between PBS and HDM treated animals (**Figure 6.16D, E**). Taken together, this data shows *il17a^{cre/cre}rosa26-rfp^{fl/fl}* animals fail to produce IL-17A and other IL-17 associated cytokines after aero-allergen challenge.

To determine the impact of IL-17A deficiency on GC B cell generation, the frequency and total number of GC B cells in the mLN and lung tissue was examined. GC B cells were identifiable within the mLN and lungs of all 3 allergen treated groups (**Figure 6.17A-C**) but not in PBS exposed counterparts (**Figure 6.17B, C**). In the mLN, GC B cell proportions were increased

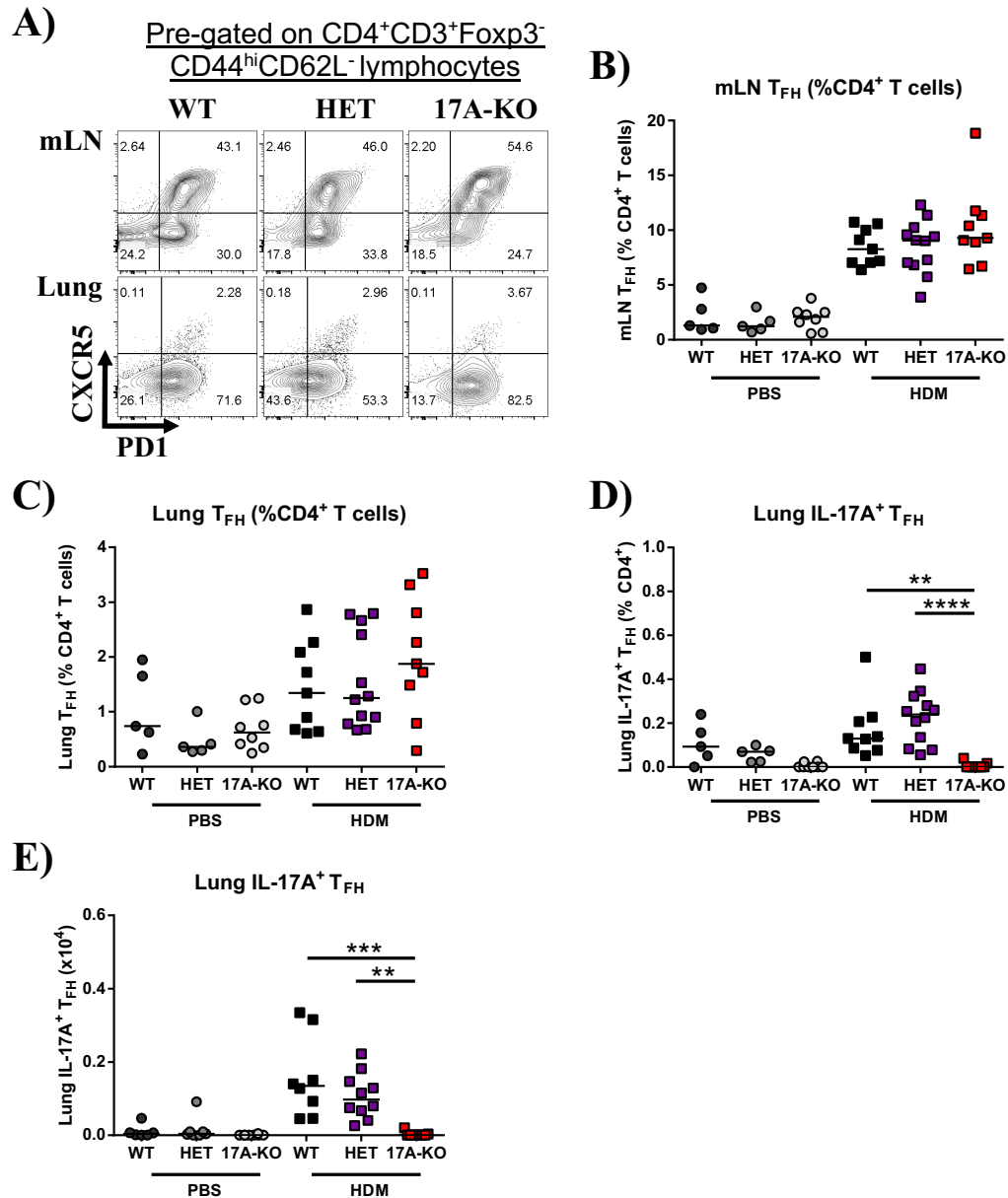


Figure 6.15: IL-17A deficiency prevents lung IL-17A production but does not impact T follicular helper cell generation

Adult *rosa26-rfp^{fl/fl}* (WT), *il17a^{cre/+}-rosa26-rfp^{fl/fl}* (HET) and *il17a^{cre/cre}-rosa26-rfp^{fl/fl}* (17A-KO) mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 5 times a week for 5 weeks. **A)** Representative flow plots of T_{FH} in the mLN and lungs are displayed and the frequency was quantified in the **B)** mLN, **C)** Lung **D-E)** Lung IL-17A⁺ T_{FH} proportion and number were assessed after PMA/ionomycin stimulation, **D)** Lung IL-17A⁺ T_{FH} proportions, **E)** Lung IL-17A⁺ T_{FH} numbers. Data is pooled from 2 independent experiments. Statistical significance was determined using Kruskal-Wallis analysis with Dunn's multiple comparison test. n=5-12.

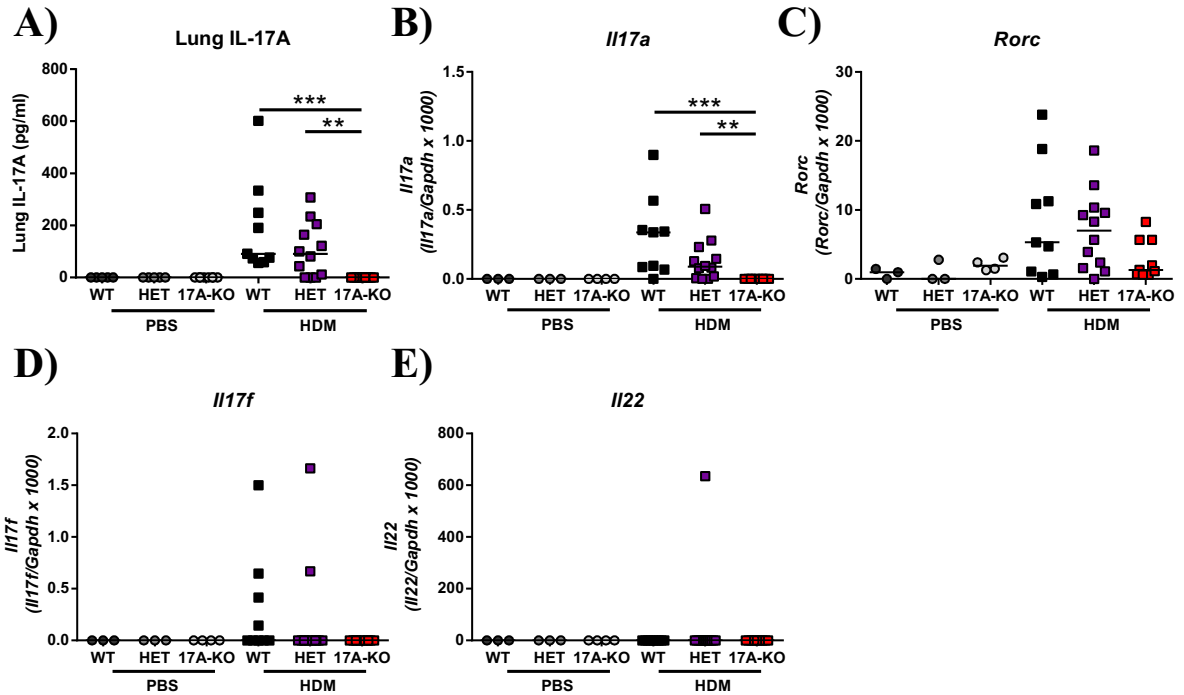


Figure 6.16: IL-17A knockout mice lack IL-17A⁺ cells and Th17 associated cytokines Adult *rosa26-rfp^{fl/fl}* (WT), *il17a^{cre/+}-rosa26-rfp^{fl/fl}* (HET) and *il17a^{cre/cre}-rosa26-rfp^{fl/fl}* (17A-KO) mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS 5 times a week for 5 weeks. **A)** Lung IL-17A was measured by ELISA, **B-E)** Transcription factors and cytokine gene expression was determined by RT-qPCR. Data is presented relative to *Gapdh*, **B)** *Il17a*, **C)** *Rorc*, **D)** *Il17f*, **E)** *Il22*. Data is pooled from 2 independent experiments. Statistical significance was determined using Kruskal Wallis analysis with Dunn's multiple comparison test. n=3-12.

in IL-17A KO animals compared to their WT and HET counterparts (**Figure 6.17A, B**) but this increase was not observed in number (**Figure 6.17C**). However, in the lungs the overall proportion and number of GC B cells were similar between all 3 HDM treated groups (**Figure 6.17D, E**). However, the HET mice did appear to show a higher proportion of lung GC B cells when compared to IL-17A KO animals (**Figure 6.17A, D**), and GC B cell populations appeared to be trending downwards in the IL-17A KO (**Figure 6.17A, D**). Therefore, although there were changes in mLN GC B cell populations in the absence of IL-17A, in the lungs despite no difference in the GC population there was trend towards decreased GC B cell generation.

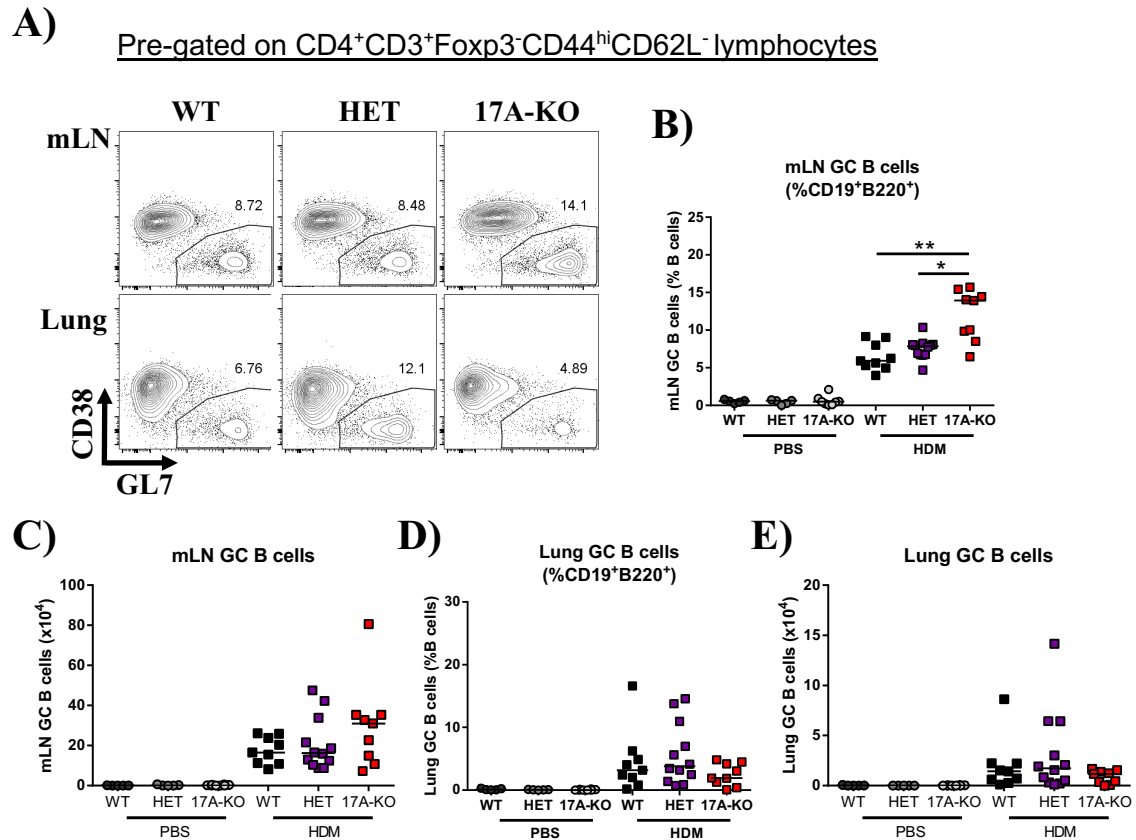


Figure 6.17: IL-17A does not impact lung germinal centre formation

Adult *rosa26-rfp^{fl/fl}* (WT), *il17a^{cre/+}-rosa26-rfp^{fl/fl}* (HET) and *il17a^{cre/cre}-rosa26-rfp^{fl/fl}* (17A-KO) mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 5 times a week for 5 weeks. **A)** Representative flow plots of GC B cells in the mLN and lung tissue. Frequency and total number of GC B cells are quantified. **B)** mLN GC B cell frequency, **C)** mLN GC B cell numbers, **D)** Lung GC B cell frequency, **E)** Lung GC B cell numbers. Data is pooled from 2 independent experiments. Statistical significance was determined using Kruskal Wallis analysis with Dunn's multiple comparison test. n=5-12 per group.

Next, the impact of loss of IL-17A on B cell aggregation was analysed. As previously observed B220⁺ B cell aggregates were not found in the PCLS sections from PBS treated animals (**Figure 6.18A**). But were clearly observed throughout the lung tissue of WT, HET and IL-17A KO HDM treated animals (**Figure 6.18A**). Overall no difference was observed in the number of B cell aggregates in the lungs of allergen treated animals (**Figure 6.18B**) but the size of each aggregate was reduced in IL-17A KO animals compared to WT and HET HDM exposed animals (**Figure 6.18C**). Given that IL-17A had been implicated in driving B cell aggregation via stimulating stromal cells to produce the B cell chemoattractant, CXCL13, the level of this chemokine in the lung was then analysed. Lung CXCL13 levels were unchanged between the

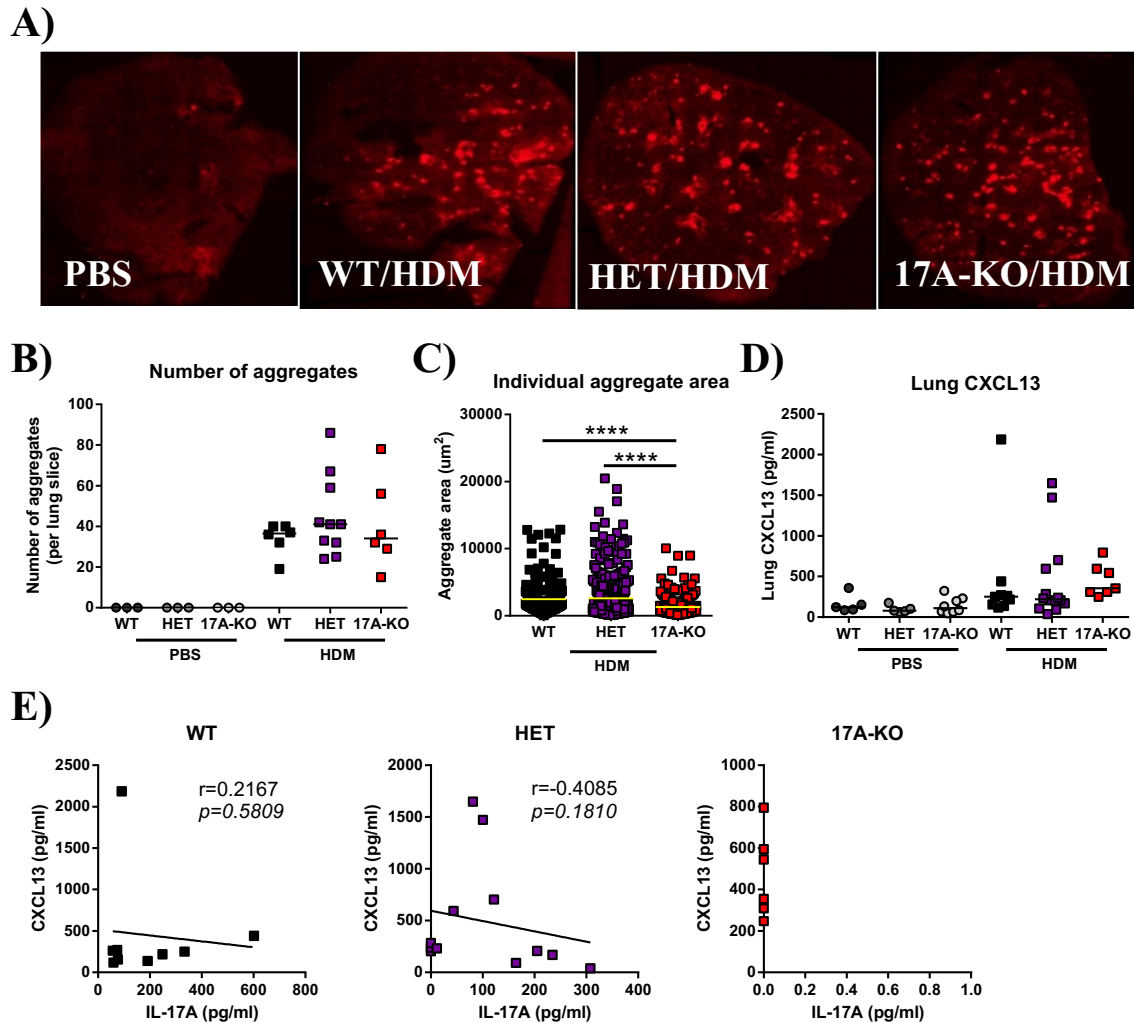


Figure 6.18: IL-17A is important for B cell aggregation

A-C) Adult *rosa26-rfp^{fl/fl}* (WT), *il17a^{cre/+}-rosa26-rfp^{fl/fl}* (HET) and *il17a^{cre/cre}-rosa26-rfp^{fl/fl}* (17A-KO) were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 5 times a week for 5 weeks. **A)** Representative wide-field imaging of B cell aggregates in whole lung slices stained with anti-B220 antibody. **B)** Quantification of the number of B cell aggregates per lung slice, **C)** Individual B cell aggregate area. **D)** CXCL13 was measured in lung homogenate by ELISA. **E)** Correlation between lung IL-17A and CXCL13 in HDM treated WT, HET and 17A-KO animals. Statistical significance was determined using Kruskal Wallis analysis with Dunn's multiple comparison test. Correlation was determined by Spearman's coefficient. Data is from one experiment. n=3-9 per group.

3 HDM treated groups (**Figure 6.18D**) and there were no significant correlations between CXCL13 and IL-17A levels in the WT, HET or IL-17A KO animals (**Figure 6.18E**).

Given the impact of IL-17A on B cell aggregation within the lung, B cell populations were examined further (**Figure 6.19**). Following allergen exposure the total number of B cells in the lungs increased and no difference was observed between the three allergen treated groups

(**Figure 6.19A**). Similar results were seen with naïve B cell numbers within the lungs (**Figure 6.19B**). However, when examining lung memory B cell populations it was clear that HET mice had the highest frequency and number of IgG1⁺ memory B cells, which trended down in the IL-17A KO mice compared to the HET (**Figure 6.19C, D**). Similar trends of non-significant decrease were observed in IgA⁺ memory B cells in IL-17A KO mice compared to HET animals (**Figure 6.19E, F**). Thus, the absence of IL-17A appeared to have subtle impact on memory B cell populations but was not sufficient to ablate these responses. Consistent with the mild effect of IL-17A on B cell populations, no significant differences were observed in allergen specific serum IgG1 or IgE (**Figure 6.20A**), lung IgG1 and IgA (**Figure 6.20B**), NAL IgA (**Figure 6.20C**) or BAL IgA (**Figure 6.20D**) in allergen exposed animals. Therefore, IL-17A deficiency did not impair local or systemic antibody responses.

Finally, the impact of IL-17A deficiency on hallmark features of AAD was examined. As expected mLN cell counts were increased in allergen treated animals compared to PBS controls but no significant differences were observed between the 3 HDM exposed animals (**Figure 6.21A**). However, in the lungs HDM induced inflammation was similar between WT and HET treated animals, but was significantly reduced in IL-17A KO mice compared to HET mice while it trended towards significance compared to WT animals (**Figure 6.21B**). No difference was observed in proportions and numbers of lung eosinophils in allergen treated animals (**Figure 6.21C**). However, consistent with the loss of IL-17A the proportion and number of neutrophils were reduced in IL-17A KO animals compared to WT and HET treated animals (**Figure 6.21D**). Therefore, the loss of IL-17A impaired allergen induced lung neutrophilia.

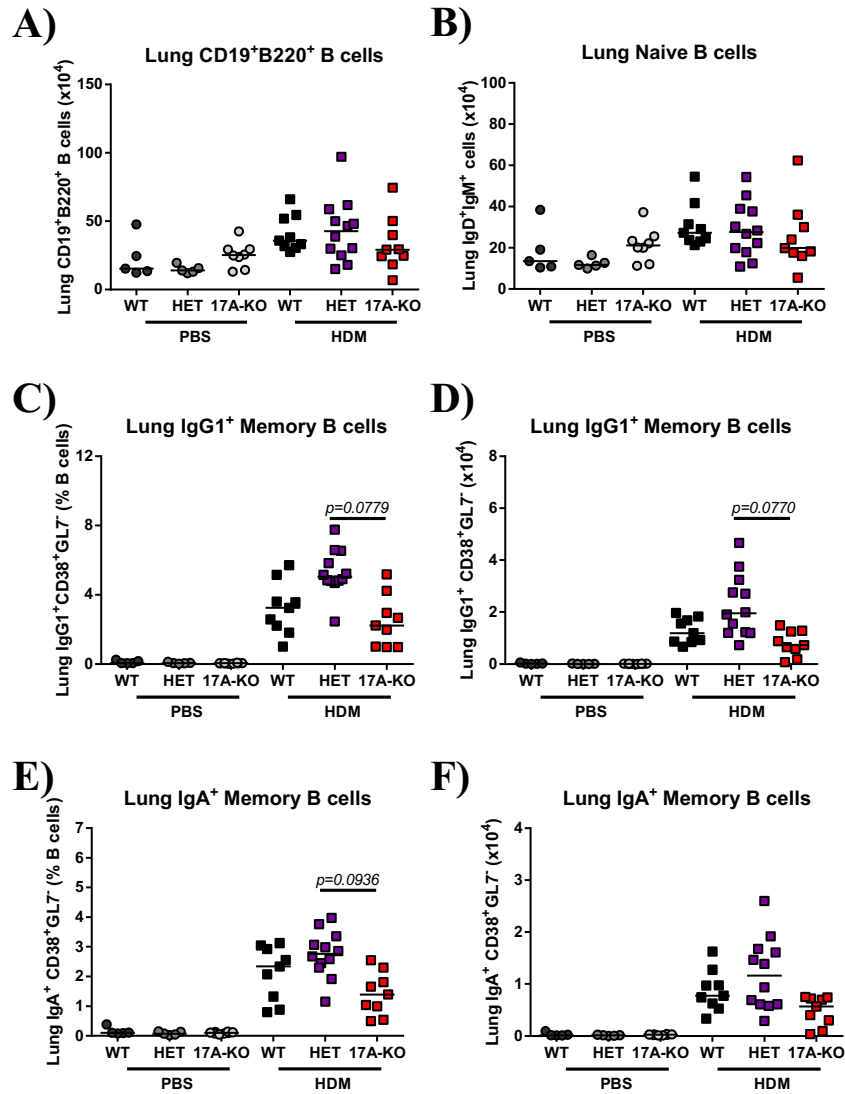


Figure 6.19: IL-17A has subtle impact on lung memory B cell populations

Adult *rosa26-rfp^{fl/fl}* (WT), *il17a^{cre/+}-rosa26-rfp^{fl/fl}* (HET) and *il17a^{cre/cre}-rosa26-rfp^{fl/fl}* (17A-KO) mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS. Flow cytometry was used to analyse B cell populations in the lung, **A)** Total B cell numbers, **B)** Number of naïve B cells, **C)** Frequency of IgG1⁺ memory B cells, **D)** Number of IgG1⁺ memory B cells **E)** Frequency of IgA⁺ memory B cells, **F)** Number of IgA⁺ memory B cells. Statistical significance was determined using Kruskal Wallis analysis with Dunn's multiple comparison test. Data is pooled from 2 independent experiments. n=5-12 per group.

