Genetic and environmental influences on the development of allergic pulmonary disease

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Copyright Declaration

Declaration

The work in this thesis was conducted by me, with the following exceptions: Lorraine Lawrence (Leukocyte Biology Histology Service) embedded, sectioned and stained all the lung sections with haematoxylin and eosin. Flexivent machines were operated by Simone Walker. Partek analysis of RNAseq results were analysed and figures generated by Tom Shea. AAV serotype administration and GFP staining was carried out by Dr. Lisa Gregory. Figure 3.6 was generated by Jessica Vassiliou. Figures 6.4 and 6.9 were generated and supplied by Janssen Pharmaceuticals Inc.

The following experiments have been verified independently by additional members of the lab: All experiments in figures 3.1 – 3.6, neonatal and adult HDM and Alternaria allergen exposure models (Fig 4.1 – 4.7 with the exception of some flow cytometric analysis), WT and $ST2^{-/-}$ adult HDM and Alt exposure models, not $IL\text{-}33^{-/-}$ (5.1-5.11 with the exception of some flow cytometric analysis). All other experiments were performed only by me and have not been independently verified.

Abstract

Allergic airway disease is a growing health concern and despite extensive research, mechanisms underlying allergen sensitisation are not fully understood. By utilising experimental mouse models of intra-nasal (i.n) allergen exposure the role of a number of factors in the onset and progression of allergic airway disease (AAD) were investigated including age, allergen type and genetic background.

Age at first allergen exposure was found to be a critical determinant of the severity of AAD. Neonatal mice exposed to i.n. house dust mite (HDM) extract developed enhanced AAD compared to adult mice. A period of non-responsiveness to HDM exposure was also identified between the ages of day 14 and 21 of life which corresponded to a natural nadir in both IL-13^+ $CD4^+$T cells and IL-13^+ innate lymphoid cells (ILCs).

The type of allergen to which mice were exposed was also revealed as a determinant of the severity of AAD that developed. The fungal allergen *Alternaria alternata* (Alt) was associated with a more severe disease phonotype, characterised by elevated IL-13 levels and steroid resistance.

Investigation of the genetic contribution to the development of AAD focussed on two genes identified by large-scale genome wide association studies (GWAS) as associated with childhood onset asthma - IL-33 and ST2. ST2, but not IL-33, was determined to be critical for sensitisation with HDM. However, neither IL-33 nor ST2 were required for the development of AAD in response to Alt exposure. Investigation of short-term exposure to Alt identified ST2 as having a critical role in early inflammatory responses to Alt but not in the development of Alt specific T cells and IgE.

Overall, work conducted in this thesis identified the age-dependent development of allergic disease, the likely existence of an alternative ligand for ST2 and provided a mechanistic explanation for the association of Alt sensitisation and steroid resistance.

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Chapter 1. Introduction

1.1 Allergic asthma

Asthma is a chronic disease characterised by eosinophilic inflammation, reversible airflow obstruction and structural airway remodelling. Asthma affects approximately 300 million people worldwide and is implicated in roughly 1 in 250 deaths (Masoli et al., 2004). Both environmental and genetic factors are known to contribute to the asthmatic state and the term 'asthma' encompasses a variety of diseases (von Mutius, 2009).

Allergic asthma commonly develops during childhood (von Mutius, 2000) and involves the infiltration of immune cells to the lung in response to inhaled allergens and the subsequent development of an antigen-specific immune response, including IgE. Although broadly defined as allergic asthma, significant phenotypic heterogeneity is known to exist in terms of inflammatory cells and mediators involved. This diversity amongst patients is becoming more evident through results of late-stage therapeutic antibody trials such as anti-IL-5 (Castro et al., 2011; Flood-Page et al., 2007; Leckie et al., 2000; Pavord et al., 2012) and anti-IL-13 (De Boever et al., 2014; Brightling et al., 2014; Gauvreau et al., 2011) which show clinical improvement only in a small subset of patients. These trials highlighted the importance of characterising individual phenotypes of patients before deciding appropriate treatment regimens and the possibility that targeting the late stage components of allergic inflammation may not be enough to reverse or halt the worsening of disease. Instead, it has been suggested that directing treatments towards inflammatory mediators upstream of the development of adaptive immune responses – such as the epithelial derived cytokines IL-33 and IL-25 – could potentially prevent the onset or worsening of the allergic response (Lloyd and Saglani, 2010).