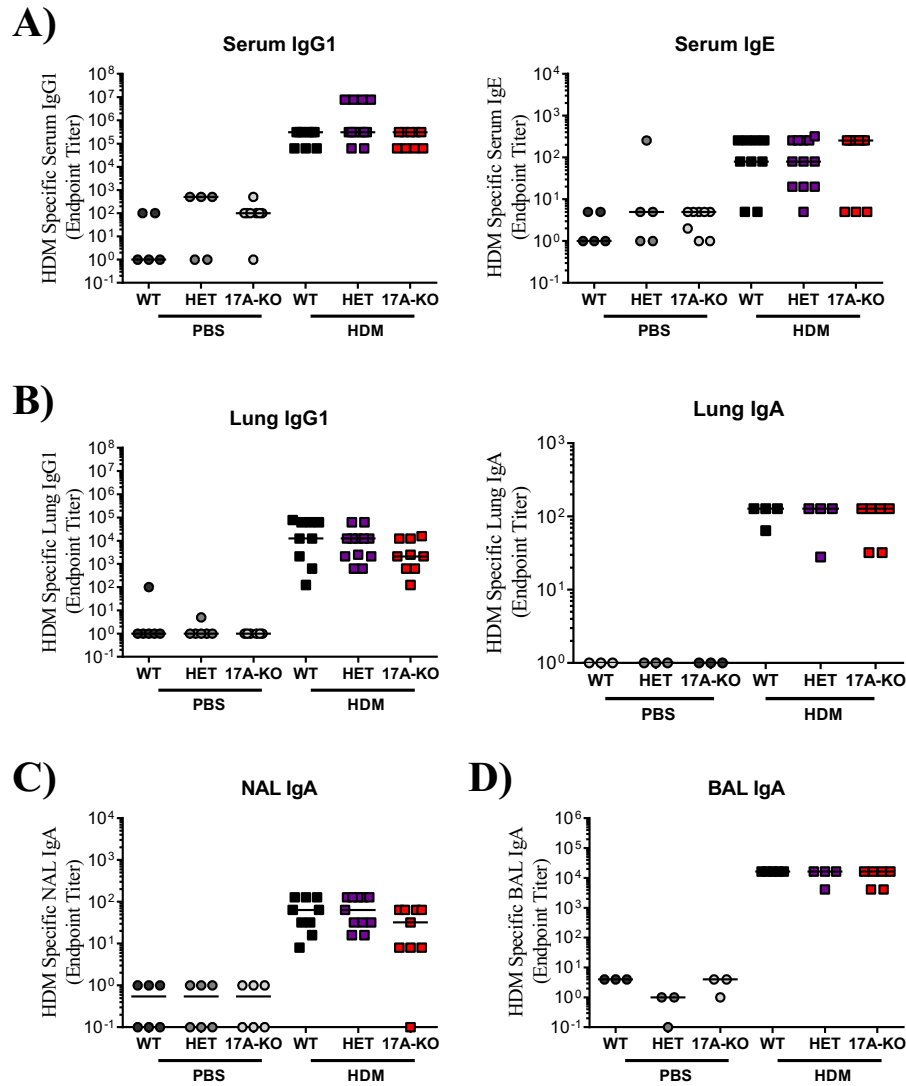


Figure 6.20: IL-17A deficiency does not impair local or systemic antibody responses Adult *rosa26-rfp^{fl/fl}* (WT), *il17a^{cre/+}-rosa26-rfp^{fl/fl}* (HET) and *il17a^{cre/cre}-rosa26-rfp^{fl/fl}* (17A-KO) mice were exposed (i.n) to 25 µg HDM or 25 µl PBS. Antibody responses were determined by ELISA in the lung, BAL, NAL and serum and endpoint titres were calculated. **A)** Serum IgG1 and IgE, **B)** Lung IgG1 and IgA, **C)** NAL IgA, **D)** BAL IgA. Mann Whitney U analysis was used to determine statistical significance. Data is pooled from 2 independent experiments. n=5-12 per group. BAL was only taken in one study.

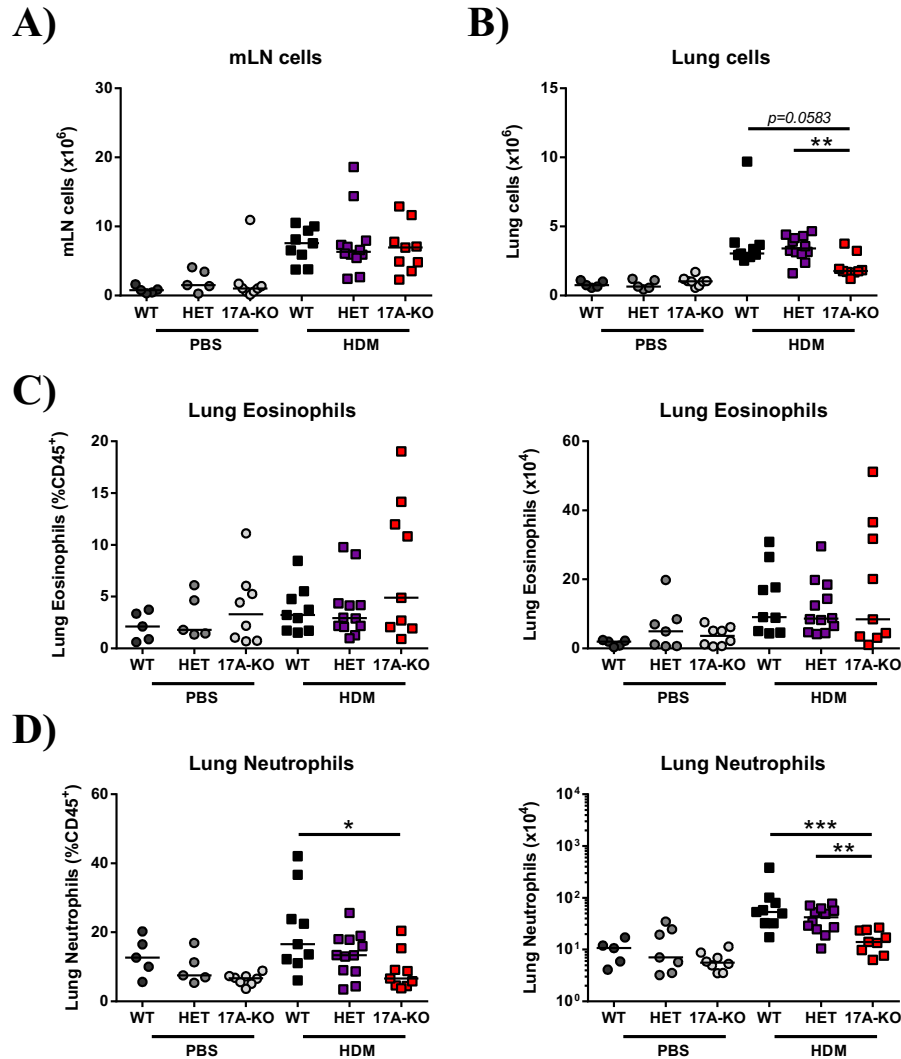


Figure 6.21: IL-17A deficiency reduces lung inflammation and neutrophilia

Adult *rosa26-rfp^{fl/fl}* (WT), *il17a^{cre/+}-rosa26-rfp^{fl/fl}* (HET) and *il17a^{cre/cre}-rosa26-rfp^{fl/fl}* (17A-KO) mice were exposed (i.n) to 25 μ g HDM or 25 μ l PBS, 5 times a week for 5 weeks. **A-B)** Total cells were counted, **A)** mLN, **B)** Lung tissue. **C-D)** Flow cytometry was used to determine the frequency and total number of different cell populations, **C)** Eosinophils, **D)** Neutrophils. Data is pooled from 2 independent experiments. Statistical significance was determined using Kruskal Wallis analysis with Dunn's multiple comparison test. n=5-12 per group.

6.3.8. T_{FH} deficient animals lack allergen induced humoral responses

To determine the role of T_{FH} during chronic AAD, *cd4^{cre}bcl-6^{fl/fl}* (T-Bcl6^Δ) animals (Hollister et al., 2013) or littermate *bcl-6^{fl/fl}* control (WT) were exposed (i.n) to HDM, 5 times a week for 5 weeks (**Figure 6.22A**). WT mice exposed to HDM generated strong mLN and lung T_{FH} populations and as expected both populations were absent in T-Bcl6^Δ animals (**Figure 6.22B**). Consistent with the dependence of GC B cells on T_{FH} for their generation, GC B cells found in the lung and mLN of WT animals were also absent in T-Bcl6^Δ animals (**Figure 6.22C**). This lack of lung TLS was clearly observed by confocal imaging of PCLS from HDM treated WT and T-Bcl6^Δ animals (**Figure 6.23A**). WT animals had large B220⁺ B cells aggregates, complete with a Ki67⁺ B cell core and CD4⁺ T cell scattered within or around this structure and CD21/25⁺ FDCs networks (**Figure 6.23A**). However, in allergen treated T-Bcl6^Δ animals B cell clusters could still be identified but they lacked Ki67⁺ cells and CD21/35⁺ FDC networks (**Figure 6.23A**). Similar results were observed in ALT treated animals, where ALT induced mLN and lung T_{FH} and GC B cells in WT animals which were absent in allergen treated T-Bcl6^Δ mice (**Figure 6.24A-E**).

WT and T-Bcl6^Δ animals treated with PBS lacked B220^{lo}CD138⁺ plasma cell populations in their lungs and mLN (**Figure 6.25A-E**). While, HDM induced plasma cells in the mLN and lungs of WT animals and these plasma cells were lost in allergen exposed T-Bcl6^Δ animals (**Figure 6.25A-E**). Consistent with the lack of GC B cells and plasma cells T-Bcl6^Δ animals lacked total serum IgG1 and IgE (**Figure 6.25F**), serum HDM specific IgG1 and IgE (**Figure 6.25G**), lung HDM specific IgG1 (**Figure 6.25H**) while lung HDM specific IgE was poorly detected in WT and T-Bcl6^Δ animals (**Figure 6.25H**), and BAL IgA was dramatically reduced (**Figure 6.25I**). ALT treated T-Bcl6^Δ animals also lacked allergen induced plasma cells (**Figure 6.26A-E**) and had impaired serum and lung IgG1 (**Figure 6.26F, G**), serum IgE (**Figure 6.26H**) and reduced BAL IgA (**Figure 3.26J**). Once again allergen specific lung IgE was poorly detected, but was generally lower in the T-Bcl6^Δ animals (**6.26I**).

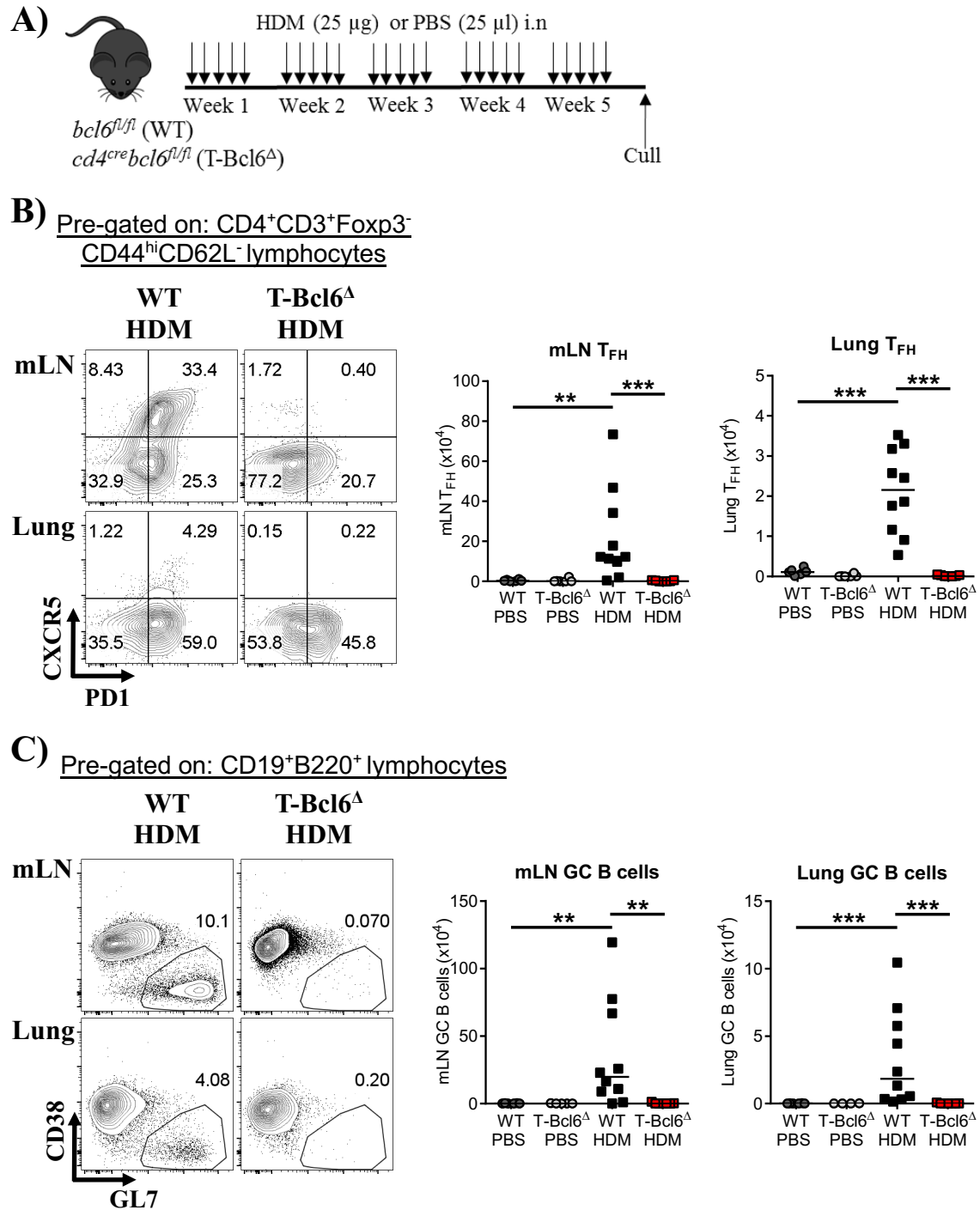


Figure 6.22: T follicular helper cells are critical for germinal centre formation

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6^Δ) and *bcl6^{fl/fl}* littermate controls (WT) controls were exposed (i.n) to 25 µg HDM or 25 µl PBS, 5 times a week for 5 weeks. Flow cytometry was used to determine the frequency and number of T_{FH} and GC B cells. **A)** Schematic of experimental design, **B)** Representative flow plots of T_{FH} in mLN and lungs of WT and T-Bcl6^Δ HDM treated animals, pre-gated on CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes. mLN and lung T_{FH} numbers were quantified, **C)** Representative flow plots of GC B cells in PBS and HDM treated animals, pre-gated on CD19⁺B220⁺ lymphocytes. mLN and lung GC B cell numbers were quantified. Data is combined from 2 experiments. n=6-10. Statistical significance was determined using Kruskal-Wallis analysis with Bonferroni correction.

A)

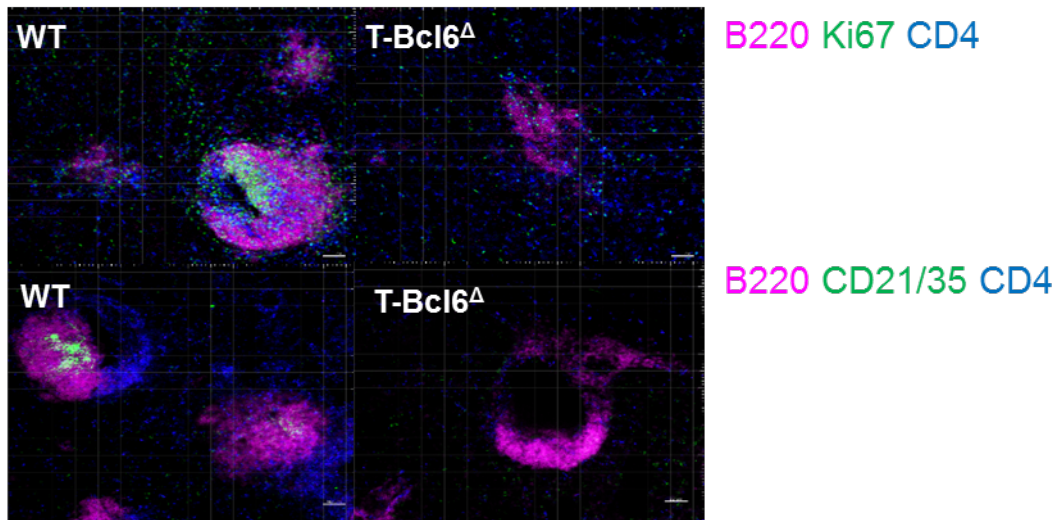


Figure 6.23: Lung GC formation is dependent on T follicular helper cells during chronic allergic airway disease.

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6 Δ) and *bcl6^{fl/fl}* littermate controls (WT) controls were exposed (i.n) to 25 μ g HDM, 5 times a week for 5 weeks. Representative confocal imaging of precision cut lung slices from HDM treated animals. B220 (B cells), CD4 (T cells), Ki67 (Proliferating cells-GC), GL7 (GC B cells), CD21/35 (Follicular dendritic cells – FDCs).

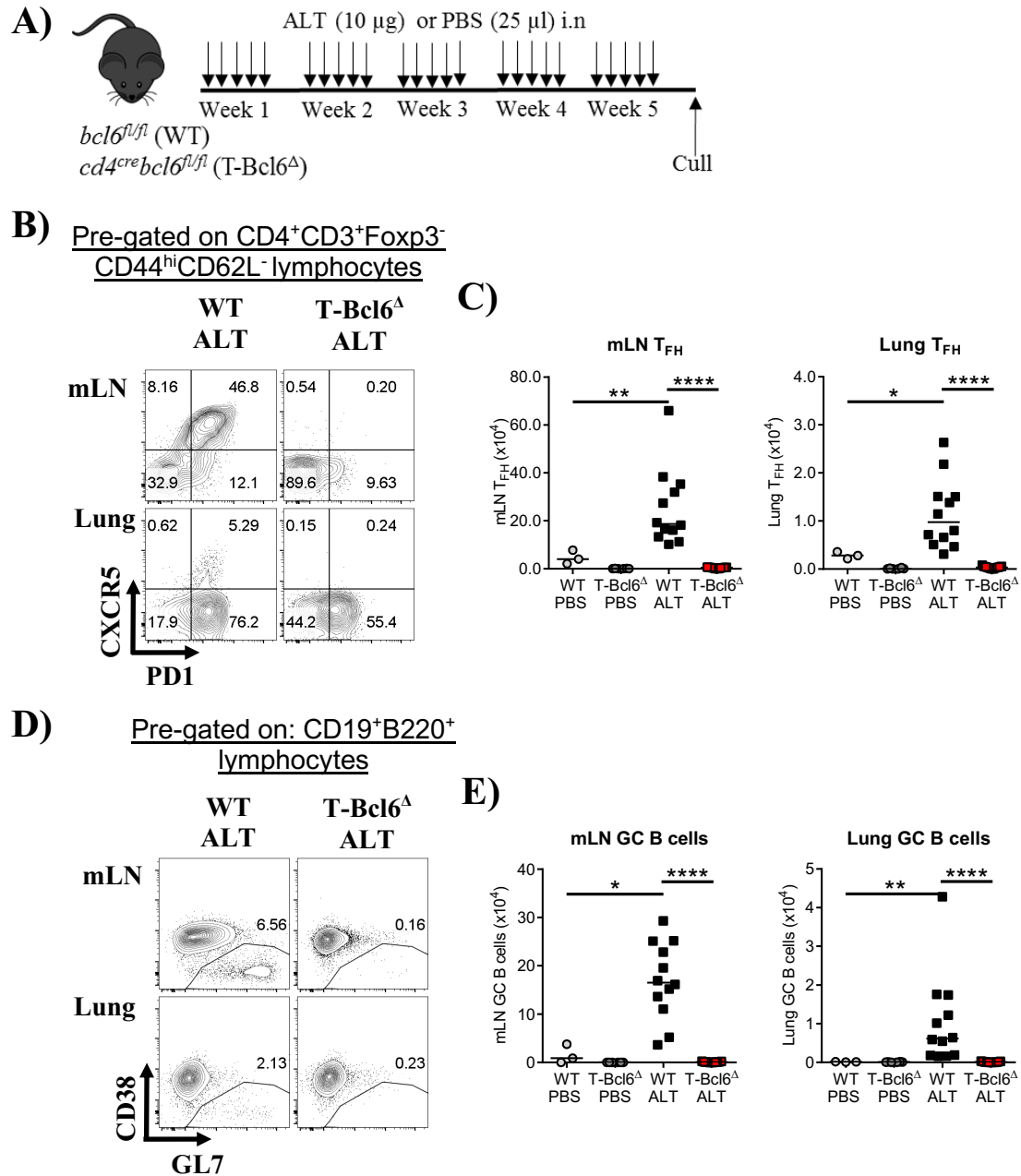


Figure 6.24: CD4⁺ T cells lacking bcl-6 fail to generate T_{FH} and lack GC B cells during ALT driven allergic airway disease.

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6^Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 10 µg ALT or 25 µl PBS, 3 times a week for 5 weeks. **A)** Schematic of experimental protocol. **B)** Representative flow plots of T_{FH} in WT and T-Bcl6^Δ ALT treated animals, pre-gated on CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes. T_{FH} numbers were quantified. **C)** Representative flow plots of GC B cells in WT and T-Bcl6^Δ ALT treated animals, pre-gated on CD19⁺B220⁺ lymphocytes. GC B cell numbers were quantified. Data is pooled from 2 independent experiments. n=3-12. Kruskal-Wallis analysis with Bonferroni correction was used to determine statistical significance.

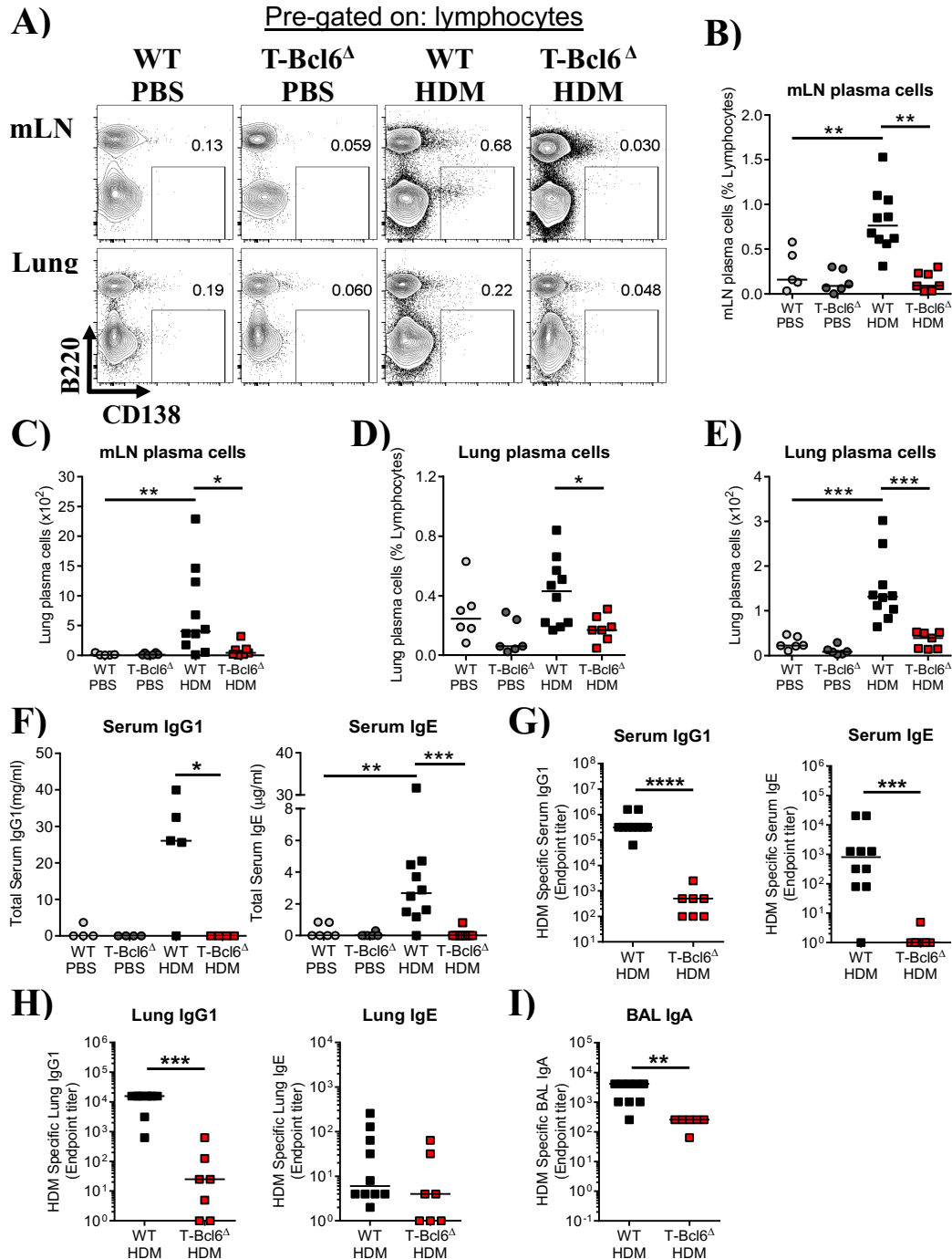


Figure 6.25: T_{FH} deficiency severely impaired HDM driven antibody responses
 Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6 Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 25 μ g HDM or 25 μ l PBS, 5 times a week for 5 weeks. **A-E)** Flow cytometry was used to determine the frequency and number of plasma cells within cellular compartments. **A)** Representative flow plots of plasma cells in PBS and HDM treated animals, pre-gated on lymphocytes, plasma cell frequencies were quantified **B)** mLN frequency, **C)** mLN total, **D)** Lung frequency, **E)** Lung total, **F)** Total serum IgG1 and IgE was determined by ELISA, **G-I)** HDM specific antibody was determined by ELISA and endpoint titers were calculated, **G)** Serum IgG1 and IgE, **H)** Lung IgG1 and IgE, **I)** BAL IgA. Data is pooled from 2 independent experiments. n=5-10. Mann Whitney U analysis was used to determine the statistical significance between 2 groups and for 3 or more groups Kruskal-Wallis analysis with Bonferroni correction was used.