A number of cell types are traditionally associated with allergic asthma, such as eosinophils and Th2 cells with the capacity to secrete IL-4, IL-5 and IL-13. In addition to these, a growing body of evidence is revealing the importance of a number of other cell types; including mast cells, other T cell subsets,

(Lloyd and Hessel, 2010), innate lymphoid cells (Chang et al., 2011) and the pulmonary epithelium (Holgate, 2007).

1.1.1 Mast cells

Mast cells are implicated in the initiation of airway inflammation and the continuation of chronic disease through the binding of antigen specific IgE. Mast cells are located at the interface between the host and the inhaled environment and respond rapidly to stimuli through the release of chemo attractants and vasodilators. The numbers of mast cells in the smooth muscle of asthmatic patients is greatly increased compared to healthy controls and also correlates with the degree of airway hyper-responsiveness in these patients, implying a role in the contraction of smooth muscle (Brightling et al., 2002). Mast cells play a dual role in the pathogenesis of asthma by responding to signals from both innate and adaptive components of the immune system. ST2 receptors and high affinity FcεR1 on the surface of mast cells allow cells to respond to both IL-33 and the cross linking of surface bound IgE respectively (Ho et al., 2007). Upon activation, mast cells release a number of mediators stored in pre-formed granules including proteases, histamine and cytokines that all contribute toward the pathogenesis of asthma. Importantly, Mast cells are unusual in the fact that they store preformed cytokine in granules and can therefore act as an early source of both IL-4, which is essential for the generation of IgE (Mosmann and Coffman, 1989) and IL-13, known to be essential for the pathogenesis of allergic asthma (Wills-Karp et al., 1998a).

1.1.2 T cells

T cells are extremely important for the control of infection through the generation of memory cells specific for bacteria and viruses. The control of parasites within the body is dependent on Th2 cells via the antigen specific secretion of cytokines such as IL-4, IL-5 and IL-13 (Finlay et al., 2014). These

cytokines, along with the interaction of T cells with B cells via antigen presentation and co-stimulation, leads to the generation of high affinity IgE, allowing the control of parasites (Coyle and Tsuyuki, 1995). Despite this vital role, Th2 cells can also be generated against innocuous antigens, such as pollen and animal dander, leading to allergic sensitisation. Th2 cells specific for inhaled allergens can lead to the secretion of Th2 cytokines in the lung and the generation of allergic asthma. Th2 cytokines in the lung attract infiltrating immune cells and also act directly on structural cells leading to contraction of smooth muscle and deposition of collagen (Murdoch and Lloyd, 2010). IL-4 leads to the generation of allergen specific IgE and also induces the expression of VCAM-1 on pulmonary endothelial cells to induce the migration of immune cells to the lung (Paul and Zhu, 2010). IL-5 is responsible for the attraction of eosinophils to the lungs and their maturation and survival. These cells are known to cause smooth muscle contraction and mucus secretion by the production of leukotrienes and platelet activation factor (Rosenberg et al., 2013). Th2 cells also secrete IL-13 in response to allergen stimulation which leads to many features of allergic asthma including airway remodelling, smooth muscle contraction and mucus production (Ingram and Kraft, 2012; Wills-Karp et al., 1998a).

In addition to Th2 cells, dysregulation of other T cell subsets have been identified as contributing towards the allergic asthma phenotype including CD8, Th1, Th17, Th9 and regulatory T cells (Tregs). The main role of $CD8^+$ T cells is the elimination of virally infected cells (Noble et al., 1995), however, they have also been implicated in the pathogenesis of asthma with both harmful and beneficial roles for increased $CD8^+$ T cells described in the literature (Betts and Kemeny, 2009). As with $CD4^+$ T cells, $CD8^+$ cells can be divided into two phenotypically distinct populations – Tc1 and Tc2 - which essentially mimic Th1 and T2 responses with Tc1 known to reduce asthmatic symptoms and Tc2 to contribute towards the generation of IgE and therefore enhance symptoms (Ishimitsu et al., 2001; Noble et al., 1995; Schaller et al., 2005). Like $CD8^+$ T cells, Th1 cells are also involved in protection against pathogens, including bacterial and fungal infections, through the production of IFNγ (Jankovic et al., 2001). However, they have also been implicated in the pathogenesis of asthma as

increased IFN-γ producing T cells have been detected in the airways of asthmatic patients in comparison to both healthy and atopic patients (Krouwels et al., 1996; Krug et al., 1996). Th17 cells have also been identified in the lungs of asthmatic patients and an increase in IL-17A has been associated with more severe, steroid resistant disease (Pène et al., 2008; Al-Ramli et al., 2009). In contrast to the clear association of Th17 cells and asthma, the association of Th9 cells with allergic disease is less well defined. Although IL-9 has been detected in biopsies from asthmatic patients, the blocking of IL-9 in mouse models has yielded variable results. In a model of OVA sensitisation, IL-$9^{-/-}$ mice developed similar allergic inflammation to WT mice (McMillan et al., 2002). However, in a model of HDM sensitisation, both direct blocking of IL-9 and the inhibition of Th9 cell differentiation prevented HDM associated pathology via actions on mast cells (Jones et al., 2012; Kearley et al., 2011). In addition to dysregulation of inflammatory cells, a role for abnormal regulatory cell populations have been shown in the pathogenesis of asthma. Both the number and suppressive capacity of $CD4^{+}CD25^{+}$ Tregs in peripheral blood and BAL of children with allergic asthma are reduced when compared to healthy controls (Hartl et al., 2007; Lin et al., 2008).

1.1.3 Innate lymphoid cells

Innate lymphoid cells (ILCs) are defined as lineage negative and ICOS positive. Three subsets of ILCs have been described to date – ILC1, ILC2 and ILC3 - as defined by transcription factors and cytokines they produce. In humans and mice ILC1 and ILC2 cells parallel TH1 and TH2 profiles with ILC1s defined by the transcription factor T-bet and secretion of IFN-γ and TNF-α while ILC2s are defined by GATA-3 and RORα transcription factors and the secretion of IL-5, IL-9 and IL-13. ILC3 cells are comparable to Th17 cells as they are defined by RORγt expression and secretion of IL-17 and IL-22 (Mjösberg et al., 2012; Walker et al., 2013). Since the recent discovery of these cells, a number of vital roles in the control against infection and maintenance of normal functions, such as epithelial

cell integrity in response to commensal gut bacteria, have been described. ILC3s have been described as a vital contributor towards intestinal barrier function and gut immune homeostasis through the production of IL-22 (Sonnenberg et al., 2012). This group demonstrated that deletion of ILCs led to the dissemination of bacteria normally confined to lymphoid tissue and the subsequent development of systemic inflammation, similar to that observed in models of Crohn's disease. In addition to controlling inflammation in response to commensal bacteria, ILC3s also have a role in the postnatal development of gut associated lymphoid tissue (GALT), allowing the production of IgA for the protection of mucosal surfaces against infection (Eberl and Littman, 2004). The direct targeting of pathogenic bacteria by cells now considered to be ILC3s has also been described in the lung and intestine (Pitt et al., 2012; Satoh-Takayama et al., 2009).