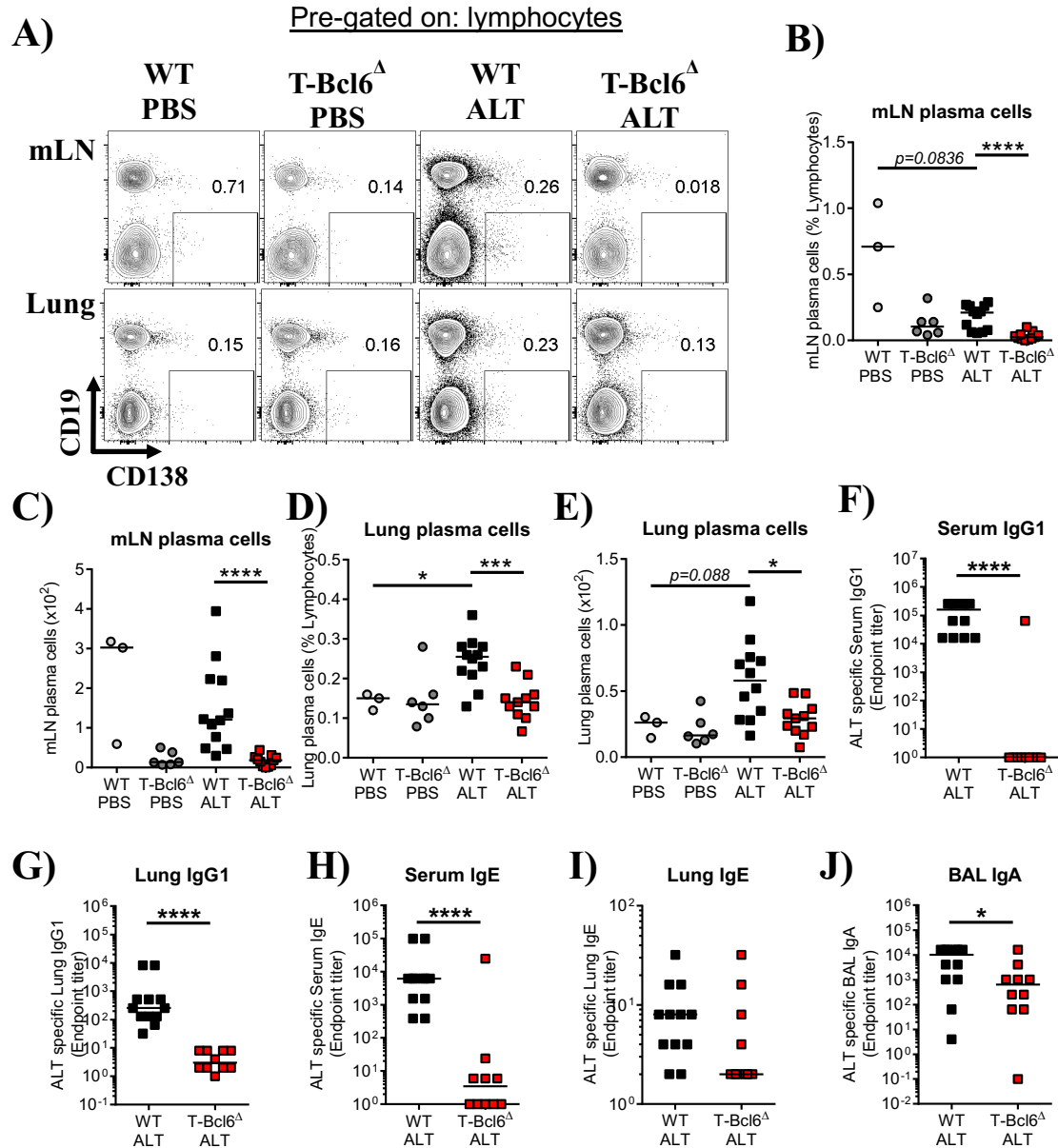


Figure 6.26: T_{FH} deficiency severely impaired ALT driven antibody responses

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6 Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 10 μ g ALT or 25 μ l PBS, 5 times a week for 5 weeks. **A-E)** Flow cytometry was used to determine the frequency and number of plasma cells within cellular compartments. **A)** Representative flow plots of mLN and lung plasma cells in PBS and ALT treated animals, pre-gated on lymphocytes, plasma cell frequencies were quantified **B)** mLN frequency, **C)** mLN total, **D)** Lung frequency, **E)** Lung total. **F-J)** ALT specific antibody was determined by ELISA and endpoint titers were calculated, **F)** Serum IgG1, **G)** Lung IgG1, **H)** Serum IgE, **I)** Lung IgE, **J)** BAL IgA. Data is pooled from 2 independent experiments. $n=3-12$. Mann Whitney U analysis was used to determine the statistical significance between 2 groups and for 3 or more groups Kruskal-Wallis analysis with Bonferroni correction was used.

Furthermore, HDM treated T-Bcl6^Δ mice lacked the allergen induced mLN and lung IgG1⁺ memory B cells seen in WT treated animals (**Figure 6. 27A, B**). Similar results were observed following ALT exposure (**Figure 6.27C, D**). In the ALT model, lung IgA⁺ memory B cells were also assessed and again allergen induced IgA⁺ memory B cells observed in the WT animals were absent in the T-Bcl6^Δ mice (**Figure 6.27E**). Taken together, it is clear that humoral responses induced during chronic AAD do not fully develop in *cd4^{cre}bcl-6^{fl/fl}* animals indicating a dependence on T_{FH} for these responses.

6.3.9. Cell-intrinsic Bcl6 expression is required for T_{FH} and loss of Bcl-6 does not increase differentiation to other lineages

Bcl-6 is a zinc finger transcriptional repressor which drives T_{FH} differentiation by targeting gene loci associated with other Th lineages, preventing differentiation down alternative pathways (Hatzi et al., 2015). Given that Bcl-6 functions to prevent differentiation down alternative lineages, it is possible that Bcl-6 loss within CD4⁺ T cells would cause increased differentiation down other Th lineages. To examine whether CD4⁺ T cells deficient in Bcl-6 have enhanced propensity to differentiate into alternative Th cell lineages, mixed bone marrow chimera were performed. To do this bone marrow cells from CD45.1 WT and CD45.2 T-Bcl6^Δ (*cd4^{cre}bcl-6^{fl/fl}*) mice were mixed at a 1:1 ratio and transferred into irradiated female C57BL/6 recipients. Mice were given 9 weeks to reconstitute their bone marrow before being exposed to HDM or PBS 5 times a week for 3 weeks. For comparison a mixed bone marrow chimera of CD45.1 WT and CD45.2 WT (*bcl-6^{fl/fl}*) was also set up (**Figure 6.28A**).

Both mixed bone marrow chimeras exposed to HDM developed chronic AAD characterised by AHR and pulmonary inflammation and no difference was observed between the two allergen treated groups (**Figure 6.28B-H**). Therefore, mixed bone marrow chimeras were still capable of developing chronic AAD.

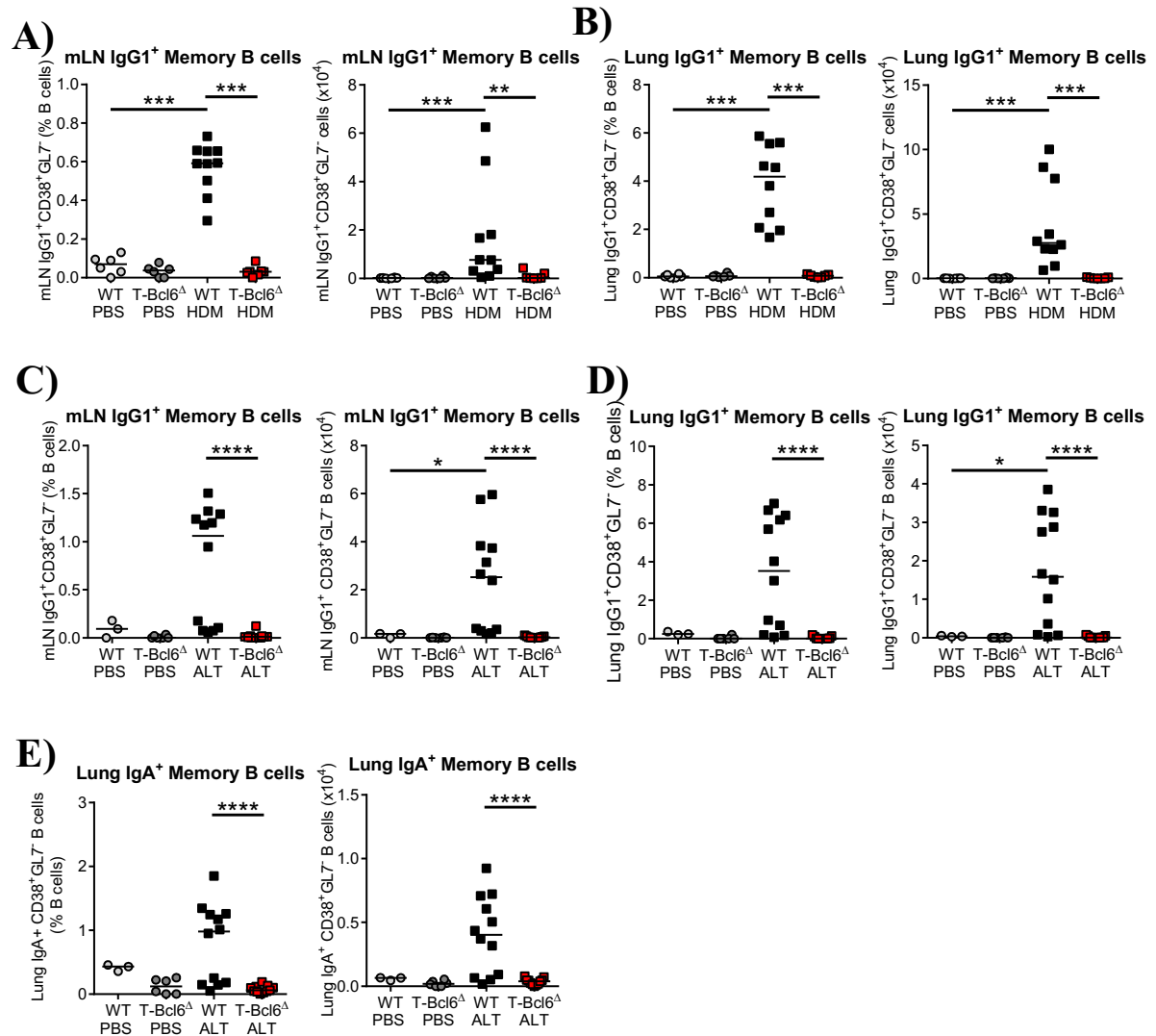


Figure 6.27: CD4⁺ T cells lacking *bcl-6* fail to generate switched memory B cells

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6^Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 25 μg HDM, 10 μg ALT or 25 μl PBS, 3 times a week for 5 weeks. Flow cytometry was used to determine the frequency and number of IgG1⁺ and IgA⁺ memory B cells. **A-B)** HDM study, **A)** mLN IgG1⁺ memory B cells, **B)** Lung IgG1⁺ memory B cells **C-F)** ALT study, **C)** mLN IgG1⁺ memory B cells, **D)** Lung IgG1⁺ memory B cells, **E)** Lung IgA⁺ memory B cells. Data is pooled from 2 independent experiments. n=6-10 for HDM study, n=3-12 for ALT study. Kruskal-Wallis analysis with Bonferroni correction was used to determine statistical significance.

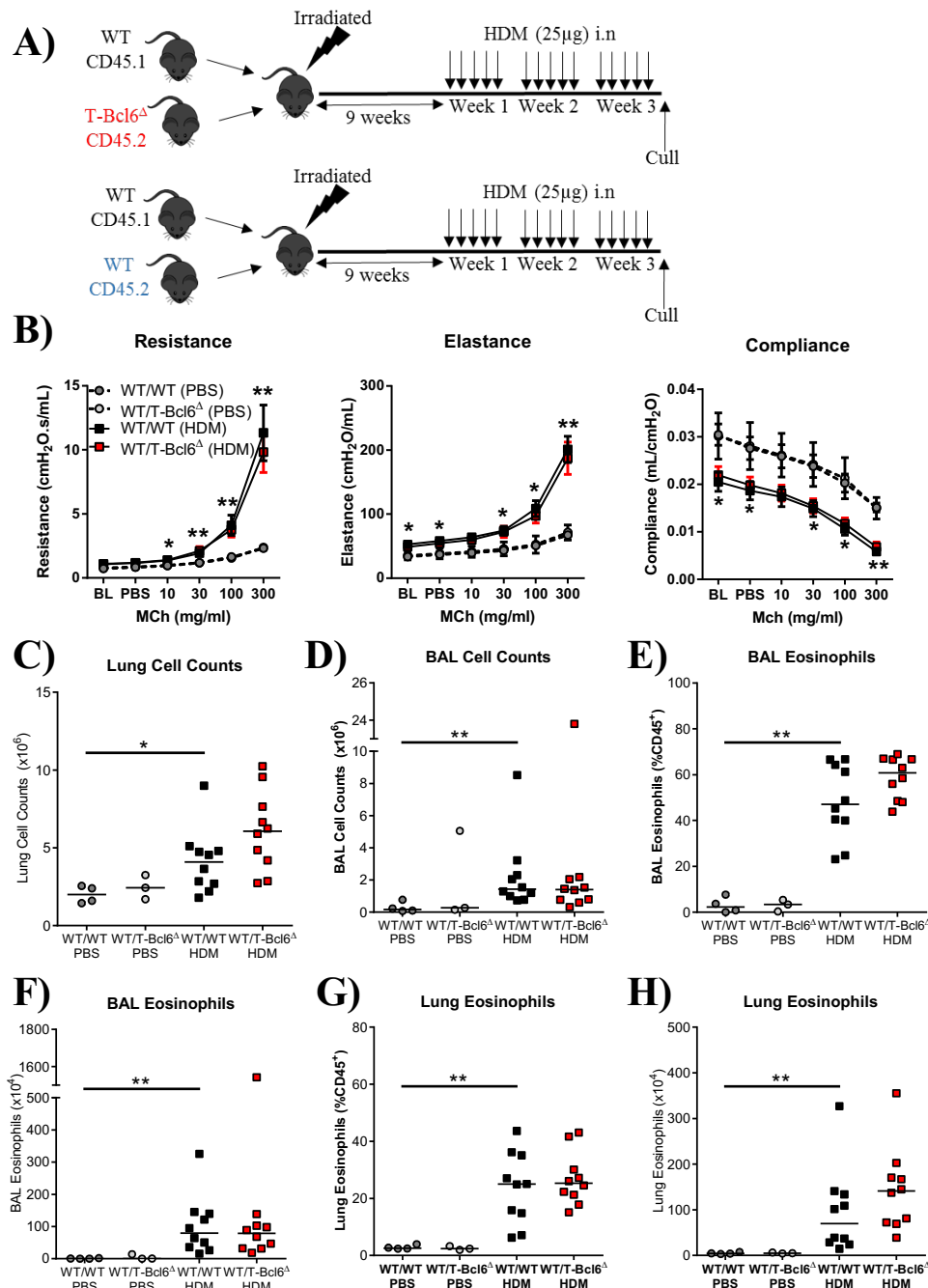


Figure 6.28: Mixed BM Chimeras develop chronic allergic disease

cd4^{cre} bcl6^{fl/fl} - CD45.2 (T-Bcl6 Δ) or *bcl6^{fl/fl}* - CD45.2 (WT) bone marrow was mixed at a 1:1 ratio with CD45.1 (WT) cells and transferred (i.v.) into irradiated C57BL/6 host. Mice were given 9 weeks to reconstitute their bone marrow then exposed to 25 μ g HDM, 5 times a week for 3 weeks. **A)** Schematic of experimental design, **B)** Airway hyper-responsiveness was measured by exposing mice to 10-300mg/ml methacholine (MCh) using the FlexiVent system. Airway resistance, elastance and compliance are displayed. **C)** Lung cell counts, **D)** BAL cell counts. **E-H)** Flow cytometry was used to determine the frequency and number of eosinophils, **E)** BAL eosinophils frequency, **F)** BAL eosinophil numbers, **G)** Lung eosinophils frequency, **H)** Lung eosinophil numbers. Data is pooled from 2 independent experiments. n=3-10. Kruskal-Wallis analysis with Bonferroni correction was used to determine statistical significance.

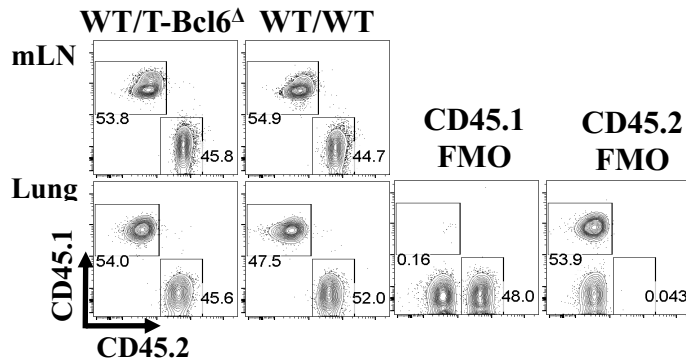
The fate of CD4⁺ T cell populations in the allergen treated WT/T-Bcl6^Δ and WT/WT bone marrow chimeras was then examined (**Figure 6.29**). CD45.1⁺ and CD45.2⁺ CD4⁺ T cells were easily detected in the mLN and lungs in WT/T-Bcl6^Δ and WT/WT animals (**Figure 6.29A**). As expected in the WT/T-Bcl6^Δ chimeras, T_{FH} were easily identified within the CD45.1⁺ WT CD4⁺ T cell fraction in the mLN and the lungs of allergen exposed animals and this proportion was dramatically reduced in the CD45.2 T-Bcl6^Δ CD4⁺ T cells fraction (**Figure 6.29B**). Whereas, in the WT/WT chimera similar proportions of T_{FH} were identified in the lung CD45.1⁺ and CD45.2⁺ WT CD4⁺ T cells. While CD45.2 cells were slightly reduced compared to their CD45.1 comparators in the mLN, but this was not to the extent seen in the WT/T-Bcl6^Δ chimera (**Figure 6.29C**). Taken together this shows CD45.2⁺ bone marrow derived cells from T-Bcl6^Δ mice to have a significantly reduced T_{FH} response.

Next the impact that this lack of T_{FH} has on Th2 and Th17 cell differentiation during chronic AAD was examined. In the WT/T-Bcl6^Δ chimera, mLN GATA3⁺ Th2 and Rorγt⁺ Th17 cells were decreased in the T-Bcl6^Δ CD45.2⁺CD4⁺ T cells compared to the CD45.1⁺CD4⁺ WT T cells (**Figure 6.30A**). While in the lungs both GATA3⁺ Th2 cells and Rorγt⁺ Th17 cells were similar between the two populations (**Figure 6.30B**). In the WT/WT mixed bone marrow chimera, similar proportions of mLN GATA3⁺ CD4⁺ T cells and Rorγt⁺ Th17 cells were observed in both CD4⁺ T cell compartments (**Figure 6.31A**), and the same result was seen in the lungs (**Figure 6.31B**). Overall, given the role of Bcl-6 as a transcriptional repressor it was expected that Bcl-6 deficient CD4⁺ T cell would have increased capacity to differentiate into other CD4⁺ T cells subsets. Instead, in the mLN these CD4⁺ T cells appear to have reduced capacity to differentiate into Th2 and Th17 cells, while no difference was observed in the lung CD4⁺ T cells.

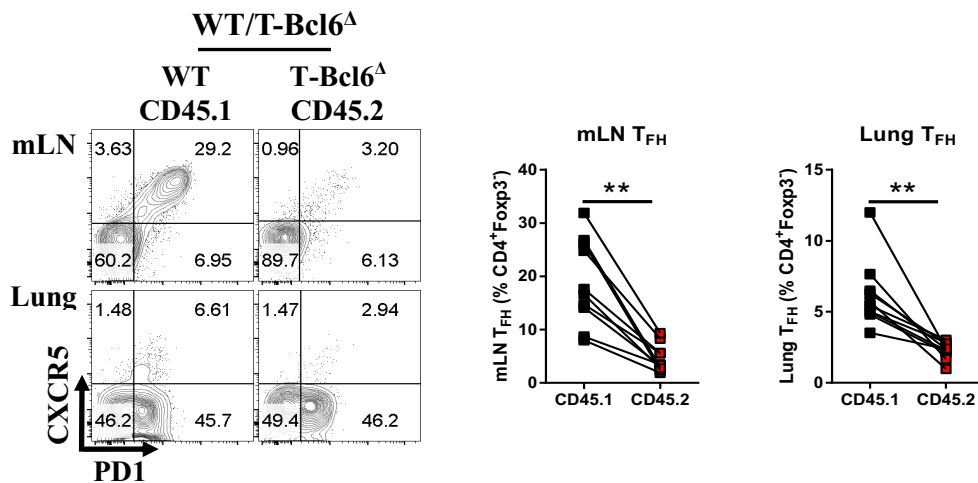
6.3.10. T_{FH} deficient animals have exacerbated chronic allergic airway disease

To examine the role of T_{FH} on hallmark features of chronic AAD, T-Bcl6^Δ and WT mice were exposed (i.n) to PBS, ALT or HDM, 5 times a week for 5 weeks. After 5 weeks of ALT exposure

A) Pre-gated on: CD4⁺CD3⁺lymphocytes



B) Pre-gated on: CD4⁺CD3⁺Foxp3⁻cells



C) WT/WT

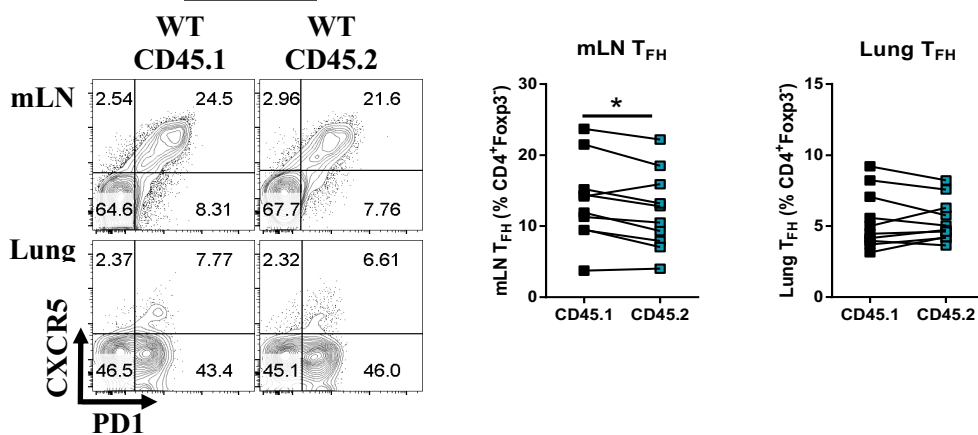
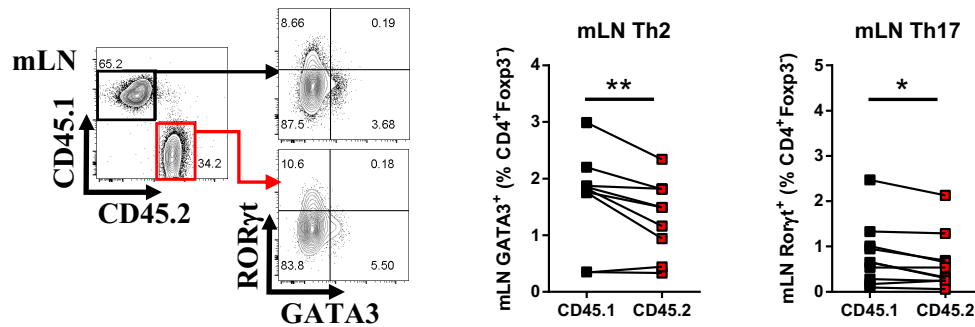


Figure 6.29: T_{FH} are decreased in Bcl-6 deficient mixed bone marrow chimeras
cd4^{cre} bcl6^{fl/fl}-CD45.2 (T-Bcl6^Δ) or *bcl-6^{fl/fl}* - CD45.2 (WT) bone marrow was mixed at a 1:1 ratio with CD45.1 (WT) cells and transferred (i.v.) into irradiated C57BL/6 hosts. Mice were given 9 weeks to reconstitute their bone marrow then exposed to 25 μg HDM, 5 times a week for 3 weeks. **A)** Representative flow plots of CD45.1 and CD45.2 CD4⁺ T cells, pre-gated on CD4⁺CD3⁺ lymphocytes, **B)** Representative flow cytometry and quantification of T_{FH} proportions in CD45.1 (WT) and CD45.2 (T-Bcl6^Δ) compartments, **C)** Representative flow cytometry and quantification of T_{FH} proportions in CD45.1 (WT) and CD45.2 (WT) compartments. Data is pooled from 2 independent experiments, n=10. Wilcoxon signed rank test was used to determine statistical significance.