As well as protecting against pathogenic bacteria in the intestine, ILCs have also been shown to be vital for the protection against intestinal parasites. ILC2s were originally discovered in a model of helminth expulsion through the utilisation of an IL13-eGFP mouse model and were found to be a vital component of the innate immune system (Neill et al., 2010). Neill *et. al*. demonstrated that the epithelial derived cytokines IL-33 and IL-25 were essential for the expansion of ILCs in the gut.

However, like T cells, the protective function of this cell type is susceptible to dysregulation and ILCs have been implicated in a number of inflammatory conditions such as intestinal and pulmonary inflammation. The production of IL-22 and IL-17 by ILC3s and IFNγ by ILC1s has been implicated in the pathogenesis of both inflammatory bowel disease and colitis in response to *Helicobacter* infection (Buonocore et al., 2010; Geremia et al., 2011). The role of ILC2s in the development of airway hyper-reactivity was recently described following both infection with influenza (Chang et al., 2011) and allergic sensitisation with Ovalbumin (OVA) (Barlow et al., 2012), *Alternaria alternata* (Alt) and ryegrass (Kim et al., 2014). In the setting of allergic asthma, IL-33 is released from the pulmonary epithelium upon allergen challenge, which leads to the migration and expansion of ILC2s in the lung. Due to the lineage negative nature of the cells they do not respond to allergen in an antigen specific

fashion but ILC2s produce IL-13, known to be essential to the progression of allergic asthma (Wills-Karp et al., 1998).

Through the production of inflammatory cytokines in response to pathogens or cytokine stimulation, ILCs have the ability to act independently of other cell subsets as an immediate innate immune cell capable of clearing bacteria and intestinal parasites and responding to allergen. ILCs have been implicated in the initiation of allergic airways disease in the absence of adaptive cells since papain and house dust mite (HDM) induced airway inflammation and mucus generation developed in the absence of T cells (Halim et al., 2012; Oboki et al., 2010). In addition to this, ILCs have also been shown to interact with other immune cells to allow the generation of adaptive, antigen specific responses. This was first suggested by Tsuji (2008) who demonstrated the interaction between RORγt^{+} innate cells (now thought to be ILC3s) and B cells in intestinal immune structures and found these cells to be critical for the synthesis of IgA in the gut. Since then, a large body of evidence has determined that ILC interaction with adaptive cells, such as T cells (Halim et al., 2014), are vital for the generation of adaptive immune responses. Experiments conducted in RORγt^{+} ILC deficient mice showed these cells were essential for the maintenance of memory CD4^{+}T cells (Withers et al., 2012). Most recently, ILC2 deficient mice were utilised to demonstrate that ILCs are not only required for the maintenance of memory T cells but also for the generation of antigen specific Th2 cells in a model of papain allergy (Halim et al., 2014). The requirement of ILC2s was found to be dependent on the production of IL-13 by these cells as administering IL-13 could overcome the loss of ILC2s. Interestingly, ablation of IL-4, which has previously been considered vital for allergic reactions, did not affect the generation of Th2 cells. ILCs have also recently been shown to have the ability to present antigens to T cells via MHCII, although not as effectively as dendritic cells (Oliphant et al., 2014).

1.1.4 The pulmonary epithelium

The airway epithelium forms the first line of defence between the environment and the host by providing both a physical barrier and a number of anti-microbial mediators. The physical barrier between host and environment is maintained by a number of different tight junctions – maintained by E-cadherin and other tight junction proteins – which prevent the crossing of the epithelial layer unless by active transport or breakdown of junctions (Sparrow et al., 1995). In addition to the physical barrier property of the epithelium, it is also considered an active contributor to host defence through the action of cilia to clear particles - such as allergens, particulate matter and pathogens, the secretion of mucus and the surface expression of a plethora of pattern recognition receptors (PRRs), toll-like receptors (TLRs) and C-type lectin receptors (CLRs) to detect pathogens and damage signals (Knight and Holgate, 2003). Once activated by the ligation of PRRs or TLRs, epithelial cells rapidly interact with local dendritic cells (DC) to initiate downstream inflammatory responses (Lambrecht and Hammad, 2009). Epithelial cells have the capacity to interact with DCs and a number of other immune cells through the direct release of chemotactic and pleiotropic immune mediators upon stimulation including TSLP, GM-CSF, IL-25 and IL-33 (Lambrecht and Hammad, 2012). Upon release of these mediators, resident DCs become activated and migrate towards draining lymph nodes to interact with B and T cells and generate adaptive immune responses against pathogens.

Dysregulation of any of the extensive control mechanisms to prevent infection and pulmonary inflammation in response to innocuous peptides can lead to the development of allergic airways disease. For example, biopsies from asthmatic airways have been found to have reduced expression of E-cadherin and therefore reduced tight junction integrity, leading to enhanced lymphocyte trafficking to the lung (de Boer et al., 2008). The function of TLR4 is the detection of a component of bacteria – LPS – therefore alerting the immune system to possible infection (Lu et al., 2008), however, components of house dust mite allergen have also been shown to activate TLR4 (Hammad

et al., 2009). Two environmental factors known to be associated with the development of asthma – cigarette smoke and viral infection – upregulate TLR4 expression on epithelial cells and may therefore cause enhanced inflammatory responses to HDM exposure (Monick et al., 2003; Pace et al., 2008). In addition to activating the TLR4 receptor HDM, as well as a number of other inhaled allergens, contain proteases that activate epithelial cells and disrupt the epithelial layer through the destruction of tight junctions. The DerP1 component of HDM acts on claudin-1 proteins of tight junctions and therefore increase epithelial permeability and enables the interaction between DCs and allergens (Wan et al., 1999). Proteases also bind to epithelial surface receptors such as protease-activated receptor-2 (PAR-2) with DerP1 ligation of PAR-2 causing the secretion of IL-6 and IL-8 from cultured epithelial cells (Asokananthan et al., 2002). In addition to proteases, the β-glucan components of HDM have also been shown to stimulate the airway epithelium and cause the rapid release of CCL20 which acts to attracts immature dendritic cells to the airway (Nathan et al., 2009). Taken together, the action of allergens on the pulmonary epithelium lead to the rapid secretion of inflammatory mediators and the attraction of DCs to take up and present allergens.

One clinical feature of patients with severe asthma is increased IL-33 expression in the bronchial epithelium (Préfontaine et al., 2009) and subepithelial cells (Saglani et al., 2013). The involvement of epithelial IL-33 release in response to allergen exposure has therefore been investigated by a number of groups in the context of allergic asthma. Both HDM and the fungal allergen *Alternaria alternata* are known to cause IL-33 secretion from epithelial cells (Llop-Guevara et al., 2014a; Snelgrove et al., 2014; Willart et al., 2012). These results indicate that asthmatic patients could have increased release of IL-33 upon allergen exposure and therefore enhanced immune infiltration to the lung. In addition to the activation of dendritic cells, epithelial derived cytokines also cause a number of innate immune cells, such as mast cells and ILCs, to be attracted to the lung where they proliferate and release inflammatory cytokines such as IL-13 and IL-4 to initiate the features of allergic asthma, as illustrated in Figure 1.