A) Pre-gated on: CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes

WT/T-Bcl6^Δ Mixed BM Chimera



B)

WT/T-Bcl6^Δ Mixed BM Chimera

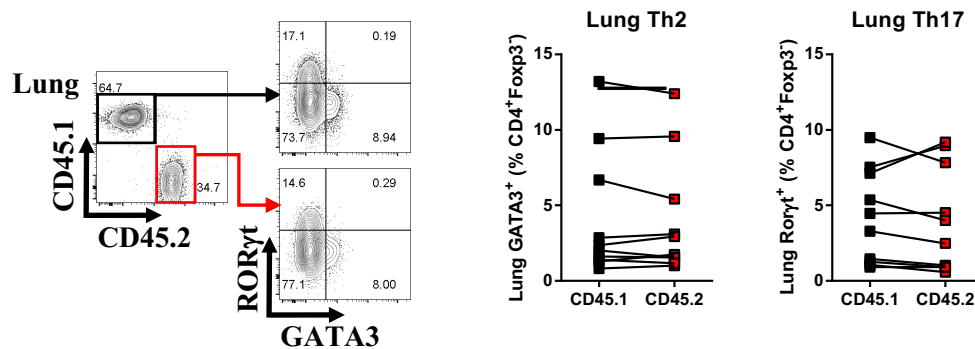
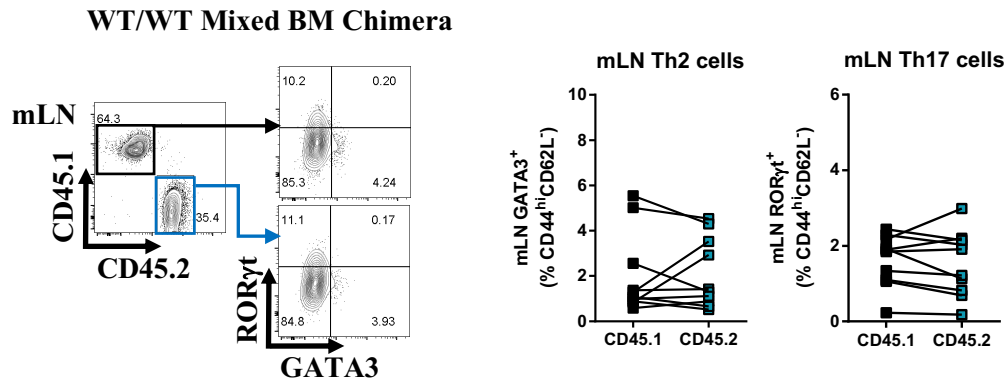


Figure 6.30: Bcl-6 deficient CD4⁺ T cells do not differentiate into Th2 or Th17 cells.

Mixed bone marrow chimeras. T-Bcl6^Δ (CD45.2) and WT (CD45.1) bone marrow was mixed at a 1:1 ratio and transferred (i.v.) into irradiated C57BL/6 host. Mice were given 9 weeks to reconstitute their bone marrow then exposed to 25 μg HDM, 5 times a week for 3 weeks. **A)** Representative flow cytometry of CD45.1⁺ and CD45.2⁺ CD4⁺ T cells in the WT/T-Bcl6^Δ chimera. mLN Th2 and Th17 cells are quantified, **B)** Representative flow cytometry of CD45.1⁺ and CD45.2⁺ CD4⁺ T cells in the WT/T-Bcl6^Δ chimera. Lung Th2 and Th17 cells are quantified. Data is pooled from 2 independent experiments, n=10. Wilcoxon signed rank test was used to determine statistical significance.

both allergen treated groups had increased airway resistance and elastance and reduced compliance compared to both PBS treated groups, and this AHR was exacerbated in ALT treated T-Bcl6^Δ animals (**Figure 6.32A**). In the HDM study, although WT HDM treated animals failed to develop AHR, airway resistance, elastance and compliance was still exacerbated in T-Bcl6^Δ HDM treated animals (**Figure 6.32B**), but not to the extent observed during ALT driven

A) Pre-gated on: CD4⁺CD3⁺CD44^{hi}CD62L⁻ lymphocytes



B) **WT/WT Mixed BM Chimera**

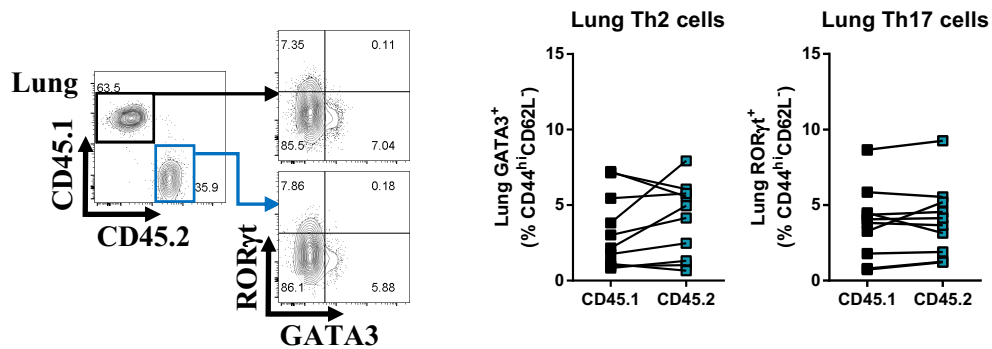


Figure 6.31: WT/WT bone marrow chimera during chronic allergic airway disease

Mixed bone marrow chimeras. WT (CD45.2) and WT (CD45.1) bone marrow was mixed at a 1:1 ratio and transferred (i.v.) into irradiated C57BL/6 host. Mice were given 9 weeks to reconstitute their bone marrow then exposed to 25 µg HDM, 5 times a week for 3 weeks.

A) Representative flow cytometry of CD45.1⁺ and CD45.2⁺ CD4⁺ T cells in the WT/WT chimera. mLN Th2 and Th17 cells are quantified, **B)** Representative flow cytometry of CD45.1⁺ and CD45.2⁺ CD4⁺ T cells in the WT/WT chimera. Lung Th2 and Th17 cells are quantified. Data is pooled from 2 independent experiments, n=10. Wilcoxon signed rank test was used to determine statistical significance.

disease (**Figure 6.32A**). Taken together, this data shows T_{FH} deficiency to drive worsened AHR and this phenotype was still sufficient to drive AHR development in mice not normally capable of developing AHR.

Next the impact of T_{FH} deficiency on pulmonary inflammation was assessed. As expected BAL and lung cell counts were increased in allergen treated animals compared to PBS treated controls (**Figure 6.33A, B**). However, while no difference were observed in BAL cell counts

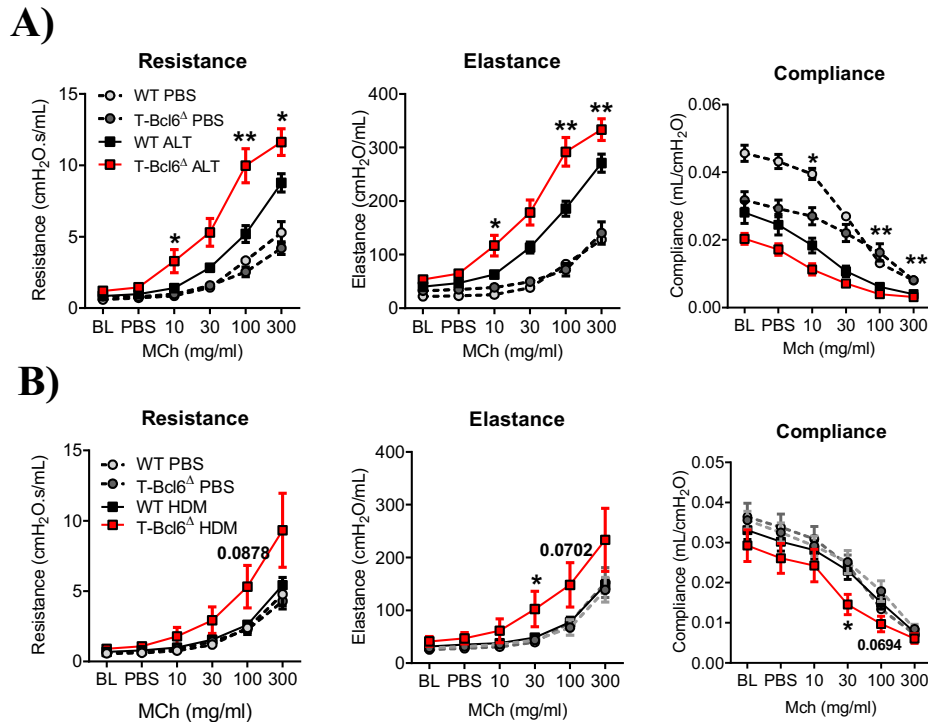


Figure 6.32: Mice lacking T_{FH} have exaggerated airway hyperresponsiveness

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6 Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 5 times a week for 5 weeks. **A-B)** Airway hyperresponsiveness was measured by exposing mice to 10-300 mg/ml methacholine (MCh) using the flexiVent. Airway resistance, elastance and compliance are displayed. **A)** ALT study, **B)** HDM study. Data from the HDM and ALT studies are pooled from 2 independent experiments. n=3-12 mice per group. Mann Whitney U analysis was used to determine statistical significance between the 2 allergen treated groups.

between allergen treated WT and T-Bcl6 Δ mice (**Figure 6.33A**), lung cell counts were decreased in allergen treated T-Bcl6 Δ animals compared to WT, while in ALT treated animals numbers were increased (**Figure 6.33B**). When proportions and numbers of eosinophils are examined it is clear that allergen induced BAL and lung eosinophils were increased in T-Bcl6 Δ animals compared to WT animals after either HDM or ALT challenge (**Figure 6.33C-F**), indicating T_{FH} deficient animals have worsened eosinophilia.

Neutrophils were also analysed. Overall, BAL and lung neutrophils were generally increased or trending upwards in HDM exposed mice compared to PBS controls (**Figure 6.34A, B**) and no significant difference was observed in the proportion or number of BAL neutrophils between

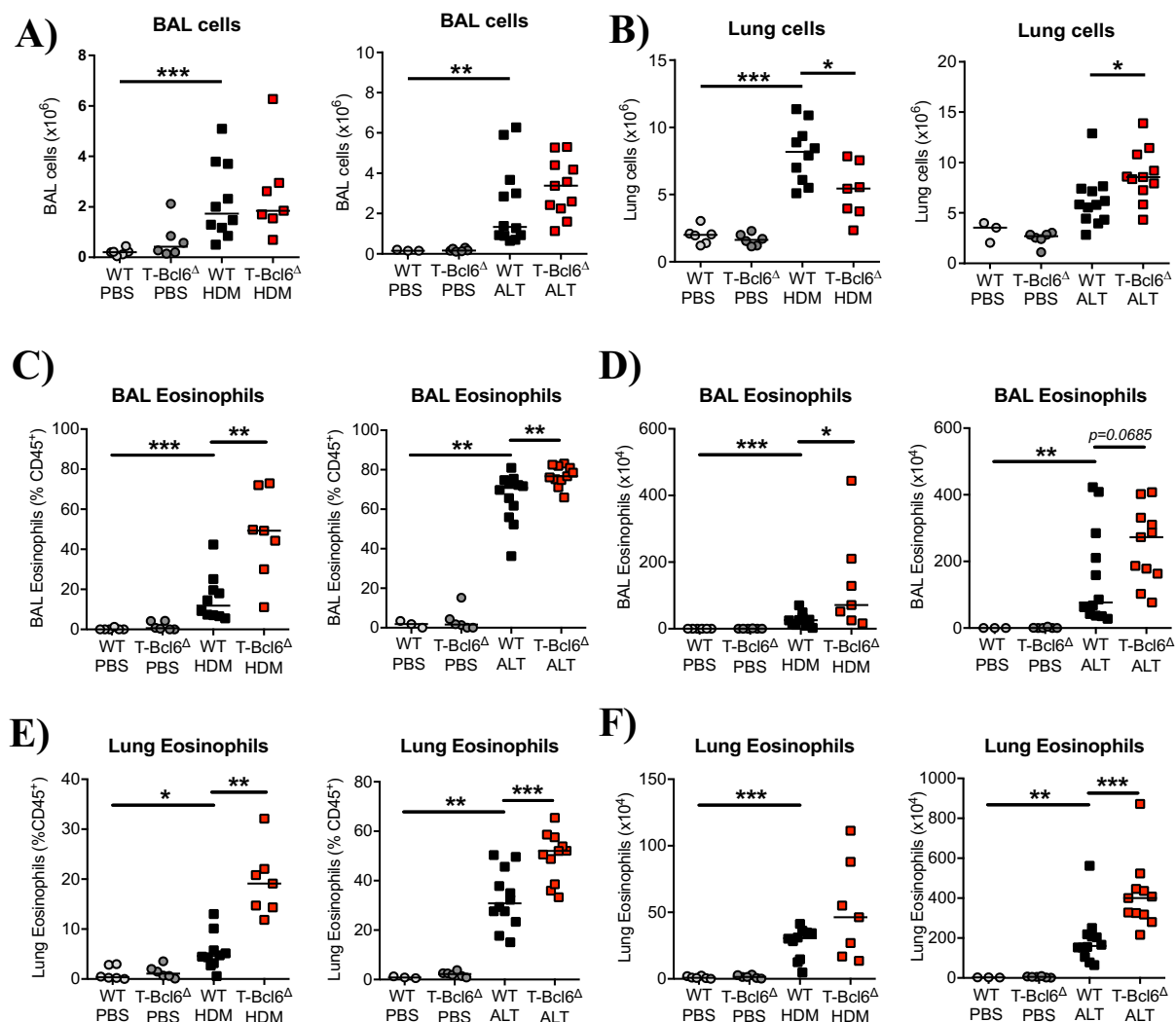


Figure 6.33: Mice lacking T follicular helper cell have worsened eosinophilia

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6^Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 25 μg HDM, 10 μg ALT or 25 μl PBS, 5 times a week for 5 weeks. **A)** BAL cell counts, **B)** Lung cell counts, **C-F)** Flow cytometry was used to determine the frequency of eosinophils in the lungs and the BAL in the HDM and ALT study, **C)** BAL eosinophils frequency **D)** BAL eosinophils numbers, **E)** Lung eosinophils frequency, **F)** Lung eosinophils numbers. Data from the HDM and ALT studies are pooled from 2 independent experiments. n=3-12 mice per group. Kruskal-Wallis analysis with Bonferroni correction was used to determine statistical significance.

WT and T-Bcl6^Δ HDM treated animals (**Figure 6.34A**). However, in the lungs neutrophils were decreased by total number but not by proportion (**Figure 6.34B**). ALT did not drive a significant BAL or lung neutrophil response compared to PBS controls and no difference were observed between WT and T-Bcl6^Δ ALT treated animals (**Figure 6.34C, D**). Overall, T_{FH} deficiency did not significantly impact neutrophil responses induced during chronic AAD.

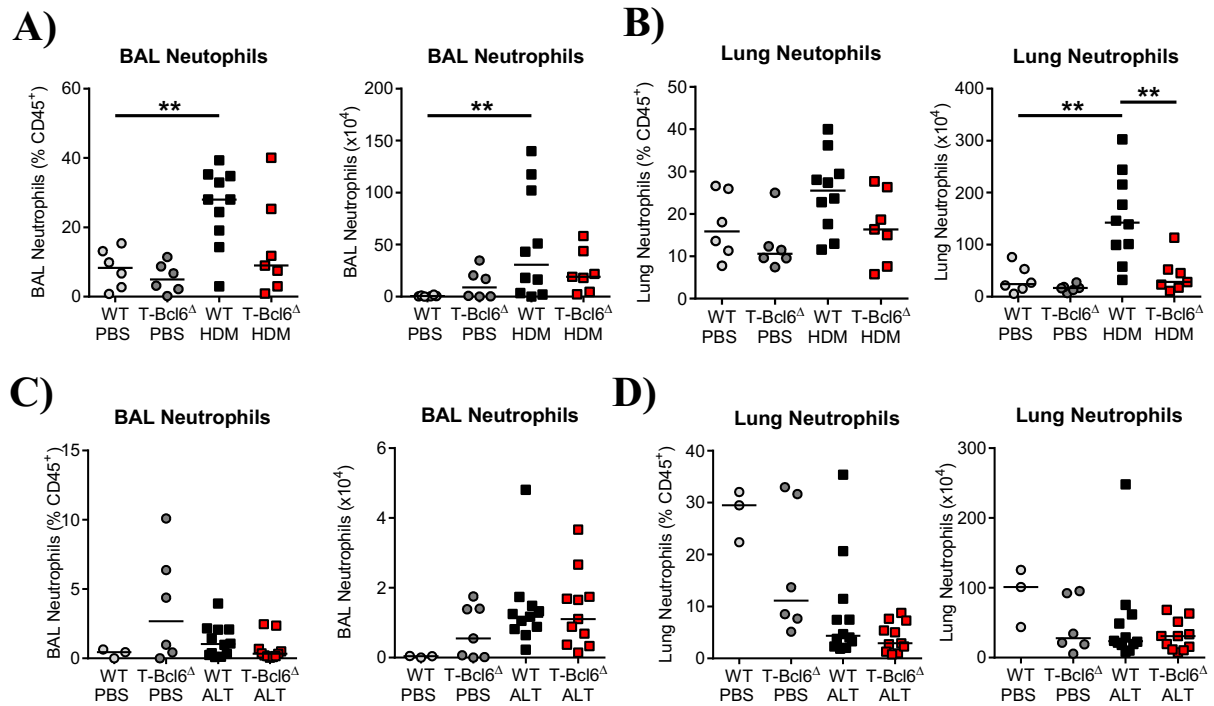


Figure 6.34: Mice lacking T follicular have normal neutrophil responses

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6^Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 25 μg HDM, 10 μg ALT or 25 μl PBS, 5 times a week for 5 weeks. **A-B)** HDM driven disease, **A)** BAL neutrophils, **B)** Lung neutrophils, **C-D)** ALT driven disease, **C)** BAL neutrophils, **D)** Lung neutrophils. Data from the HDM and ALT studies are pooled from 2 independent experiments. n=3-12 mice per group. Kruskal-Wallis analysis with Bonferroni correction was used to determine statistical significance.

As AHR and eosinophilia were worsened in these animals, this suggested that allergen exposed T-Bcl6^Δ mice were experiencing enhanced type 2 disease. As AAD is generally considered a Th2 driven pathology, the impact of T_{FH} deficiency on other CD4⁺ T cell populations during chronic AAD was examined. Firstly, Th2 cells were examined during chronic AAD and as expected in WT animals following ALT treatment GATA3⁺ CD4⁺ T cells were identified with the mLN and the lung tissue (**Figure 6.35A**). However, when T-Bcl6^Δ animals were treated with ALT, GATA3⁺ Th2 cells were increased compared to their WT counterparts both in the mLN and the lung tissue (**Figure 6.35A**). After HDM treatment, no dramatic difference was observed in GATA3⁺ populations in the mLN, while in the lung tissue GATA3⁺ CD4⁺ T cells were dramatically increased in T-Bcl6^Δ HDM animals compared to WT animals (**Figure 6.36B**). As expected GATA3⁺ CD4⁺ T cells were not observed in the mLN and lung tissue of PBS treated animals, and no differences observed between WT and T-

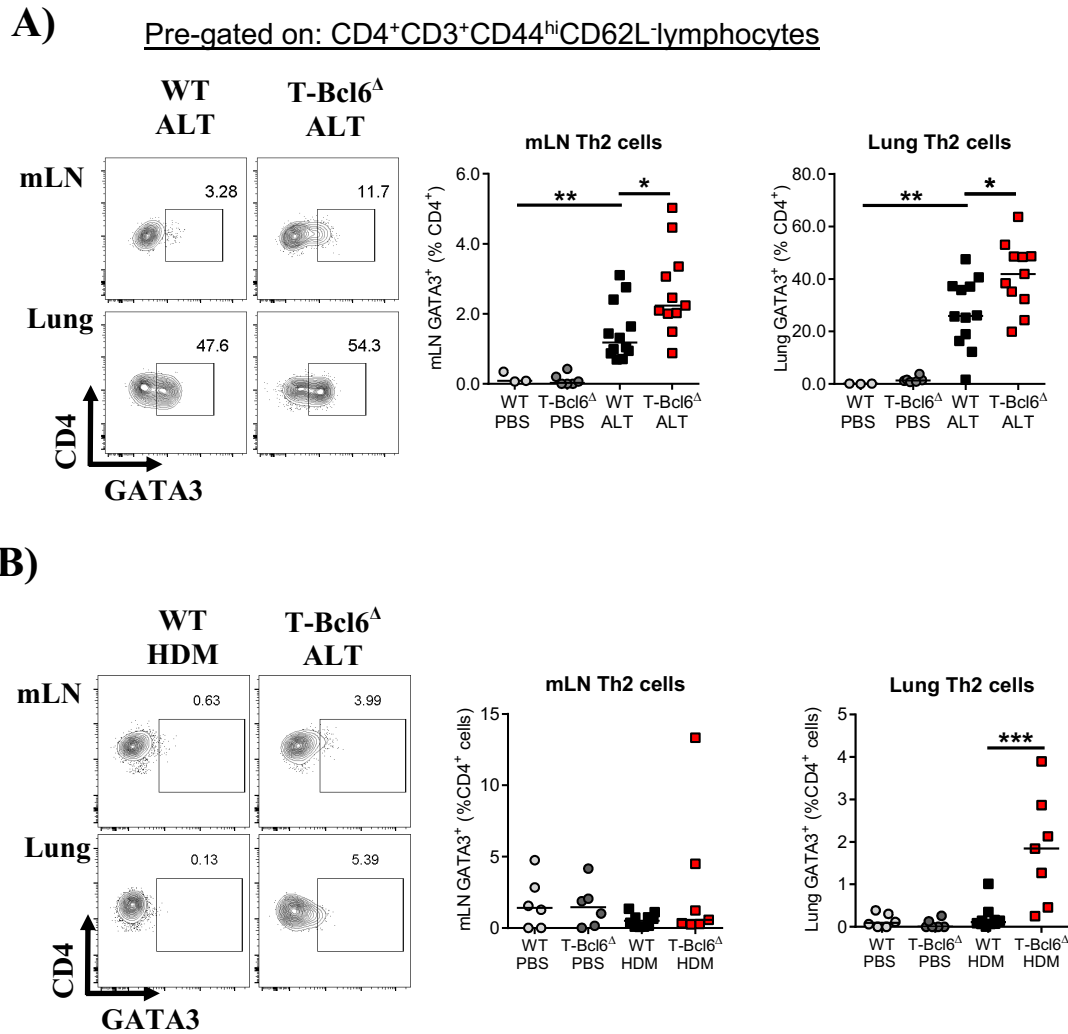


Figure 6.35: Allergen exposed T_{FH} deficient animals have a increased Th2 cells

Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6^Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 10 µg ALT, 25 µg HDM or 25 µl PBS, 5 times a week for 5 weeks. Flow cytometry was used to analyse Th2 cell populations. **A)** Representative plots of GATA3⁺ CD4⁺ T cells in the mLN and lung tissue of ALT treated mice. GATA3⁺ CD4⁺ T cell frequency are quantified. **B)** Representative plots of GATA3⁺ CD4⁺ T cells in the mLN and lung tissue of HDM treated mice. GATA3⁺ CD4⁺ T cell frequency are quantified. Data is pooled from 2 independent experiments. n=3-12 mice per group. Kruskal Wallis analysis with bonferroni correction was used to determine statistical significance.

Bcl6^Δ PBS controls (**Figure 6.35A, B**). Overall, the data from the two models showed that GATA3⁺ Th2 cells were increased in T_{FH} deficient animals compared to WT animals.

Given the strong Th17 response induced during chronic AAD driven by HDM, IL-17A⁺ CD4⁺ T cells were also analysed. As expected few IL-17A⁺CD4⁺ T cells were identified in the mLN or lung tissue of PBS treated WT or T-Bcl6^Δ animals (**Figure 6.36A**). IL-17A⁺CD4⁺ T cells were

increased by proportion and total number in the mLN in HDM treated animals compared to PBS controls but no differences were observed between the two allergen treated groups (**Figure 6.36A**). While, in the lungs a strong IL-17A⁺CD4⁺ T cell population was induced in WT animals exposed to HDM and this was dramatically reduced by proportion and number in T-Bcl6^Δ animals (**Figure 6.36A**). During ALT driven chronic AAD, there was a limited IL-17A⁺CD4⁺ T cell response in the mLN compared to PBS treated animals (**Figure 6.36B**). A small population of IL-17A⁺CD4⁺ T cells were induced in the lung tissue compared to PBS controls but no significant differences were observed between WT and T-Bcl6^Δ allergen treated groups (**Figure 6.36B**). Taken together, this data shows T_{FH} deficiency to be associated with a dramatic reduction of IL-17A⁺ CD4⁺ T cells in the HDM driven allergic response but not ALT.

To determine if this increase in Th2 cells had any impact on cytokine production, type 2 cytokines were assessed by ELISA. Consistent with the increase in Th2 cells, following ALT treatment T-Bcl6^Δ animals had increased BAL and lung IL-4 and IL-13 compared to the WT animals (**Figure 6.37A, B**). However, not all type 2 cytokines were increased and BAL and lung IL-5 were similar between ALT treated WT and T-Bcl6^Δ animals (**Figure 6.37C**). In the HDM model, allergen treated T-Bcl6^Δ animals had increased lung IL-4, IL-5 and IL-13 compared to WT allergen treated controls (**Figure 6.37D-F**). However, this data was only from one experiment as these cytokines were not detected in WT or T-Bcl6^Δ in the repeat. To examine if only type 2 cytokines were impacted by T_{FH} deficiency the levels of IL-17A, IL-17F and IL-21 were also assessed. Following ALT treatment BAL IL-21, IL-17A and IL-17F were increased but these cytokines were not impacted by T_{FH} deficiency (**Figure 6.37G-I**). In the HDM model, IL-17A was strongly induced in the lungs following allergen exposure and although level of the cytokine trended downwards, the levels were not significantly different between WT and T-Bcl6^Δ HDM treated animals (**Figure 6.37J**). Consistent with increased type 2 disease, eotaxin-2 was increased in T-Bcl6^Δ HDM treated animals compared to controls and alongside this serum MCPT1 was elevated which was also suggestive of enhanced type 2 disease (**Figure 6.37K, L**).

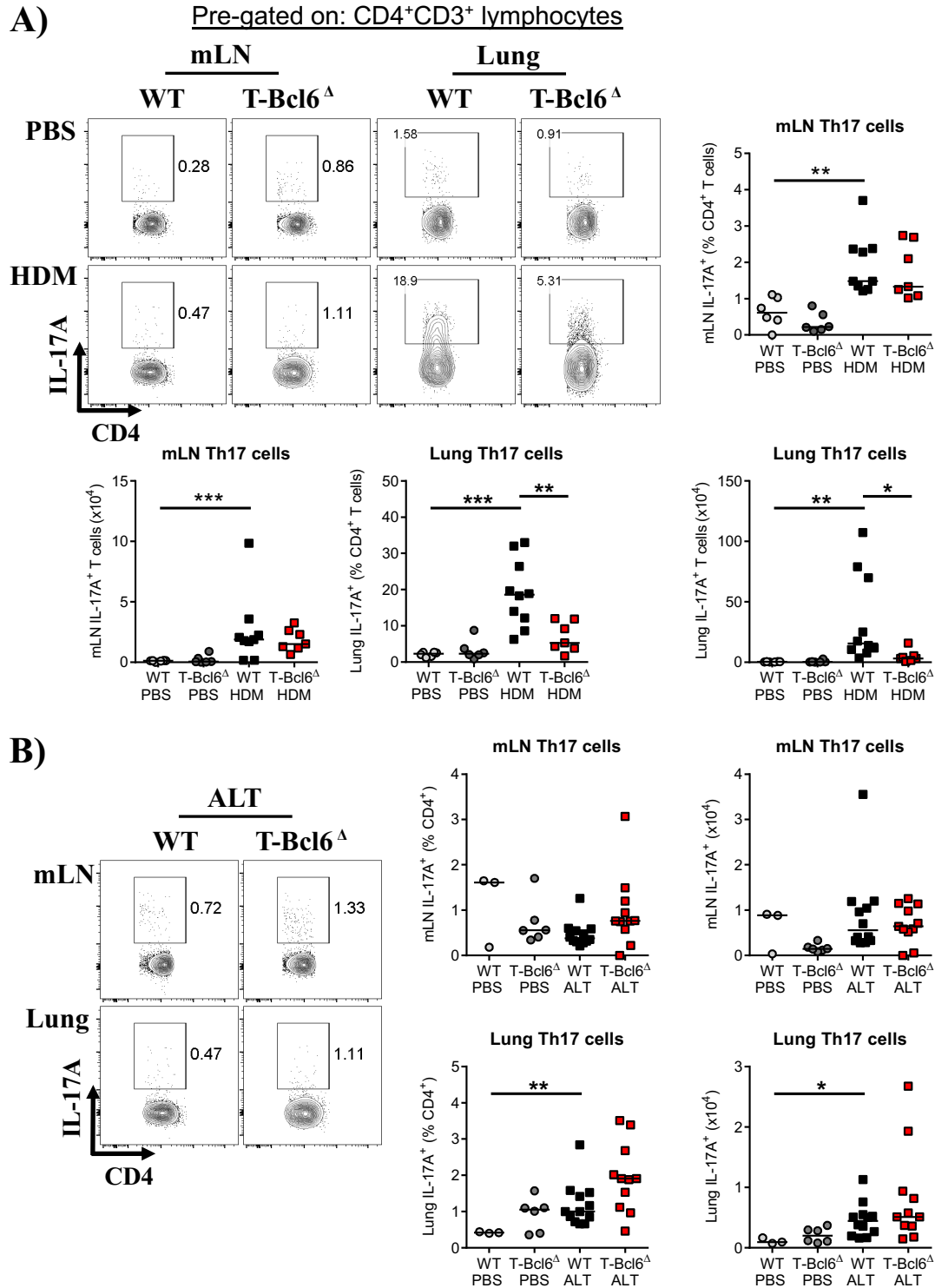


Figure 6.36: HDM exposed T_H17 deficient mice have impaired Th17 responses
 Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6^Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 25 μg HDM, 10 μg ALT or 25 μl PBS, 5 times a week for 5 weeks. Flow cytometry was used to determine the frequency and number of IL-17A⁺ CD4⁺ T cells in the mLN and lungs. Representative flow cytometry plots of IL-17A⁺ CD4⁺ T cells are displayed and data is quantified for **A)** HDM study, **B)** ALT study. Data from the HDM and ALT studies are pooled from 2 independent experiments. n=3-12 mice per group. Kruskal-Wallis analysis with Bonferroni correction was used to determine statistical significance.

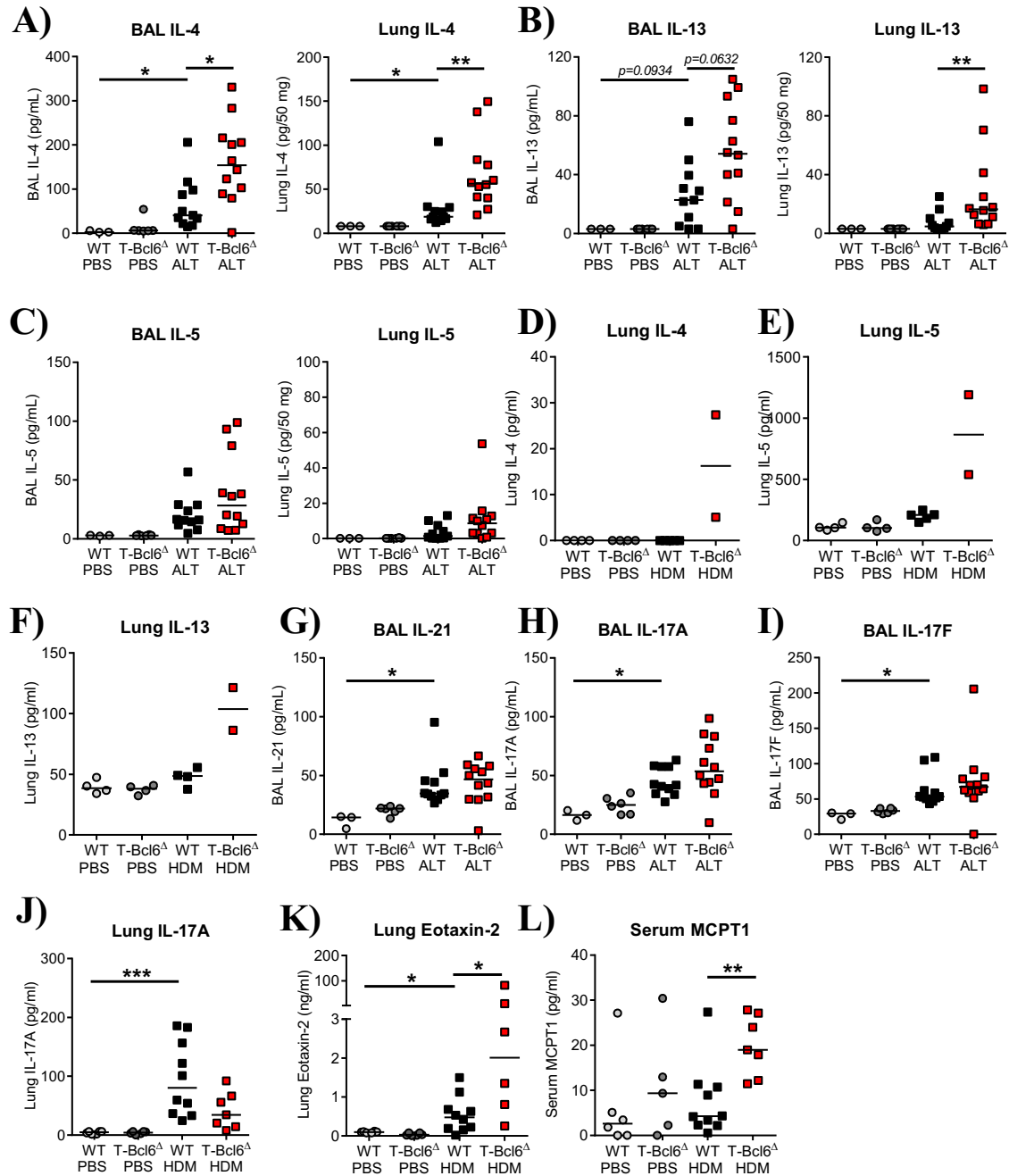


Figure 6.37: Mice lacking T follicular helper cell have increased type 2 cytokines
Adult *cd4^{cre} bcl6^{fl/fl}* (T-Bcl6 Δ) and *bcl6^{fl/fl}* littermate controls (WT) were exposed to 25 μ g HDM, 10 μ g ALT or 25 μ l PBS, 5 times a week for 5 weeks. **A)** BAL and lung IL-4 - ALT, **B)** BAL and lung IL-13 - ALT, **C)** BAL and lung IL-5 - ALT, **D)** Lung IL-4 - HDM, **E)** Lung IL-5 - HDM, **F)** Lung IL-13 - HDM, **G)** BAL IL-21 - ALT, **H)** BAL IL-17A - ALT, **I)** BAL IL-17F - ALT, **J)** BAL IL-17A - HDM, **K)** Lung eotaxin-2 - HDM, **L)** Serum MCPT1 - HDM. Data from the HDM and ALT studies are pooled from 2 independent experiments. n=3-12 mice per group, apart from panel D-F where n=1 experiment. Kruskal-Wallis analysis with Bonferroni correction was used to determine statistical significance.

Taken together this data shows that mice deficient in T_{FH} have worsened AAD phenotype characterised by increased AHR, eosinophilia, $GATA3^+CD4^+$ T cells and type 2 cytokines. Overall, this implies T_{FH} to have two major roles during chronic allergic disease, both critically driving humoral responses and regulating type 2 responses in a cell extrinsic manner.

6.4. Discussion

The aim of this chapter was to understand the role that T_{FH} , IL-17A and lung resident GC responses play in the development of chronic allergic disease. The data presented in this chapter clearly showed that T and B cells in the lungs exist in large lymphoid aggregates with B and T cell zones and FDC networks. These structures contain GCs that actively undergo CSR and differ from their mLN counterparts in their antibody repertoire, being unique sites of IgA switch and memory. IL-17A deficiency appeared to have a subtle role in B cell aggregation and memory B cell generation. But the formation of local and systemic GCs and the subsequent allergen specific antibody responses was dependent on T_{FH} . T_{FH} were found to be critical regulators of chronic allergic airway disease and in their absence animals displayed a worsened type 2 disease phenotype.

6.4.1. T and B cells form TLS during chronic allergic airway

From this thesis it is clear that lung T_{FH} and GC B cell responses form after aero-allergen exposure. Therefore, one of the key aims of this chapter was to examine whether these T and B cells organise into lung resident TLS similar to observations in other respiratory disorders (Hogg et al., 2004, Moyron-Quiroz et al., 2004, Rangel-Moreno et al., 2006, Ulrichs et al., 2004). In this study, after allergen treatment there is a large infiltration of T and B cells into the lung tissue where they form large lymphoid clusters. These large clusters form around large airways and blood vessels rather than within the parenchymal space, suggesting that similar to iBALT these structures form at specific sites (Randall and Mebius, 2014). B cells represent the predominant lymphocyte in these clusters and most of the B cells in the allergic lung are incorporated into these lymphoid structures. While, most lung B cells in these structures are naïve, a central core of highly proliferative $Ki67^+$ or $GL7^+$ B cells are present, along with FDC networks indicating the presence of lung resident GCs. $CD4^+$ T cells are predominantly identifiable around the outer perimeter of these B cell clusters but are also amongst the B cells themselves. Therefore, after allergen exposure large structures with defined T and B cell

zones and GCs, reminiscent of SLOs and suggestive of iBALT form within the lung tissue at peribronchial and perivascular locations, consistent with observations in other chronic diseases, including COPD, TB and RA (Rangel-Moreno et al., 2011a, Rangel-Moreno et al., 2006, Slight et al., 2013, Hogg et al., 2004) .

The development of these large lung resident structures during chronic AAD is partly dependent on access to a circulating pool of naïve B and T cells. Therefore, when lymphocyte egress is stopped during allergen re-challenge using FTY720, circulating lymphocyte populations severely fall and the resulting B cell clusters are considerably smaller. This indicates that for large full sized TLS to form, lymphocyte recruitment into the lungs must occur. However, the fact that the structures still form in the absence of lymphocyte entry into the lungs highlights the presence of a resident pool of cells capable of driving the formation of these structures.

The organisation of T and B cells into specific areas normally involves the interplay of ectopically expressed homeostatic chemokines secreted by stromal or reticular cells. CXCL13 is important for B cell organisation in iBALT structures in humans and mice and can be secreted by pulmonary fibroblasts and/or FDCs (Rangel-Moreno et al., 2007, Hwang et al., 2016, Eddens et al., 2017). As the lung TLS contain FDC networks and lung CXCL13 is detectable in this model, it is easy to suggest the FDCs to be the source of this chemokine. However, as aeroallergen exposed T_{FH} deficient animals still have lung resident B cell aggregates despite lacking FDC networks this suggests another source of CXCL13 exists, such as the pulmonary fibroblasts consistent with work by Eddens and colleagues (Eddens et al., 2017). Thus, both the location and the identity of CXCL13⁺ cells needs to be assessed along with the location of CXCL13 deposits. Overall, although, not all iBALT features were examined in this study such as chemokine networks or the presence of HEVs, it is clear that structures suggestive of iBALT form in the lungs of chronic allergen exposed animals.

Given the finding of CSR transcripts within lung B cells, it is easy to presume that this event is occurring within local lung GCs that form in these TLS. Sorted lung B cells had detectable levels of either SCT or GLT for IgE and IgG1 at similar or higher levels than their mLN

counterparts. This is particularly interesting because local *IgE* SCT have been found in bronchial biopsies from asthmatic patients (Takhar et al., 2007), while *IgE*⁺ germinal centres have been identified in an allergic patient (Slavin et al., 1992). Although, *IgE* GLT are detectable in the murine lung in this model they are found at a greater level in the mLN indicating *IgE* switch to occur at a higher scale within the mLN. It is worth noting that germline transcription occurs prior to the physical switching process and therefore cannot define that successful switch has taken place which can only be confirmed by the detection of SCT (Chaudhuri and Alt, 2004). Interestingly, *IgA* SCT is uniquely identifiable in lung B cells indicating *IgA* switch to occur within this site. Alongside this, *IgA*⁺ GC B cells and memory B cells are exclusively seen in the lungs, but not the mLN, indicating the lungs to represent a particularly important site for *IgA* generation during chronic AAD. Taken together, it is easy to presume this *IgA* switch is occurring in GC B cells in these lung TLS, allowing the generation of local memory *IgA*⁺ B cells and eventually a long-lasting supply of *IgA* via antibody secreting plasma cells. This fits with *IgA* being detectable within the BAL and lung tissue following allergen exposure in this study. However, it should be noted that although the formation of local GC and generation of SCT suggests switch to occur at these sites the possibility of these events occurring outside of the GC cannot be ruled out.

IgA is important at mucosal surfaces and has protective roles during infection to neutralise viruses, bacteria and toxins, preventing their adhesion to mucosal surfaces and limiting their entry into mucosal sites (Macpherson et al., 2008). *IgA* has also been implicated to be protective in allergic disease and treating mice with allergen specific *IgA* can protect them from AAD symptoms, including eosinophilia and AHR (Schwarze et al., 1998). Consistent with this one of the hallmark features of successful AIT is the generation of allergen specific *IgA* (Scadding et al., 2010, Pilette et al., 2007). Therefore, this local source of *IgA* has the potential to have protective roles during allergic disease, attempting to prevent or resolve disease.

It is worth noting that one of the particularly interesting findings from these study is that the lungs contain a large pool of memory B cells. Both *IgG1*⁺ and *IgA*⁺ memory cells are identifiable by flow cytometry suggesting that *IgE*⁺ memory cells may also exist. Recently, a study using

model antigens found the lungs to be a long lasting reservoir of antigen specific memory B cells ready to respond upon secondary challenge (Vu Van et al., 2016). A lung resident memory B cell population also exists within ectopic lymphoid structures in response to viral infection providing a rapid source of IgG and IgA plasma cells during secondary infection (Adachi et al., 2015, Onodera et al., 2012, Joo et al., 2008). Although, true memory B cells have not been analysed in this thesis using markers such as PDL2, CD73 and CD80, in chapter 3 following a period of rest lung resident IgG1⁺CD38⁺GL7⁻ B cells could still be identified by flow cytometry suggesting memory B cells to remain in the lungs long after disease resolution. Given the organisation of almost all B cells within the lymphoid aggregates following antigen exposure, it is expected that these lung memory B cells will also reside within the large lung TLS. However, in this chapter B cell aggregates disappear after allergen cessation suggesting memory B cells to relocate themselves within the lung when the TLS are no longer present. It is possible that lung resident memory B cell population reside within the or below the lung epithelium like TRM T cell populations (Takamura, 2018). This would enable them to be well positioned to respond to antigen upon allergen rechallenge and differentiate into antibody secreting plasma cells.

As the lung GC B cells phenotyped using flow cytometry in this thesis clearly reside within these organised structures, it is expected that lung T_{FH} would also be found here. Indeed, in previous chapters, Bcl6⁺ CD4⁺ T cells are readily identifiable by flow cytometry in the lungs, and their numbers correlate with the frequency of lung GC B cells. Despite this however, attempts to stain for Bcl6⁺ CD4⁺ T cells in PCLS and image them by confocal microscopy have proven challenging. This could potentially due to high levels of auto-fluorescent extracellular matrix present in the lung, combined with lower expression of Bcl6 by lung T_{FH} compared to their lymphoid counterparts. However, traditionally T_{FH} are found in close proximity to B cells to facilitate their role in driving antibody responses, in particular due to high expression of CXCR5 which allows them to follow CXCL13 gradients (Vinuesa et al., 2016, Kim et al., 2001, Breitfeld et al., 2000, Schaerli et al., 2000). Furthermore, it is easy to presume that the T cells found amongst the B cells within the structures are T_{FH}, similar to T_{FH} found in lung TLS during

TB (Slight et al., 2013). In the future, more work can be done to try and perfect this staining to attempt to identify these cells or other markers such as CXCR5 in combination with CD4 or CD3 can be used as other groups have done (Slight et al., 2013).

Overall, the data shows allergen exposure to induce the recruitment of naïve T and B cells into the lungs to generate ectopic lymphoid structures complete with GCs, FDCs and a memory B cell pool. These lung TLS are a site of active class switching generating both protective and pathogenic antibody.

6.4.2. T_{FH} are critical for local and systemic germinal centre formation

T_{FH} are known to be critical for GC and T-dependent antibody responses (Vinuesa et al., 2016). During chronic AAD mice with Bcl-6 deficient CD4⁺ T cell do not have local or systemic T_{FH} populations indicating lung resident T_{FH} require Bcl-6 for their development like bona fide SLO T_{FH} (Johnston et al., 2009, Nurieva et al., 2009, Yu et al., 2009). T_{FH} deficient animals critically do not develop GCs within SLOs or within the lung TLS. Although lung GC formation is T_{FH} dependent, lung B cell clustering still occurs in their absence although aggregates do appear smaller. This suggests B cell recruitment and clustering to be a largely T_{FH} independent while T_{FH} are absolutely required for GC formation.

Alongside this loss in GCs, both local and lung resident switched memory B cells and plasma cells are absent in T_{FH} deficient animals and these mice also severely lack both local and systemic IgE and IgG1. This data is similar to studies by other groups showing T_{FH} to be essential in the production of these immunoglobulins (Kobayashi et al., 2017, Noble and Zhao, 2016). However, neither of these studies examine the impact of T_{FH} deficiency on mucosal allergen specific IgA. Here, T_{FH} deficiency results in a dramatic fall but not complete ablation of BAL IgA. This implies that during chronic AAD a significant proportion of IgA generation is TD, driven by the action of T_{FH}, but there remains a source of T_{FH} independent IgA. Overall, TD IgA is generally of higher affinity and has longer latency than that produced by TI mechanisms and therefore is probably critical for lung host defence (Cerutti, 2008).