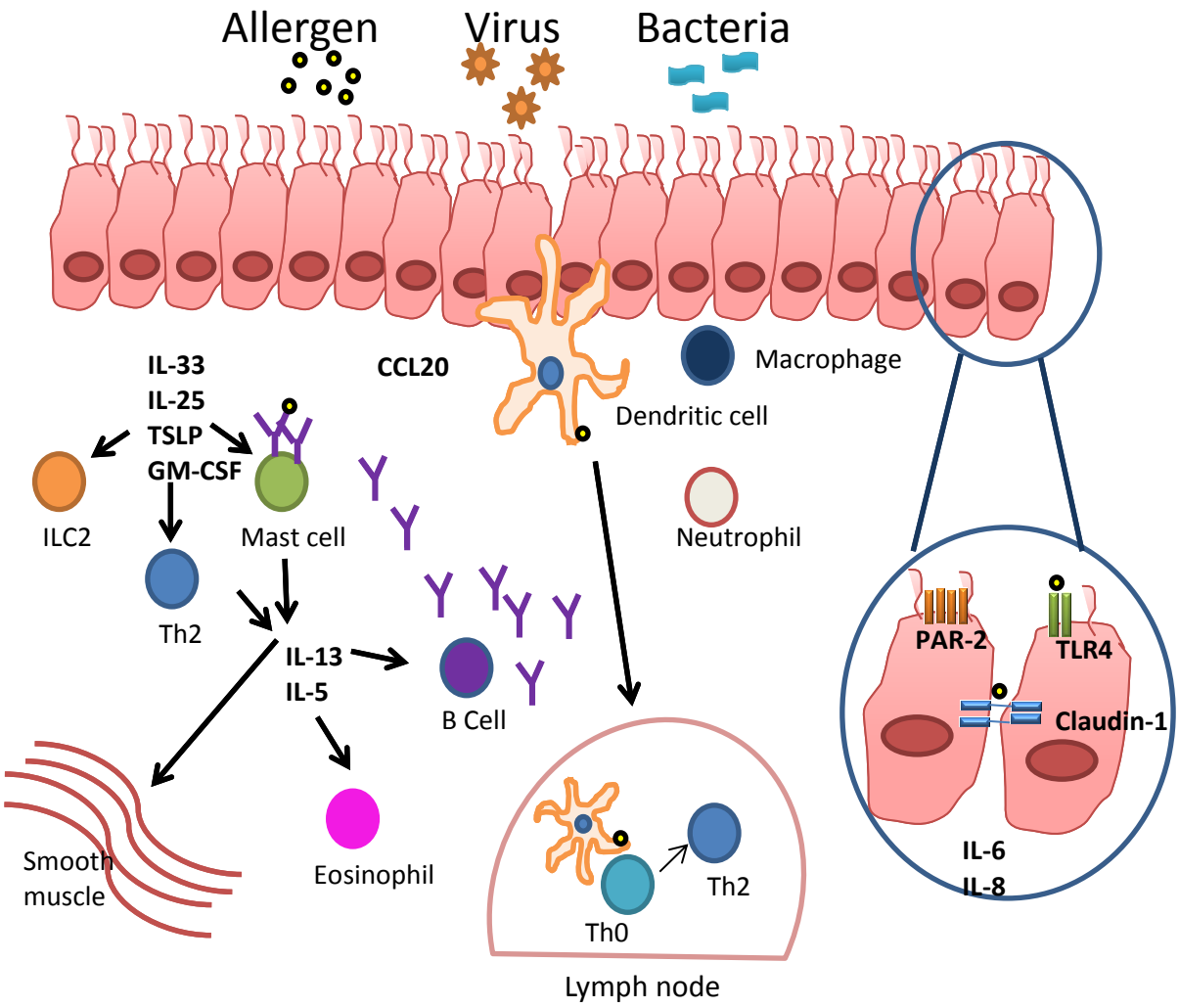

Figure 1.1 Illustration of cells and inflammatory mediators in the asthmatic airway.

An illustration showing the recruitment and activation of innate immune cells and the polarisation of Th2 cells in response to inflammatory mediators released from the epithelium. In response to stimulation by allergen, bacteria and viruses, the epithelium secretes cytokines such as IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) and the chemokine CCL20. Dendritic cells present antigen and polarise T helper0 (Th0) cells to Th2 cells. Along with Th2 cells, Innate lymphoid cell 2s (ILC2s), and mast cells secrete IL-13, which activates B cells and smooth muscle. Allergens also act on epithelial cells via toll-like receptor 4 (TLR4), proteinase-receptor 2 (PAR2) ligation and the disruption of tight junctions through breakdown of claudin-1.