Although, T_{FH} deficiency impairs local and systemic humoral immunity during chronic allergic disease, it is difficult to distinguish the contribution of the lung T_{FH} response versus the more typical mLN response. Therefore, to understand this further the drug FTY720 was given during allergen re-challenge to examine the impact of excluding systemic lymphocytes including T_{FH} and B cell populations from the recall response (Matloubian et al., 2004). Interestingly, upon allergen re-exposure both groups exhibit a similar allergen recall response, displaying cellular increases in the lung and BAL, and a similar trend upwards in AHR (Cates et al., 2004, Fattouh et al., 2005). Thereby, indicating the lung resident population to sufficiently drive these allergen rechallenge responses. However, no change in GC populations or antibody generation occurs upon allergen re-challenge regardless of lymphocyte egress status, making it difficult to conclude on whether the local response was sufficient to drive humoral antibody responses. Lung T_{FH} unlike their mLN counterparts are uniquely capable of producing IL-17A. Given the critical roles that IL-17A plays in iBALT formation in other models (Fleige et al., 2014, Rangel-Moreno et al., 2011a, Al-Kufaidy et al., 2017) and the probable location of allergen induced T_{FH} within the lungs, it was hypothesised that T_{FH} derived IL-17A could help drive TLS formation. This is particularly interesting because the transfer of T_{FH17} cells into T cell deficient animals can drive iBALT formation far better than Th17 cells (Rangel-Moreno et al., 2011a). Nonetheless, IL-17A deficient animals lack IL-17A⁺ CD4⁺ T cells in their lungs along with IL-17A⁺ T_{FH} and although IL-17A⁺ $\gamma\delta$ T cells were not assessed they are also expected to be absent. Despite the loss of IL-17A these mice did not appear to have major deficiencies in lung GC formation, but did have a mild reduction in lung memory B cell populations and lung B cell aggregates were smaller in its absence. This suggests that IL-17A could be playing a role in memory B cell generation during AAD, potentially by promoting B cell differentiation. This aligns with the cytokine being shown to drive B cell differentiation during autoimmune disease (Subbarayal et al., 2016). Furthermore, this also indicates that although IL-17A is not essential for B cell aggregation and iBALT formation as shown during other models, including TB, LPS exposure and viral infection (Rangel-Moreno et al., 2011a, Fleige et al., 2014, Gopal et al., 2013), this cytokine must be playing a subtle role in either maintaining or continuing the

B cell aggregation process. This is different from work by Rangel-Moreno and colleagues who show that IL-17A is important for the initiation but not the maintenance of iBALT formation in their LPS exposure model, and instead show LT α to be important for both stages (Rangel-Moreno et al., 2011a). The role of LT signalling has not been examined in this thesis but potentially could have a more critical role in lung TLS formation than IL-17A.

Although, IL-17A leads to subtle changes in B cell aggregation and memory B cell populations it did not alter systemic or local antibodies. Recently, Eddens and colleagues show that iBALT structures driven by pneumocystis infection are dependent on CXCL13 production by pulmonary fibroblasts, stimulated by the synergistic action of IL-13 and IL-17A (Eddens et al., 2017). Therefore, it is possible that in the heavy IL-13 environment in the lungs of allergen exposed mice, IL-17A deficiency is not sufficient to disrupt lung GC formation, consistent with data from IL-13 KO animals in chapter 5 which still have normal GC B cell responses. Thus, loss of both may be required to completely abrogate lung GC formation.

Overall, following allergen exposure, T_{FH} are critical for lung and mLN humoral responses by driving the formation of SLO and lung resident GCs. In their absence, there are no antibody secreting mLN and lung plasma cells and there is a severe defect in allergen specific antibody production. Although, lung T_{FH} uniquely express IL-17A, this appears to not be critical for lung resident GC formation but potentially plays more subtle roles in B cell aggregation and B cell differentiation during disease.

6.4.3. T_{FH} regulate chronic allergic airway disease

The impact of T_{FH} deficiency on hallmark features of allergic disease was also examined. Alongside having a dramatic defect on allergen specific antibody generation, T_{FH} deficient mice also display severe increases in their allergic disease symptoms. To start with these mice display exaggerated AHR during both ALT and HDM driven AAD compared to their respective wild type littermate controls. The AHR exacerbation in T_{FH} deficient animals is likely due to the dramatic increase in type 2 immune responses in these animals following allergen exposure.

T_{FH} deficient animals show increases in eosinophilia in the lung and BAL, along with mLN and lung GATA3⁺ Th2 cells, the type 2 cytokines IL-4, -5 and 13 and the eosinophil chemoattractant, eotaxin-2. Thus the increase in AHR is probably driven by IL-13 (Grünig et al., 1998, Wills-Karp et al., 1998), while the IL-5 and eotaxin-2 facilitate the increases in eosinophilia (Forssmann et al., 1997, Foster et al., 1996). Lastly the IL-4 may further propagate allergic disease by aiding the differentiation of more Th2 cells (Zhu et al., 2010, Le Gros et al., 1990).

Given the role of BCL-6 as the master regulator of T_{FH} differentiation, partly by actively preventing differentiation down other lineages (Yu et al., 2009, Nurieva et al., 2009, Johnston et al., 2009) it might not be surprising to observe increases in Th1, Th2 or Th17 differentiation in its absence. Type 2 disease skewing has been observed in complete Bcl-6 deficient animals but this has been suggested to be predominantly due to increased secretion of pro-inflammatory cytokines by myeloid cells (Mondal et al., 2010, Toney et al., 2000, Ohtsuka et al., 2011). Indeed this type 2 disease skewing is not observed in mice with a specific loss BCL-6 within CD4⁺ T cells (Hollister et al., 2013). Furthermore, *cd4^{cre}bcl-6^{fl/fl}* despite naïve CD4⁺ T cells displaying increases in *Gata3* mRNA no skewing is observed in type 2 cytokine secretions or after *in vitro* Th2 differentiation (Hollister et al., 2013). In fact, in this study after 24 hours in anti-CD3/CD28 culture, IL-4 levels decreased in Bcl-6 deficient T cells compared to WT cells (Hollister et al., 2013). Fitting with this Bcl-6 deficient T cells in WT/T-Bcl6^Δ bone marrow chimeras also did not display increases in Th2 differentiation, indicating loss of Bcl6 does not favour cell intrinsic skewing toward Th2 cell fate, even after *in vivo* exposure to aeroallergen.

Furthermore, the mixed bone marrow chimeras do not show a dramatic increase in HDM induced Th17 cells in the mLN or lungs, instead the mLN population show a reduction. This indicates that T cell BCL-6 deficiency does not enhance Th17 differentiation as seen in complete BCL-6 KO animals where BCL-6 deficient macrophages produce high levels of Th17 differentiation promoting cytokines (Mondal et al., 2010). Instead when Th17 responses are induced during chronic AAD it was shown to decrease, more consistent with a shifting immune

response to a more Th2 driven disease. Given that a strong Th17 response is only induced in response to HDM but not ALT but both models show exacerbated allergic disease, this implies the enhanced Th2 response and not the loss of Th17 cells to be the major driver of enhanced AAD. Taken together, the data here shows the T_{FH} deficient animals to display worsened AAD suggesting T_{FH} to act as regulators of type 2 disease.

6.4.4. Concluding remarks

This chapter demonstrates that following allergen exposure TLS reminiscent of iBALT form within the lungs. These are highly organised structures with T and B cell zones complete with active germinal centres and a large memory B cell pool. These structures present a site for local production of IgE, IgG1 and uniquely IgA. The formation of GCs within these structures and traditional SLO is completely dependent on T_{FH} . During allergic disease T_{FH} are critical for driving local and systemic antibody responses and negatively regulating type 2 allergic disease either by production of protective IgA and/or novel regulatory molecules.

Chapter 7: General Discussion

7.1. Summary of findings

T_{FH} are critical regulators of humoral immunity and are essential for fully functioning antibody mediated immunity. T_{FH} are however also implicated in the pathology of several diseases, including chronic infections, autoimmune disorders and immunodeficiencies. T_{FH} balance is critical and thus heightened responses can drive autoimmunity while limited responses result in immunodeficiency (Vinuesa et al., 2016). Given that one of the hallmark features of allergic asthma is the generation of allergen specific IgE, it was important to examine the contribution of T_{FH} to this humoral dysregulation. In particular, understanding whether T_{FH} responses are dysregulated during allergic disease, for example in terms of their phenotype or quantity is critical, in addition to whether T_{FH} can contribute to allergic pathogenesis outside of humoral immunity. It was therefore important to examine these features using a clinically relevant chronic models of AAD which drives airway inflammation, remodelling and AHR (Johnson et al., 2004, Gregory et al., 2009b). Thus the overall goal of this thesis was to study the role of T_{FH} during chronic AAD.

It is now clear that chronic allergen exposure induces both local and systemic T_{FH} responses. T_{FH} responses arise quickly in the lung draining mLN and then within the lungs and spleen with longer exposure times. These cells are bona fide T_{FH} expressing the key phenotypic markers, CXCR5, PD1, BCL-6 and ICOS, while secreting IL-4 and IL-21. However, lung T_{FH} differ from their more traditional mLN counterparts and express Ror γ t and secrete IL-17A. mLN and lung resident T_{FH} exist alongside GC B cells which are dependent on T_{FH} for their generation, and thus T_{FH} are also critical for local and systemic allergen specific antibody production during chronic AAD. Following allergen treatment lung resident T and B cells form large TLS which contain active GCs, where B cells undergo CSR to IgE, IgG1 and IgA. IgA switch is unique to the lung tissue and does not occur in mLN B cells, and therefore IgA⁺ GC B cells and memory B cells are identifiable in the lung tissue but not the mLN. Therapeutic targeting of T_{FH} after the development of chronic AAD using antibody blockade improves key AAD hallmark features, including AHR, cellular inflammation and allergen specific IgE. Thus,

using this antibody blockade method to limit T_{FH} responses could be beneficial to allergic disease patients. Interestingly however, T_{FH} deficiency during the development of AAD highlighted that T_{FH} can also negatively regulate the development of type 2 inflammation during chronic AAD in a cell-extrinsic manner.

7.2. Exploring T_{FH} in the context of allergic airway disease

7.2.1. T_{FH} are found in the lungs during allergic disease

Here it was found that allergen exposure induces T_{FH} populations in the SLOs (mLN and spleen), in addition to the lung tissue, and this lung population has now also been reported by another group (Vroman et al., 2017). Both mLN and lung T_{FH} populations exist alongside GC B cells, memory B cells and plasma cell populations. Furthermore, large lung resident TLS containing GCs form and actively undergo CSR following allergen exposure. From the literature it is known that lymphoid aggregates suggestive of TLS are found in post mortal lung tissue from asthma patients, and they appear larger than their healthy comparators (Elliot et al., 2004). This suggests that chronic lung inflammation could be driving the formation of these structures as in other chronic diseases such as COPD, RA or TB (Hogg et al., 2004, Rangel-Moreno et al., 2006, Ulrichs et al., 2004). T_{FH} and GC B cell populations accumulate over time as chronic AAD develops, therefore this accumulation could play a role in why aggregates are larger in asthmatics than healthy individuals (Elliot et al., 2004). Consistent with this although not formally quantified in this thesis, B cell aggregates which form in the lungs after 5 weeks of allergen exposure appear larger and more frequently than those forming after 3 weeks. Therefore, although it is particularly hard to examine human T_{FH} populations due to their tissue localisation, it is expected that in allergic asthma patients T_{FH} will be found within SLOs and lung TLS.

7.2.2. T_{FH} are critical for antibody production during allergic disease

It is clear that T_{FH} are critical for the generation of humoral immune responses during chronic allergic disease. Firstly, mLN, lung and splenic T_{FH} and GC B cell frequencies and numbers

positively correlate during allergic disease. T_{FH} depletion during ongoing disease decreases GC B cell populations and T_{FH} deficient animals fail to generate GC B cell populations. Thereby indicating that similar to protective immunity studies, protein immunisation and disease models, GC B cell responses are highly dependent on T_{FH} (Baumjohann et al., 2013, Hollister et al., 2013, Vinuesa et al., 2016).

Secondly, during allergic disease, T_{FH} are critical for IgE generation and thus T_{FH} deficient animals fail to generate allergic specific IgE both within the lung tissue and the serum. Thus fitting with work from other groups showing impairment in IgE in the absence of T_{FH} (Noble and Zhao, 2016, Kobayashi et al., 2017) and with human data showing IL-4⁺ and IL-21⁺ Derp1 specific c T_{FH} in asthmatics to positively correlate with serum IgE (Gong et al., 2018).

Thirdly, T_{FH} do not just drive allergen specific IgE responses. T_{FH} deficient animals have severe defects in their mLN and lung memory B cell and plasma cells populations, in addition to allergen specific IgG1 and IgA. While allergen specific IgG1 is completely TD in this model, airway allergen specific IgA levels are dampened but not completely lost. Thus, indicating TI IgA responses exist in the model, potentially via innate immune cells, such as neutrophils, monocytes and mast cells producing innate cytokines, such as BAFF and APRIL or via cell to cell interactions (Puga et al., 2012, Vinuesa and Chang, 2013, Merluzzi et al., 2010). Interestingly, the type of antibody driven by T_{FH} drive appears to vary depending on the site with both the mLN and lung population driving the production of IgG1 and IgE, and the lung additionally generating IgA. Nonetheless, it is clear that during allergic disease, chronic allergen exposure induces T_{FH} population which drive humoral immune responses resulting in the generation of allergen specific antibody responses. T_{FH} and their role in humoral immunity during chronic AAD is summarised in **Figure 7.1**.

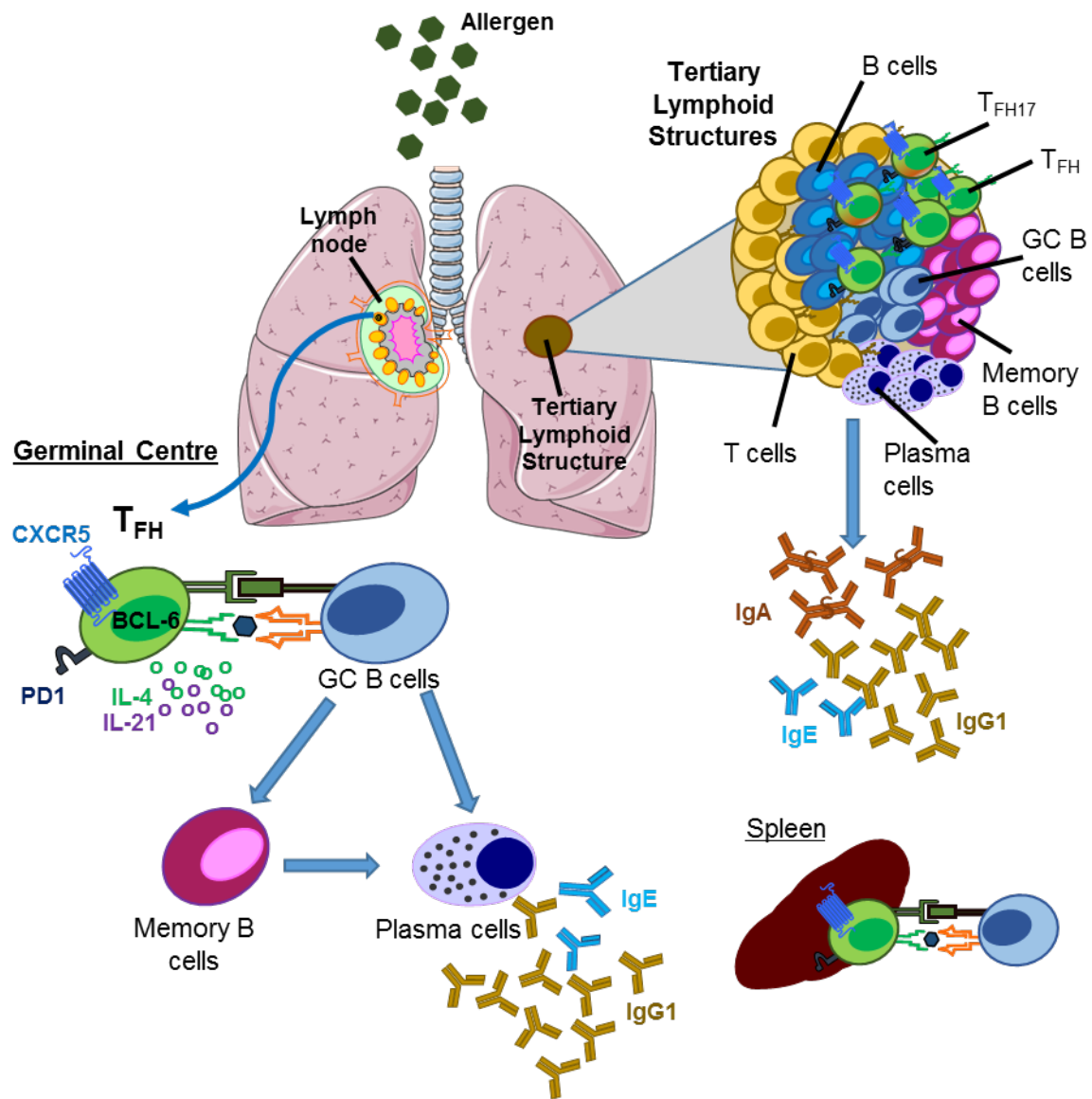


Figure 7.1: T_{FH} and their role in humoral immunity during chronic allergic airway disease

T_{FH} populations are induced in the lung draining mediastinal lymph node (mLN) and lung tissue. T_{FH} are first induced in the mLN where they reside within the B cell zone and interact with germinal centre (GC) B cells within GCs. T_{FH} drive the proliferation and differentiation of GC B cells into memory B cells and plasma cells which go on to drive the production of allergen specific IgE and IgG1. In the lung tissue allergen exposure drives the generation of tertiary lymphoid structures, complete with T cell and B cell zones. Here, GC B cells, memory B cells, plasma cells and T_{FH} populations reside. Within the lungs, T_{FH} again interact with GC B cells and drive the eventual generation of allergen specific IgA in addition to IgE and IgG1. Splenic T_{FH} and GC B cells are also induced during chronic AAD.

7.2.3. T_{FH} are not dysregulated Th2 cells driving type 2 disease

Whether allergen exposure drives the induction of dysregulated T_{FH} populations capable of contributing to allergic disease pathogenesis is a key question of this thesis. Here, both local and systemic T_{FH} populations express the canonical T_{FH} markers of CXCR5, PD1, Bcl-6 and ICOS (Vinuesa et al., 2016). Similar to stereotypical T_{FH}, allergen induced T_{FH} are also dependent on Bcl-6 expression for their generation (Johnston et al., 2009, Nurieva et al., 2009, Yu et al., 2009) and require continuous ICOS signalling to maintain their phenotype even during ongoing disease (Weber et al., 2015, Choi et al., 2011). Furthermore, they secrete the signature cytokines, IL-4 and IL-21 (Vinuesa et al., 2016) and thus allergen exposure induces “bona fide” T_{FH} populations.

One way T_{FH} dysregulation could occur during allergic disease is by T_{FH} taking on features of Th2 cells such as GATA3 expression and production of type 2 cytokines, including IL-5 and IL-13, thereby driving allergen specific IgE and allergic disease. This is particularly interesting given studies show that the context of T_{FH} induction determines the effector cytokines T_{FH} produce and the isotype switching they drive (Reinhardt et al., 2009). Thus, a Th1 environment drives IFN- γ and IgG2a switch (Luthje et al., 2012) and helminth infection drives *gata3* expression in T_{FH}, in a model that favours IgG1 and IgE (Zaretsky et al., 2009). In AAD, following allergen exposure, T_{FH} do not take on a T_{FH2} phenotype and thus do not express GATA3 or produce IL-5, -9 or 13. Indeed, CXCR5 and GATA3 expression are mutually exclusive in CD4⁺ T cells, and CCR4⁺ T_{FH} populations do not develop during chronic AAD. These observations are true for both mLN and lung T_{FH} indicating that skewing towards T_{FH2} is not the mechanism by which T_{FH} are driving IgE production. In support of this; allergic rhinitis patients show T_{FH} skewing to cT_{FH2} (Kamekura et al., 2015, Yao et al., 2018), although no further skewing occurs in the blood of allergic rhinitis patients with co-morbid asthma, suggesting this to be a feature of allergic rhinitis (Kamekura et al., 2015).

The separation of T_{FH} and Th2 cell lineages is particularly interesting when examining this thesis in the context of work by other groups using murine models of AAD. Ballesteros-Tatos

and colleagues suggest IL-4⁺ T_{FH} generation to occur prior to Th2 cells in the mLN, and these mLN T_{FH} to differentiate into Th2 cells which migrate to the lungs and drive allergic disease (Ballesteros-Tato et al., 2016). However, chronic allergen exposure does not appear to drive a Th2 cell population which are critically dependent on T_{FH} for their generation. Instead, Th2 populations increase in the absence of T_{FH}, alongside other hallmarks of allergic disease, including airway inflammation and AHR. Furthermore, chronic allergen exposure also induces lung T_{FH} populations showing Th2 cells to not be the only IL-4⁺ population in the lung tissue. Thus, data from this thesis appears to be in disagreement with the Ballesteros-Tatos study (Ballesteros-Tato et al., 2016) and instead aligns more with the work of Hondowicz and colleagues whom show Bcl-6 KO bone marrow deficient animals to have increases in lung TRM Th2 cells (Hondowicz et al., 2016). Alongside this, Coquet and colleagues show neither the transfer of IL-21⁺ nor IL-21⁻ T_{FH} to generate Th2 cells (Coquet et al., 2015), consistent with others showing T_{FH} transfer to not drive IL-5 or IL-13 production (Kobayashi et al., 2017). Concomitantly, Liang and colleagues show IL-4 producing CD4⁺ T cells that can or cannot co-produce IL-13 after helminth infection, and show IL-13 co-producing cells to reside in the lungs but not the mLN where they find their T_{FH} population (Liang et al., 2011). Thus, our data appears to align more with the latter studies showing a T_{FH} to not act as Th2 cell precursors and actually suggests T_{FH} to regulate rather than drive allergic airway disease.

During chronic AAD, a subpopulation of lung T_{FH} populations take on features of Th17 cells, expressing Ror γ t and producing IL-17A. Thus, demonstrating a link between T_{FH} and Th17 rather than Th2 cells at this site. This is particularly interesting given the differentiation pathways of T_{FH} and Th17 cells are quite similar, with both relying on IL-6, but Th17 cells additionally requiring TGF- β and IL-23 signalling (Bettelli et al., 2006, Veldhoen et al., 2006, Mangan et al., 2006, Nurieva et al., 2008). Furthermore, both are reliant on STAT3 signalling (Nurieva et al., 2008, Laurence et al., 2007, Yang et al., 2007) and their differentiation is limited by IL-2 (Laurence et al., 2007, Ballesteros-Tato et al., 2012, Johnston et al., 2012). Thus, it is feasible to speculate that there is potential for cells to switch between the two lineages easily

and these T_{FH17} cells to represent an intermediate population. This is not the first account of T_{FH17} cells *in vivo* and these cells are found in the PPs of mice and are suggested to differentiate out of the Th17 cell lineage (Hirota et al., 2013). Consistent with this idea of plasticity, in the strong Th17 inducing environment of HDM driven AAD, T_{FH} deficiency also results in clear reductions in Th17 cells. Furthermore, the transfer of *in vitro* differentiated T_{FH17} into T cell deficient animals drives more efficient iBALT formation than the transfer of Th17 cells (Rangel-Moreno et al., 2011a). This is interesting given that during chronic AAD IL-17A appears to have a subtle effect on B cell aggregation. Thus, it is possible that given the proximal location of the lung T_{FH17} and their ability to help B cells that these T_{FH17} cells could be propagating further B cell aggregation within the lungs. Nonetheless, exploring the relationship between T_{FH} and Th17 cells further in allergic disease could be of interest.

Overall, it is clear that allergen exposure induces T_{FH} populations locally and systemically that do not resemble Th2 cells, and thus although they drive IgE production, they do not directly drive type 2 disease via production of IL-5 or IL-13. However, lung T_{FH} and Th17 cells share characteristics and may have a reciprocal relationship. The relationship between T_{FH} and other CD4⁺ T cells during chronic AAD is outlined in **Figure 7.2**.

7.2.4. Do T_{FH} drive or regulate allergic disease?

Another interesting finding from this thesis is that although interfering with T_{FH} responses prior to allergic disease results in exacerbations in type 2 disease, the opposite is true when T_{FH} depletion occurs after disease establishment. The studies with complete T_{FH} deficiency utilise *cd4^{cre}bcl-6^{fl/fl}* animals which are incapable of generating T_{FH}, while the therapeutic studies use antibody mediated ICOS-L blockade, which aims to selectively deplete T_{FH} after disease establishment. From a technical point of view the *cd4^{cre}bcl-6^{fl/fl}* mouse more selectively excludes T_{FH} from the model and thus the differences in the results could simply result from one targeting T_{FH} with less side-effects on other cellular populations. However, there is also the potential that the timing of the T_{FH} response is critical to the role of this cell during AAD.

The *cd4^{cre}bcl-6^{fl/fl}* studies examine the role of T_{FH} in the initiation of allergic disease, while the ICOS-L blockade studies look at the role of T_{FH} once disease is already present. Thus, it is possible that T_{FH} only negatively regulate allergic disease during the initiation phase and once you are past that point T_{FH} no longer fulfil this role. This is interesting given that the Ballesteros-Tato study shows that depleting T_{FH} responses during allergic sensitisation severely impairs type 2 disease, while inferring after sensitisation has no impact (Ballesteros-Tato et al., 2016). Although, these authors suggest this to be due to T_{FH} providing precursor cells for Th2 cells, which does not support the data from the chronic allergen exposure studies carried out here, it does suggest the timing of T_{FH} response to be critical during allergic disease.

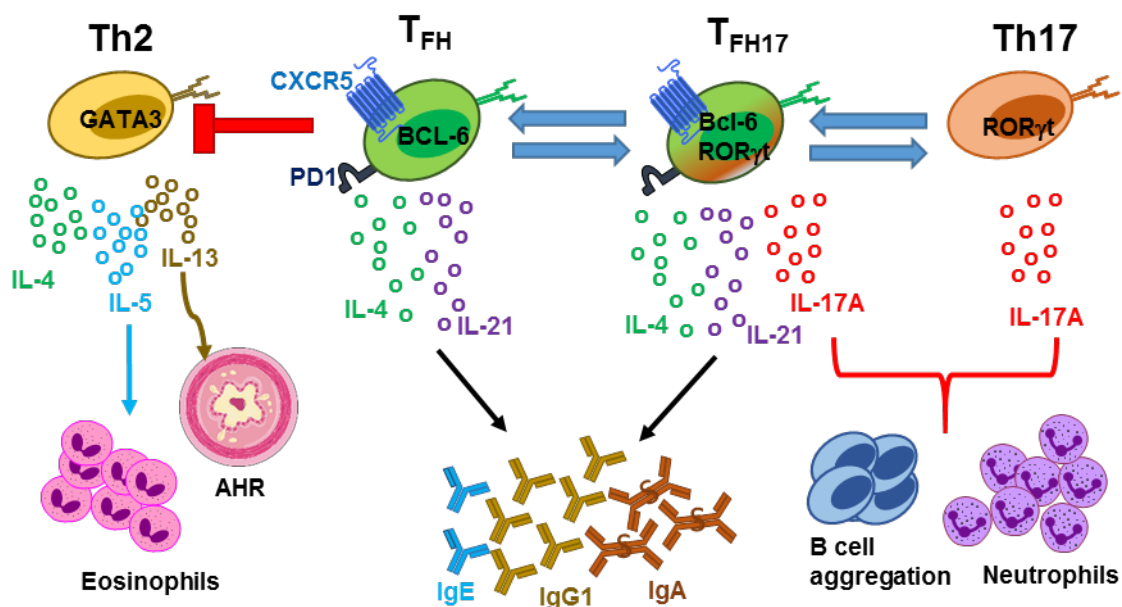


Figure 7.2: T_{FH} and their relationship with other CD4⁺ T cells

Lung and mLN T_{FH} express CXCR5 and PD1 and are dependent on Bcl-6 for their generation. They also secrete IL-4 and IL-21, and are critical for driving antibody production. T_{FH} and Th2 cells are distinct cells, and T_{FH} neither express GATA3 nor secrete IL-5 or IL-13. T_{FH} do not appear to provide precursor cells for Th2 cell differentiation and instead limit Th2 cell responses. In contrast, Th2 cells do express GATA3 and secrete IL-4, 5 and 13, driving allergic airway disease. However, lung T_{FH} contain a subpopulation of T_{FH17} cells expressing ROR_{γt} and secreting IL-17A, suggesting the presence of an intermediate population between T_{FH} and Th17 cells. This implies cells may be able to transition between these two cell types during allergic airway disease. IL-17A appears to play a subtle role in B cell aggregation and drives neutrophilia during disease.

7.2.5. How do T_{FH} regulate chronic allergic disease?

T_{FH} deficient animals with chronic AAD show T_{FH} to play a role in suppressing Th2 biased inflammation, with allergen treatment of T_{FH} deficient animals enhancing type 2 disease, as characterised by increased AHR, eosinophilia, Th2 cells and type 2 cytokines. Bcl-6 deficient bone marrow chimera studies show that this T_{FH} regulation of Th2 cell differentiation occurs in a cell-extrinsic rather than an intrinsic Bcl-6 dependent manner. This leaves key remaining questions such as, how do T_{FH} regulate allergic disease? Is this regulation of type 2 disease due to loss of T_{FH} mediated humoral immunity or via a novel mechanism of T_{FH} action? Or both?

To start with T_{FH} deficiency results in loss of local and systemic GCs and plasma cells along with allergen specific IgG1, IgE and IgA. In humans IgE is generally considered a driver of allergic disease. However, in the mouse model, it is well known that *Aspergillus*, OVA or HDM exposed IgE^{-/-} mice still develop allergic disease (Mehlhof et al., 1997, Oettgen et al., 1994, McKnight et al., 2017). This is partly because although mouse models are ideal for analysing allergen driven IgE generation, in mice IgE does not appear to contribute to the pathology. This is obviously different to allergic asthma patients where reductions in IgE can be highly beneficial and the anti-IgE monoclonal antibody, omalizumab has proven to be a successful treatment for some patients with inadequately controlled asthma (Humbert et al., 2014). Nonetheless, as reducing IgE is generally a sign of good therapeutic intervention or disease improvement, it is unlikely to be a driver of the worsened type 2 disease seen in T_{FH} deficient animals. Consistent with this, MCPT1 levels are higher in T_{FH} deficient animals, indicating that the downstream events of mast cell degranulation usually considered a consequence of IgE are still occurring via IgE independent mechanisms, such as TLR stimulation (Oettgen et al., 1994, Yu et al., 2016).

IgG1 is also severely reduced in allergen exposed T_{FH} deficient (*cd4^{cre}bcl-6^{fl/fl}*) mice, but similar to IgE, allergen specific IgG is generally linked to pathology and thus its loss is unlikely to be beneficial (Williams et al., 2012). Interestingly, T_{FH} deficient animals have a dramatic reduction

in IgA levels. IgA is known to function at mucosal sites to neutralise bacteria, viruses and toxins (Macpherson et al., 2008). Given the findings here that IgA is uniquely produced locally within the lungs following aero-allergen exposure in a T_{FH} dependent manner, it is tempting to speculate that IgA could function to neutralise allergen components prior to their interaction with the epithelial barrier where they are known to cause damage and drive allergic disease. This implies that T_{FH} could regulate allergy disease via production of IgA and thus any reduction in this antibody could potentially be an exacerbating factor (**Figure 7.3**). This has precedence because the transfer of allergen specific IgA has been shown to protect animals from AAD symptoms, including eosinophilia and AHR in a ragweed driven model of AAD (Schwarze et al., 1998). Also, severe asthma patients have been shown to have lower levels of IgA (Balzar et al., 2006) and patients with selective IgA defects have increased frequencies of allergic diseases, including asthma and food hypersensitivity (Yel, 2010, Janzi et al., 2009, Urm et al., 2013, Aghamohammadi et al., 2009).

A potential caveat to the protective mechanism of IgA is that OVA/alum (i.p.) sensitised and OVA (i.n.) challenged IgA^{-/-} mice have improved allergic disease, suggesting a pathogenic role for IgA (Arnaboldi et al., 2005). However, it is known that IgA^{-/-} mice have a general defect in class switching making it difficult to unpick the unique contribute of IgA to disease (Harriman et al., 1999, Arnaboldi et al., 2005). Therefore, it would be interesting to examine the contribution of IgA to the type 2 exacerbation in T_{FH} deficient animals.

Outside of their role in humoral immunity, T_{FH} could also be directly dampening AAD by the secretion of regulatory cytokines such as IL-10, which can directly limit Th2 cell differentiation (Coomes et al., 2016). cT_{FH} sorted from human blood and stimulated with anti-CD3 can produce IL-10 (Breitfeld et al., 2000), and PD1⁺ cT_{FH} from both non-cancer and ovarian cancer patients can produce IL-10 after anti-CD3/CD28 stimulation, with higher production from cells from cancer patients (Li et al., 2018). Furthermore, after influenza A infection T_{FH} in mice can produce IL-10 (Luthje et al., 2012), implying T_{FH} to be capable of producing IL-10 during disease. In chronic AAD the production of regulatory cytokines would allow T_{FH} to directly drive

the suppression of Th2 cells cell-extrinsically, and they could regulate disease by a cell-to-cell interaction using co-inhibitory molecules (**Figure 7.3**).

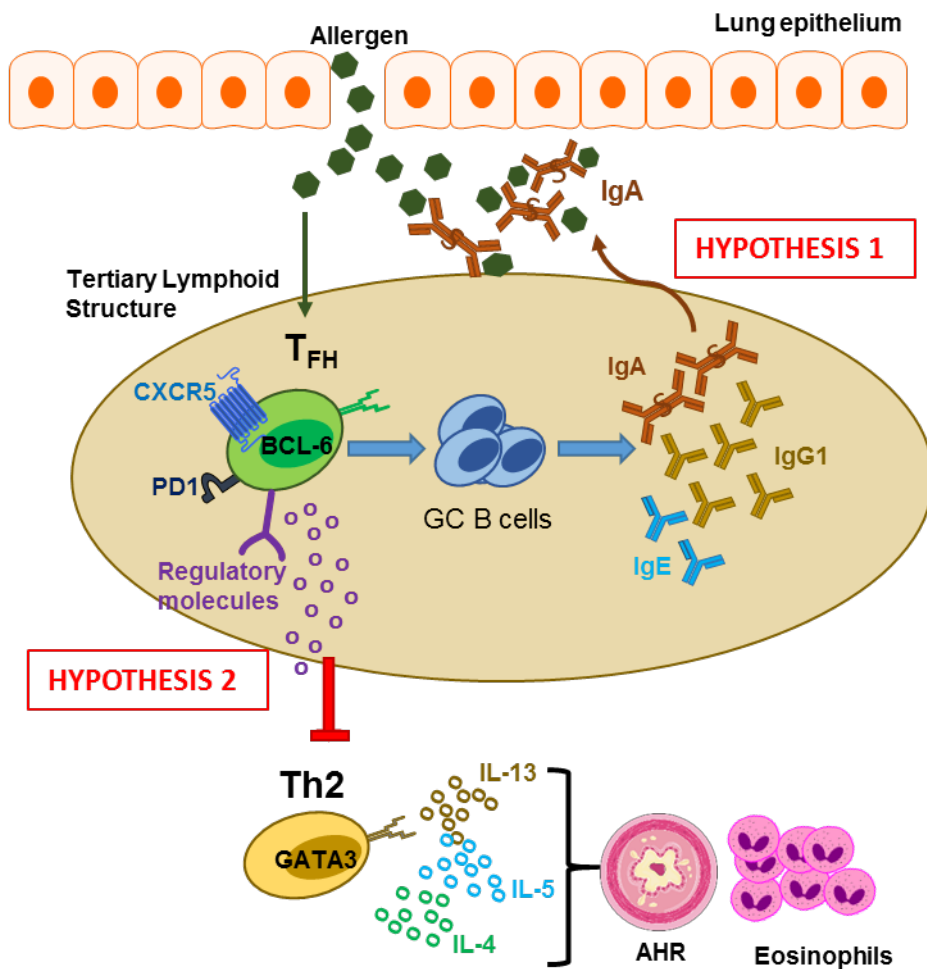


Figure 7.3: Potential mechanism of T_{FH} mediated regulation of type 2 inflammation
Allergen exposure drive T_{FH} populations and the formation of tertiary lymphoid structures within the lung tissue. **Hypothesis 1:** T_{FH} populations drive local IgA production within the lung tissue able to dampen allergic disease by neutralising allergens and limiting their ability to drive allergic disease. Thus loss of T_{FH} driven allergen specific IgA drives worsened allergic disease in T_{FH} deficient animals. **Hypothesis 2:** T_{FH} produce regulatory molecules, such as cytokines or co-inhibitory molecules that limit Th2 cell differentiation, preventing their ability to drive type 2 disease. Thus loss of T_{FH} mediated cytokine or cell to cell regulation drives worsened allergic disease in T_{FH} deficient animals.

Lastly, *cd4^{cre}bcl-6^{fl/fl}* animals are not just deficient in T_{FH} but also T_{FR} which are also reliant on Bcl-6 for their differentiation (Linterman et al., 2011, Chung et al., 2011), therefore the contribution of these cells cannot also be ruled out. However, given that the role of T_{FR} is to regulate T_{FH} and GC B cell responses, it is unlikely that results would be different if T_{FR} were still present. However, Tregs themselves are important regulators of AAD protection and their transfer can dampen disease via production of IL-10 (Kearley et al., 2005). Nevertheless, while the frequency of T_{FH} dramatically increases during AAD the frequency of T_{FR} remains roughly similar, and is particularly low in the lungs themselves, limiting the likelihood they play a substantial role. But this could be understood further by using *foxp3^{cre}bcl6^{fl/fl}* animals which exclusively lack T_{FR} without impacting T_{FH} populations (Wu et al., 2016).

7.2.6. How important is local immunity?

A key question that arose in this thesis was the importance of the local humoral response in comparison to the systemic one, and whether its presence was detrimental or protective. It is simple to presume that because TLS structures are larger in asthmatics patients in comparison to healthy individual (Elliot et al., 2004) that they are detrimental. However, asthmatics could develop these structures at any stage during disease for multiple reasons. These structures could develop as an early preventative measure, during pathology to drive disease, during more severe disease or as a late protective measure. This is interesting given that the antibody repertoire varies between the systemic and local response, with the lung response driving IgA production, in addition to IgG1 and IgE.

One thing worth noting from the literature is that iBALT formation occurs more readily in neonatal mice than after weaning or in adulthood (Rangel-Moreno et al., 2011a, Foo et al., 2015). Furthermore, iBALT are frequently found in the lungs of children and infants (Gould and Isaacson, 1993, Ersch et al., 2005, Tschernig et al., 1995). This is particularly pertinent in the context of allergic asthma which most people develop as children (Guerra and Martinez, 2009). Given that iBALT structures develop as a result of an inflammatory stimuli (Hwang et

al., 2016) and there is a link between viral infections in early life and asthma development (Busse et al., 2010) it is feasible that iBALT development early in life could be important. For instance neonatal respiratory syncytial virus (RSV) infection results in type 2 disease skewing when mice are re-infected in adult life, thereby linking infection and type 2 disease (Culley et al., 2002). Taken together, this implies that events driving TLS formation could potentially more important in neonatal mice and children. Thus, further exploration of TLS in the context of age during chronic AAD could be very interesting.

Another important question is whether lung T_{FH} require SLOs for their generation. In particular, it would be interesting to know if T_{FH} are activated in the mLN and travel via the lymphatics and bloodstream to generate local lung GC in these TLS or instead differentiate from other local effector $CD4^+$ T cells or alternatively undergo local T cell priming within TLS. After chronic allergen challenge circulating T_{FH} are not detected in the bloodstream of mice in significant frequencies suggesting this transit route to the lungs to be unlikely. Alternatively, T_{FH} are flexible cells, capable of taking on features associated with other $CD4^+$ T cells and can differentiate from other lineages (Fahey et al., 2011, Harker et al., 2011, Zaretsky et al., 2009, Ballesteros-Tato et al., 2016, Hirota et al., 2013), therefore differentiation from other lineages may be feasible. Furthermore, local iBALT formation has been shown to occur in response to influenza A and TB infection in mice lacking both lymph nodes and the spleen (Moyron-Quiroz et al., 2004, Day et al., 2010), indicating that local T cell priming is possible. Following chronic allergen exposure, lung T_{FH} responses are also slow to develop requiring 3 weeks of allergen exposure, in comparison to 1 week for mLN T_{FH} . Therefore, it is possible that local T_{FH} responses due to local priming take longer to develop, presumably due to the time taken to form the ectopic structure or the transit time from the mLN. While work to determine this is beyond the scope of this thesis, given the fact that T_{FH} are critical for humoral immunity, it is logical to presume that the presence of this local T_{FH} response in the lung resident lymphoid structures is driving local GCs and TD antibody switch. Thereby, allowing the generation of the potentially protective high affinity mucosal IgA, in addition to IgE and IgG1.

Another important question is what happens to this local TLS in asthma patients during disease progression and re-challenge. Especially, given allergic asthma patients are initially sensitised by chronic allergen exposure, but may undergo periods of allergen withdrawal and re-challenge throughout their lives. From the literature it is clear that lung allergen specific TRM Th2 cell populations are sufficient to drive allergic disease upon allergen re-challenge (Hondowicz et al., 2016). But the impact that a local lung T_{FH} population makes to memory is harder to unpick. In BALB/c mice allergen re-challenge drives a rapid increase in lung T_{FH}, but when similar challenges were carried out in C57BL/6 mice this did not occur. However, although lung T_{FH} do not rapidly increase in re-challenged C57BL/6 mice, T_{FH} populations are still seen and this is not altered when FTY720 is given to mice to prevent lymphocyte egress. This therefore supports the idea that tissue resident T_{FH} may contribute to secondary responses but their influence may be dependent on background genetics, allergen dosing regime and timing.

It is also clear from the thesis that the formation of B cell aggregates is highly responsive to allergen, with initial aggregation occurring following exposure, disappearing upon allergen cessation, and rapidly re-forming upon allergen rechallenge. As asthmatics regularly experience periods of allergen cessation and re-challenge, or exacerbations following respiratory viral infections (Busse et al., 2010), asthmatics may also have highly dynamic and responsive TLS which could reduce in size and increase depending on the current state of disease. Given that B cell aggregate re-formation can occur in the absence of circulating B cell entry into the lung, this suggests the maintenance of a significant lung resident memory B cell population able to aggregate and potentially drive humoral immunity. Given the lung population drives the generation of not just IgE and IgG1, but also IgA, there is potential for the re-challenge responses to be providing protection, in addition to pathogenic antibodies. The contribution of memory B cell populations to secondary antibody responses could therefore be interesting to examine in future work.

How crucial the systemic response is to allergic disease once the local response is in place is also therefore an important question. It is easy to speculate that the systemic mLN is critical

for providing cells that initially contribute to disease, including Th2, Th17 cells and T_{FH}, driving hallmark symptoms, including airway inflammation, AHR and allergen specific IgE. This could be critical to humoral immunity and explain why the response first develops in the lung draining mLN. Studies using FTY720 in this thesis show similar disease to develop in mice upon allergen rechallenge when lymphoid egress to tissues is prevented, thus indicating the lung cellular population is sufficient to drive disease. Work by other groups show that after OVA or HDM sensitisation, FTY720 treatment in the challenge phase can still drive similar eosinophilia and AHR to that seen in mice when lymphoid egress is intact (Hondowicz et al., 2016, Yeon et al., 2017). Thus, once the lung response is established subsequent re-challenges could be driven by the local lung resident cellular population, including TRM Th2 cells, memory B cells and fast responsive T_{FH} populations. Alongside this, long lived plasma cells which home to the bone marrow should provide a source of circulating antibody repertoire able to drive disease (Luger et al., 2009).

7.2.7. Therapeutic potential of targeting T_{FH}

SLO and lung T_{FH} have not been assessed in asthmatics due to the limited access to these tissues. However, cT_{FH} populations are found in the blood of allergic asthma patients at a higher frequency than healthy controls and these IL-4⁺ cT_{FH} and IL-21⁺ cT_{FH} correlate with total serum IgE levels (Gong et al., 2018). Therapeutic targeting T_{FH} after chronic AAD establishment by administering α -ICOS-L antibodies reduces serum allergen specific IgE levels and decreases hallmark symptoms including airway inflammation and AHR. This finding along with T-Bcl6^Δ data showing the absence of T_{FH} to abrogate IgE responses during chronic AAD, aligns with other studies showing T_{FH} to be critical for allergen specific IgE generation (Kobayashi et al., 2017, Noble and Zhao, 2016). Given the importance of IgE in some patients with allergic asthma this suggests that T_{FH} are contributing to pathology and could represent a significant therapeutic target.

To date few studies examine T_{FH} populations in the context of human allergic asthma (Gong et al., 2018) and given how new T_{FH} are as a $CD4^+$ T cell subset the impact of currently approved monoclonal antibody strategies on T_{FH} populations is not yet known. However, a few studies have now looked at the impact of AIT on T_{FH} populations in allergic rhinitis patients. Interestingly, administration of AIT to patients with allergic rhinitis reduces cT_{FH} proportions in comparison to non-treated patients (Schulten et al., 2017). Furthermore, AIT also decreases $Derp1^+IL-4^+$ cT_{FH} in allergic rhinitis patients with HDM allergy and this reduction in cT_{FH} correlates with symptom improvements and medical scores (Yao et al., 2018). Thus, suggesting T_{FH} to be pathogenic during human allergic disease and a beneficial therapeutic target.

It is however worth noting that one of the hallmarks of successful AIT is the induction of Tregs and cytokine driven skewing of the antibody response away from allergen specific IgE towards IgG4 and IgA (Scadding et al., 2010, Radulovic et al., 2008, Pilette et al., 2007). Furthermore, a Phase IIb clinical trial of a new grass pollen vaccine has just been completed and this also shows an increase in grass pollen specific IgG4 antibody responses after treatment (Niederberger et al., 2018). Although, mice do not produce IgG4, this thesis clearly shows T_{FH} are important for the production of allergen specific IgA. Therefore, whether these immunotherapy based interventions re-educate human T_{FH} populations to drive IgG4 and IgA production is an interesting question. Multiple studies show allergic rhinitis patients to have cT_{FH} population skewed towards a T_{FH2} phenotype (Kamekura et al., 2015, Yao et al., 2018) and these T_{FH2} to drive IgE generation (Yao et al., 2018). Therefore, it is possible that AIT changes the cT_{FH} to alternative phenotypes such as the T_{FH17} cells found in the lungs of allergen exposed mice in this thesis. Alternatively AIT could drive regulatory T_{FR} populations which dampen the T_{FH} responses, as recently suggested by Schulten and colleagues (Schulten et al., 2017).

7.3. Future work

7.3.1. T_{FH} mediated allergic disease regulation

One of the most interesting findings from this thesis is that T_{FH} deficiency during chronic AAD results in disease exacerbation. This is particularly interesting because it shows T_{FH} to be regulators of allergic disease. One of the first experimental proposals is to confirm that T_{FH} are sufficient to prevent this exacerbation by adoptively transferring allergen induced T_{FH} into *cd4^{cre}bcl-6^{fl/fl}* animals and then subsequently allergen exposing these animals. Furthermore, a potential mechanism that is proposed in this thesis by which T_{FH} could regulate allergic disease is via local production of allergen specific IgA. Therefore, it is important to examine whether transfer of IgA into the airways is also sufficient to dampen allergic disease exacerbation in T_{FH} deficient animals. In this study IgA will be purified from the BAL of allergen treated animals and transferred into *cd4^{cre}bcl-6^{fl/fl}* mice with chronic AAD as a therapeutic intervention. The outcome of this transfer on hallmarks of allergic disease will then be assessed. This would fit with work by Schwarze and colleagues showing transfer of allergen specific IgA to protect mice from AAD in a ragweed allergen sensitisation and challenge model (Schwarze et al., 1998). This is consistent with transfer of viral specific IgA conferring protection to mice from Influenza A (H1N1) infection by binding to the virus and reducing their eventual shedding (Renegar and Small, 1991).

Alternatively T_{FH} could be regulating allergic disease via the production of regulatory molecules, such as IL-10. An RNA-sequencing experiment was carried out during the PhD to examine the transcriptional profiles of mLN and lung T_{FH} populations and compare them to their respective T_{EFF} populations. This sequencing has been run but not yet analysed, however this data should allow identification of potential regulatory molecules or novel pathways that T_{FH} could be using to limit disease which can be further explored in mouse models.

Lastly, the distinction in the results when T_{FH} depletion occurs before or during disease is an interesting finding suggesting that timing of the T_{FH} response to be critical to its role. Therefore generating an inducible T_{FH} deficient animal, would allow the removal of T_{FH} both during

disease inception and after establishment. The inducible mouse could be created using either a CD4-DTR (Greczmiel et al., 2017) or tamoxifen inducible CD4cre (Aghajani et al., 2012). Alternatively, if the mouse does not become available, WT and *cd4^{cre}bcl-6^{fl/fl}* animals can be treated with α -ICOS-L antibodies prior to disease and the outcome on allergic disease can be examined in comparison to the effects after disease establishment.

7.3.2. Understanding T_{FH} and TLS responses in neonatal mice

A key question that arises from this thesis is the contribution of the local response to chronic AAD. It is known from the literature that neonatal mice have a type 2 skewing in disease development (Culley et al., 2002) and they possess a greater propensity to develop lung TLS (Rangel-Moreno et al., 2011a). Given that this is the age that most people develop asthma (Guerra and Martinez, 2009), it is important to examine T_{FH} responses in the context of age. The Lloyd lab has a well-established neonatal mouse model, where mice are exposed to allergen from day 3 of life and after 3 weeks develop chronic AAD (Sagani et al., 2009). Utilising this model would allow the study of T_{FH} and TLS during neonatal disease. Exploration of the site and timing of T_{FH} generation in the neonatal response is important, in particular in terms of whether a lung T_{FH} response exists. In an OVA protein immunisation model, neonatal mice generate less SLO GCs than adults, produce less antibody and have more T_{FH} located in the interfollicular region of the mLN than within GC (Debock et al., 2013). This indicates that T_{FH} responses can differ in the neonatal immune response in comparison to adults. Given the propensity for neonates to form TLS and the weakened immune environment in these animals the ability to form local TLS may be critical (Rangel-Moreno et al., 2011a). Thus, examining TLS along with their accompanying GC B cells, memory B cells and plasma cell populations using the neonatal chronic AAD model is critical. Moreover, the heavy skewing of the neonatal immune environment to type 2 disease (Zaghouani et al., 2009) may result in different T_{FH} populations to those seen in after challenge of adult mice. Therefore, it will be interesting to

phenotype neonatal T_{FH} populations. Lastly, whether T_{FH} deficiency still promotes disease exacerbation in early life is also important to determine.

7.3.3. Examining lung and mLN T_{FH} populations

As stated above, during the PhD an RNA sequencing experiment was set up to examine lung and mLN T_{FH} populations and compare them to T_{EFF} cell populations. The plan from this study is compare mLN and lung T_{FH} populations for known T_{FH} markers that could not be assessed during the course of the PhD, such as CD40-L and to determine if there are any differences between these two populations. Furthermore, this sequencing will allow the comparison between T_{FH} and their respective T_{EFF} cell populations and examine how closely related they are to other non-T_{FH} populations. This is would be interesting because T_{FH} can overlap with other Th lineages, such as Th17 cells in the PPs (Hirota et al., 2013) and Th1 cells following influenza A infection (Luthje et al., 2012). Additionally, pathway analysis using Ingenuity will allow the examination of both known and novel pathways of action. The RNA sequencing will therefore allow more in depth analysis of these cellular populations, and thus help further knowledge on T_{FH} biology during chronic AAD.

From a functional point of view, although it is easy to presume based on T_{FH} populations in the mLN and lung tissue expressing many of the same markers by flow cytometry that lung T_{FH} can provide B cell help this has not been confirmed. Therefore, setting up *in vitro* B cell co-cultures with mLN or lung T_{FH} (Cubas et al., 2013, Morita et al., 2011) will allow the examination of their ability to provide help and whether there are variations in the antibody outcome depending on the location that the T_{FH} normally resides.

7.3.4. T_{FH} and B cell memory

Memory B cell populations form during chronic AAD but their contribution and phenotype is not understood. Furthermore, similar to after Schistosoma egg antigen immunisation, mLN T_{FH} populations generated during chronic AAD are long lasting and are found long after

allergen withdrawal (Fairfax et al., 2015). However, following allergen exposure, the longest resolution period in this thesis was 5 weeks, a time period at which the GC response in the mLNs, but not the lungs, is still largely present. Thus, examining memory B cell populations and T_{FH} responses in the absence of an active GC would be interesting. Furthermore throughout this thesis, memory B cells are defined as isotype switched $CD38^+GL7^-$ B cells but have not been further subdivided based on key markers such as CD80, CD73 and PDL2 (Tomayko et al., 2010). This is something that would be interesting to do, in particular to observe how the phenotype of these cells change in different conditions, such as allergen re-challenge. Furthermore, the relative contribution of lung and mLN memory B cells is important to understand. In particular is the transfer of either memory B cell population sufficient to drive or regulate allergic disease. Memory B cell and T_{FH} responses could be studied further by parabiosis (Bunster and Meyer, 1933). Attaching a previously allergen treated animal with an allergen naïve animal, would allow examination of whether T_{FH} and memory B cells are tissue resident after disease resolution, similar to work done with Th2 cells (Hondowicz et al., 2016). Furthermore rechallenge of parabiotic animals will allow examination of the importance of a local response.

7.3.5. Human studies

Although work with human asthmatics examining cT_{FH} populations is now starting to emerge in the literature a lot of work still needs to be done. It is clear that cT_{FH} populations are higher in asthmatic individuals than non-asthmatics (Gong et al., 2018), but these populations have neither been examined for phenotype nor correlated with disease severity. Thus, it would be interesting to examine cT_{FH} proportion in non-asthmatics, and mild, moderate and severe asthma patients to allow cellular frequency and composition to be understood. However, a caveat of cT_{FH} studies is that how these cells relate to bona fide tissue resident T_{FH} is not understood. Although they express CXCR5 and CD4, their expression of other T_{FH} markers, including ICOS and PD1 is variable and they generally do not express Bcl-6 (He et al., 2013b,

Morita et al., 2011, Bentebibel et al., 2013). Furthermore, although cT_{FH} frequencies can correlate with antibody and plasmablast responses during disease or after vaccination (Bentebibel et al., 2013, Gong et al., 2018, Simpson et al., 2010), whether they correlate with GC responses is less clear. Given that the role of cT_{FH} during disease is not truly understood the ideal place to examine bona fide T_{FH} responses is still by examining human tissue which can be hard to acquire. Nonetheless, there is scope to get tissue samples from lung biopsies from healthy and asthmatic patients, or biobank histology samples that will allow examination of T_{FH} and TLS. Thus, phenotyping T_{FH} locations and TLS composition and frequencies in these samples would be an interesting continuation of this study.

7.4. Concluding remarks

T_{FH} are important regulators of antibody mediated immunity, and allergic asthma is a disease of chronic airway inflammation and remodelling, characterised by the generation of allergen specific IgE. Prior to this work T_{FH} responses have not been examined using a clinically relevant model of chronic AAD. It is now clear that during allergic disease T_{FH} populations are induced both in SLOs and the local lung tissue. T_{FH} are distinct from Th2 cells and are critical for driving humoral immune responses during AAD. Interestingly, lung T_{FH} do not exist in isolation and correlate with the formation of TLS containing GC B cells, memory B cells and plasma cell populations. Furthermore, T_{FH} appear to be critical regulators of allergic disease suppressing type 2 inflammation during disease inception, but potentially not after disease establishment. Taken together, this thesis highlights the importance of understanding T_{FH} responses during allergic disease.

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Appendix I: Publications arising from this thesis

Uwadiae, F.I., Pyle, C.J., Walker, S.A., Lloyd, C.M. and Harker, J.A., 2018. Targeting the ICOS/ICOS-L pathway in a mouse model of established allergic asthma disrupts T follicular helper cell responses and ameliorates disease. *Allergy*. doi: 10.1111/all.13602. Published online ahead of print on the 16th of September 2018.

Abstract:

Background: Allergic asthma is characterized by chronic inflammation and remodelling of the airways, associated with dysregulated type 2 immune responses and allergen-specific IgE. T follicular helper cells (T_{FH}) are crucial in T-dependent B cell responses and have been implicated in allergic airway disease (AAD). T_{FH}, unlike other CD4⁺ T cells are uniquely reliant on continuous ICOS signalling to maintain their phenotype after T cell priming, therefore disrupting this signal can impair T_{FH} responses. However, the contribution of T_{FH} to disease during chronic aero-allergen exposure and the therapeutic potential of targeting these cells has not been evaluated.

Methods: To establish AAD, female BALB/c mice were repeatedly exposed to house dust mite or *Alternaria alternata* three times a week for up to 5 weeks. To examine the impact of T_{FH} on AAD, mice were allergen exposed for 5 weeks and co-administered anti-ICOS-Ligand targeted antibodies, 3 times for the last 2 weeks.

Results: T_{FH} were first observed in the lung draining lymph nodes and with further exposure were also found locally within the lungs. T_{FH} accumulated with sustained allergen exposure, alongside germinal centre (GC) B cells. Blockade of ICOS signalling after AAD establishment successfully depleted T_{FH} but did not affect the differentiation of other CD4⁺ T cell subsets. This reduced GC responses, allergen-specific IgE, inflammation, pulmonary IL-13 and airway hyper-responsiveness.

Conclusions: T_{FH} are crucial in the regulation of AAD and the ICOS/ICOS-L pathway could represent a novel therapeutic target in allergic asthma.

Appendix II: Conference abstracts arising from this thesis

1) Oral presentation at the European Congress of Immunology, September 2018, Amsterdam, Netherlands

Ectopic lung resident germinal centres are formed during chronic house dust mite driven allergic airway disease in a T follicular helper cell dependent manner.

F. I. Uwadiae¹, R. Siroya¹, A. Gerasimov¹, C. Spilsbury¹, C. J. Pyle¹, S. A. Walker¹, C. M. Lloyd¹, J. A. Harker¹.

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Introduction: Allergic asthma is a disease of chronic allergen exposure, characterised by airway inflammation, airway hyperresponsiveness and allergen specific IgE. Germinal centres (GC) are anatomically distinct structures, located primarily in secondary lymphoid organs, and are critical for antibody generation. Lymphocyte aggregates have been identified in the lungs of asthmatic patients, but their formation and contribution to disease is not understood. This study aims to further understanding of these structures during chronic allergic disease.

Method: To establish chronic allergic airway disease mice were repeatedly exposed to intranasal house dust mite for up to 5 weeks.

Results: GC B cells and Tfh were found in the mediastinal lymph nodes (mLN) and the lungs after 3 weeks of allergen exposure. Intravascular staining confirmed both cellular populations to reside in the lung tissue, and large B cell aggregates containing GC B cells, T cells and follicular dendritic cell (FDC) networks were identified by confocal microscopy, indicative of an active GC. Sorting resident B cell from the lungs and mLN revealed that although transcripts indicative of IgG1 and IgE switch could be detected in both compartments by qPCR, IgA transcripts were only identified in the lung. Thus suggesting the lungs to also provide a source of protective antibodies. Ectopic GC formation was Tfh dependent and absent in Tfh deficient mice (*Cd4^{cre}Bcl-6^{fl/fl}*), which also lacked allergen specific IgE. Despite this, *Cd4^{cre}Bcl-6^{fl/fl}* mice had exaggerated Th2 cell biased allergic disease. Mixed bone marrow chimeras revealed this was due to the need of Tfh, rather than an intrinsic role for Bcl6 in regulating Th2 cell differentiation.

Conclusions: This data shows lung resident GCs to form during chronic allergen exposure in a Tfh dependent manner and indicates the importance of Tfh in regulating allergic airway disease.

2) Oral Presentation at the British Society of Immunology annual Congress, December 2017, Brighton, UK and published in Immunology supplement.

Ectopic lung resident germinal centres are formed during chronic house dust mite driven allergic airway disease in a T follicular helper cell dependent manner.

F. I. Uwadiae¹, R. Siroya¹, A. Gerasimov¹, C. Spilsbury¹, C. J. Pyle¹, S. A. Walker¹, C. M. Lloyd¹, J. A. Harker¹.

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Background:

Allergic asthma is a disease of chronic allergen exposure, characterised by airway inflammation, airway hyperresponsiveness and allergen specific IgE. Germinal centres (GC) are anatomically distinct structures, located primarily in secondary lymphoid organs. GCs consist of GC B cells, T follicular helper cells (Tfh) and follicular dendritic cells (FDCs) and are critical for antibody generation. Lymphocyte aggregates have been identified in the lungs of asthmatic patients, but their formation and contribution to disease is not understood. This study aims to understand whether lung GCs form during chronic allergic airway disease.

Method:

To establish chronic allergic airway disease C57BL/6 or *Cd4^{cre}Bcl-6^{fl/fl}* were repeatedly exposed to intranasal house dust mite for up to 5 weeks.

Results:

GC B cells and Tfh were found in the mediastinal lymph nodes (mLN) and the lungs after 3 weeks of allergen exposure. Intravascular staining confirmed both cellular populations to reside in the lung tissue, and large B cell aggregates containing GC B cells, T cells and FDC networks were identified by confocal microscopy. Sorting resident B cell from the lungs and mLN revealed that although transcripts indicative of IgG1 and IgE switch could be detected in both compartments by qPCR, IgA transcripts were only identified in the lung. Thus suggesting the lungs to also provide a source of protective antibodies. Ectopic GC formation was Tfh dependent and was absent in Tfh deficient mice (*Cd4^{cre}Bcl-6^{fl/fl}*), which also lacked allergen specific IgE. Despite this, *Cd4^{cre}Bcl-6^{fl/fl}* mice had exaggerated Th2 cell biased allergic disease. Mixed bone marrow chimeras revealed this was due to the need of Tfh, rather than an intrinsic role for Bcl6 in regulating Th2 cell differentiation.

Conclusions:

This data shows lung resident GCs to form during chronic allergen exposure in a Tfh dependent manner and indicates the importance of Tfh in regulating allergic airway disease.

3) Oral presentation at the London Immunology Group Meeting, November 2017, London, UK

Therapeutic blockade of inducible T-cell co-stimulator (ICOS) depletes Tfh and improves established house dust mite driven allergic airway disease

Faith I Uwadiae, Chloe J Pyle, Rohit Siroya, Simone A Walker, Clare M Lloyd, James A Harker

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Allergic asthma is a disease of chronic airway inflammation and remodelling, characterised by allergen specific antibody generation. T follicular helper cells (Tfh) are specialised CD4⁺ T cells that function to direct B cell responses and drive the production of allergen specific IgE. However, whether T_{FH} represent a therapeutic target during ongoing disease has yet to be explored. To establish chronic allergic airway disease (AAD), female BALB/c mice were repeatedly exposed to intranasal house dust mite (HDM) for 5 weeks. Tfh require continuous signalling via ICOS to maintain their phenotype, therefore to reduce Tfh, mice were also administered an ICOS-ligand blocking antibody, α-ICOS-L, or isotype control intraperitoneally three times a week, between week 3 and 5. Tfh were identified in the mediastinal lymph nodes (mLN) and lungs of HDM exposed animals and were successfully depleted during ongoing AAD. This diminished the subsequent germinal centre B cell and HDM specific antibody response. α-ICOS-L treatment also dampened airway hyperresponsiveness and reduced the severity of pulmonary infiltration into the lungs and airways, which coincided with reduced IL-13. Thus, Tfh responses can be ablated even during ongoing disease and suggests that disrupting ICOS/ICOS-L interactions to represent a potential clinical strategy for patients with allergic disease.

4) Poster presentation at the 19th International Conference on Lymphatic Tissues and Germinal Centres in Immune Reactions (GCC), September 2017, San Servolo, Venice, Italy.

Ectopic lung resident germinal centres are formed during chronic house dust mite driven allergic airway disease in a T follicular helper cell dependent manner.

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Allergic asthma is a disease of chronic allergen exposure, characterised by airway inflammation, hyper-responsiveness and allergen specific IgE. Germinal centres (GC) are anatomically distinct structures, located in secondary lymphoid organs. GCs consist of GC B cells, T follicular helper cells (Tfh) and follicular dendritic cells (FDCs) and are critical for antibody generation. Lymphocyte aggregates have been identified in the lungs of cadavers from asthmatic patients, but their formation and contribution to disease is not understood. We established chronic allergic airway disease by repeated intranasal exposure of C57BL/6 mice to house dust mite (HDM) for up to 5 weeks. GC B cells and Tfh were found in the mediastinal lymph nodes (mLN), but also in the lungs, by 3 weeks. Intravascular staining confirmed both cellular populations to reside in the lung tissue, and large B cell aggregates containing GC B cells, CD4⁺ T cells and FDC networks were identifiable by confocal microscopy. Sorting resident B cell from the lungs and mLN revealed that although transcripts indicative of IgG1 and IgE switch could be detected by qPCR in both compartments, only within the lungs were IgA transcripts identified. Thus, suggesting the lungs to also provide a source of protective antibodies. Ectopic GC formation was Tfh dependent and were absent in Tfh deficient mice (*Cd4^{cre}Bcl-6^{fl/fl}*), which also lacked allergen specific IgE. Importantly despite this *Cd4^{cre}Bcl-6^{fl/fl}* mice had increased IL-13⁺CD4⁺ T cells, and an exaggerated Th2 biased allergic disease. Mixed bone marrow chimeras revealed this was due to the need of Tfh, rather than an intrinsic role for Bcl6 in regulating Th2 cell formation. Taken together this data shows lung resident GCs to form during chronic allergen exposure in a Tfh dependent manner and indicates the importance of Tfh in regulating allergic airway disease.

5) Oral presentation at the EAACI Winter School, January 2017, Sierra Nevada, Spain.

T follicular helper cells; Janus cells of chronic allergic airway disease

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Introduction: T follicular helper cells (T_{FH}) are a specialised subset of $CD4^+$ T cells which are important for the generation of both protective and pathogenic antibody responses. In the context of allergic airway disease, T_{FH} are indispensable to the development of allergen specific IgE. However, their contribution to the overall allergic disease phenotype is controversial, with both protective and pathogenic roles being described. To date current models of studying T_{FH} and allergy have focused on using sensitisation and challenge mouse models, rather than more clinically relevant chronic exposure regimes with common aeroallergens.

Method: To assess the T_{FH} response induced during allergic airway disease BALB/C mice were chronically exposed to inhaled house dust mite extract (HDM) for up to 5 weeks. To determine the impact of impaired T_{FH} responses on the development of allergic airway disease, $CD4^{cre}Bcl-6^{fl/fl}$ (T_{FH} KO) or littermate controls (WT) mice were exposed to HDM for 5 weeks.

Results: T_{FH} appeared in the mediastinal lymph nodes of HDM exposed animals within one week and in the lungs after 3 weeks. T_{FH} accumulated at both sites with continued allergen challenge consistent with the emergence of germinal centre B cells and allergen specific IgE and IgG1 from 3 weeks onwards. As has previously been described T_{FH} loss during allergic airway disease development resulted in complete loss of allergen specific IgE and IgG1. Despite this T_{FH} KO mice also displayed worsened lung and airway eosinophilia, Th2 cells and airway hyper-responsiveness compared to WT controls.

Conclusions: Chronic HDM exposure resulted in local and systemic T_{FH} responses. Loss of T_{FH} responses was associated with impaired antibody responses but enhanced T_H2 based inflammation. Together this implicates T_{FH} as both critical drivers and regulators of distinct aspects of chronic allergic airway disease.

6) Oral Presentation at the British Society of Immunology annual Congress, December 2016, Liverpool, UK and published in Immunology supplement.

Therapeutic blockade of inducible T-cell co-stimulator (ICOS) depletes Tfh and improves established house dust mite driven allergic airway disease

Faith I Uwadiae, Chloe J Pyle, Rohit Siroya, Simone A Walker, Clare M Lloyd, James A Harker

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Background: T follicular helper cells (Tfh) function to direct B cell responses and are heavily implicated in diseases with antibody abnormalities. Tfh require continuous signalling via ICOS to maintain their phenotype and disrupting this interaction selectively depletes Tfh. Allergic asthma is a disease of chronic airway inflammation and remodelling, characterised by allergen specific antibody generation. However neither the location nor the impact of the Tfh response has been assessed in the context of clinically relevant models of chronic allergic airway disease (AAD).

Aim: To assess Tfh responses induced during chronic AAD

Method: To establish chronic AAD, female BALB/c mice were repeatedly exposed to house dust mite (HDM) intranasally for up to 5 weeks. To reduce Tfh, mice were also administered an ICOS-ligand blocking antibody, α -ICOS-L, or isotype control intraperitoneally three times a week, between 3 and 5 weeks post the initial dose.

Results: Tfh appeared in the mediastinal lymph nodes (mLN) of HDM exposed animals within one week, and locally within the lungs after 3 weeks. Tfh accumulated at both sites with continued allergen challenge correlating with germinal centre B cells. α -ICOS-L administration during established AAD successfully depleted lung and MLN Tfh and subsequent GC B cell responses but failed to alter HDM specific antibody responses. However, α -ICOS-L treatment dampened airway hyperresponsiveness and reduced the severity of pulmonary infiltration into the lungs and airways. This coincided with reduced IL-13 and IL-17A and was associated with fewer lung IL-13⁺ CD4⁺ T cells but not ILC2s.

Conclusions: Chronic HDM exposure generates both local and systemic Tfh response, which can be ablated with therapeutic α -ICOS-L treatment. Disrupting ICOS/ICOS-L interactions improves hallmark features of established ongoing allergic disease, even after only 2 weeks of intervention. Targeting this pathway may represent a potential clinical strategy for patients with allergic disease.

7) Oral Presentation at the Keystone on T Follicular Helper Cells and Germinal Centers, February 2016, Monterey, California, USA.

Chronic exposure to inhaled allergen results in sustained T follicular helper cell generation in both the lungs and peripheral lymph nodes, which potentially contributes to allergic airway disease

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T follicular helper cells (Tfh) function to direct B cell antibody responses and are heavily implicated in diseases associated with abnormal antibody production. Despite this, the contribution of Tfh to the inflammatory environment and pathogenic immunoglobulin E (IgE) generated during allergic asthma has been little studied. Allergic airway disease (AAD) is associated with chronic exposure to inhaled allergens. To model this we repeatedly treated adult BALB/c mice with house dust mite (HDM) extract intranasally for up to 5 weeks. Tfh appeared in the mediastinal lymph nodes (MLN) of HDM exposed animals within one week, and continued to accumulate throughout challenge. Interestingly, Tfh also amassed in the lungs of HDM treated mice, together with lung resident germinal centre B cells. Importantly, lung Tfh were also functionally distinct from their lymphoid resident counterparts. While MLN Tfh were maintained with cessation of HDM inhalation, lung Tfh were rapidly reduced along with both local and systemic allergen specific IgE. HDM re-exposure resulted in the re-appearance of pulmonary Tfh and lung and serum IgE, supporting a link between lung resident Tfh and IgE production. Perturbation of Tfh responses during established AAD, using ICOS-L blockade improved airway function and pulmonary inflammation. Together this data shows that chronic allergen exposure results in escalating tissue resident and lymphoid Tfh responses with distinct functions. Interfering with the Tfh response was associated with improved disease implicating Tfh as critical regulators of AAD.

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Blockade of Inducible T cell costimulatory molecule (ICOS) function dampens established house dust mite driven allergic airway disease by targeting both T cells and innate lymphoid cells.

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Introduction: Allergic asthma is a disease of chronic airway inflammation characterised by abnormal production of allergen specific IgE. T follicular helper cells (Tfh) are a specialised subset of CD4⁺ T cells which function to direct B cell antibody responses and have been recently implicated in allergic disease pathology. Blocking ICOS (Inducible T cell costimulatory molecule) signalling in murine models of acute inflammation has been demonstrated to eliminate Tfh responses without affect other CD4⁺ T cell subsets. However, the impact of losing Tfh function in established allergic airway disease is not currently understood.

Method: To establish allergic airway disease female BALB/c mice were repeatedly exposed to house dust mite (HDM) intranasally for 5 weeks. Mice were then given an ICOS-Ligand (ICOS-L) blocking antibody, HK5.3, or isotype control, 2A3, intraperitoneally three times a week from the beginning of the fourth week. Pathological and immunological responses were measured at week 5.

Results: HK5.3 treatment dampened HDM driven airway hyperresponsiveness compared to isotype controls and reduced the severity of pulmonary infiltration into the lung and airways. Blocking ICOS/ICOS-L interactions ablated lung and mediastinal lymph node Tfh and corresponding germinal centre B cells numbers, resulting in diminished serum and lung HDM specific IgE. The intervention not only impacted Tfh responses but also decreased IL-13⁺CD4⁺T cells and IL-13⁺ and IL-17A⁺ innate lymphoid cells numbers. This was accompanied by reduced IL-13 and IL-17A in both the lung and bronchoalveolar lavage fluid.

Conclusion: Blocking ICOS/ICOS-L interactions was able to reduce hallmark symptoms of allergic disease after only 2 weeks of intervention by targeting ILCs and Th2 cells in addition to Tfh and IgE responses. In the context of chronic inflammation interfering with ICOS signalling has wide-ranging and beneficial implications on allergic airway disease resolution. Therefore, disrupting this pathway may represent a potential clinical strategy for patients with allergic disease.