1.2 Age and susceptibility

1.2.1 Adult vs. childhood onset asthma

Asthma most commonly develops during childhood with around 95% of asthmatics developing symptoms by age 6 (Masoli et al., 2004). Disease that develops during childhood is commonly allergic asthma (von Mutius, 2000) while asthma that develops later in life is often linked to factors such as occupational exposure to irritants (Dykewicz, 2009), obesity (Sutherland, 2014) or stress (Lietzén et al., 2011). Adult onset asthma is often associated with a poor prognosis and rapid decline in lung function from time of diagnosis with a much lower rate of remission in patients than those who develop asthma in childhood. A clear genetic factor is also lacking in adult onset asthma and often no family history of the disease exists (Nijs et al., 2013). In addition, approximately one third of adults with severe asthma have no history of childhood respiratory disease or atopy, further identifying childhood-onset and adult-onset asthma as phenotypically different (Miranda et al., 2004). Despite the apparently more complex nature of adult onset asthma, the majority of research into the condition is carried out in adult models of disease to avoid difficulties of an immune system and pulmonary structures that are not fully developed. This has left a large gap in knowledge as to how asthma develops in neonates and infants in the absence of fully functional T and B cells at this age (Adkins et al., 2004). Interestingly, the likelihood of developing asthma differs between males and females and susceptibility changes with age. In childhood and up to age 16, the prevalence of asthma is twice as high in males as it is in females (Schaubel et al., 1996; Venn et al., 1998). In contrast, during adulthood the female gender is associated with a greater risk factor of developing asthma characterised by a higher incidence as well as a more severe disease phenotype (van den Berge et al., 2009; Strachan et al., 1996). The influence of sex hormones may therefore also play a role in the development of asthma at different ages.

1.2.2 Innate Immune cells in early life

The development of the immune system during early life occurs in the presence of constant exposure to new antigens at mucosal surfaces such as the gut and pulmonary epithelium. A predisposition to both Th2-type and regulatory responses at birth is thought to allow the development of tolerance towards these antigens and therefore avoid inflammatory responses to mostly innocuous particles such as dust, pollen and animal dander (Adkins et al., 2003; Al-Garawi et al., 2011; Krishnamoorthy et al., 2012). The composition of the neonatal innate immune system differs from an adult in that antigen-presenting cells (APCs) are primed to induce Th2 rather than Th1 responses. Studies comparing cord blood to adult peripheral blood identified a higher ratio of plasmacytoid dendritic cells over myeloid dendritic cells in neonates and also a much lower population of NKT cells in neonates, both factors leading to a reduced production of Th1 responses (Belderbos et al., 2009; Eger et al., 2006). In addition to the differences in overall cell populations between neonates and adults, the responses of individual cells to identical stimulation also differ. For example, TLR4 stimulation via LPS of cord blood cells results in higher IL-10 and lower IL-12 than equal stimulation of adult peripheral blood cells (Belderbos et al., 2009). Recently, a common precursor for all lineages of ILC – ILC1, ILC2 and ILC3 - was identified in both foetal liver and adult bone marrow (Constantinides et al., 2014). Interestingly, the authors noted that although the cells isolated from foetal and adult tissue had identical surface receptors, a much higher proportion of RORγt expressing ILCs were identified in foetal liver and this population was barely detectable in adult bone marrow. RORγt^{+} ILCs are involved in the protection against gut pathogens and produce IL-17 and IL-22 (Sawa et al., 2011). Studies conducted by this group also identified that ILC3s evolve from liver precursors alongside the colonisation of the intestine with the microbiome and therefore are present to maintain gut homeostasis upon microbiome colonisation (Sawa et al., 2010).

1.**2.3 Development of T cells**

In addition to an innate cell composition skewed toward an anti-inflammatory response, the contribution of B and T cells to host defences differ vastly between neonates and adults as neonates are born without the ability to induce memory responses. Traditionally it was believed that neonates did not have any T cells unless maternally transferred although now it is widely accepted that a neonatal T cell population exists, although in much lower numbers than T cells in adults (Garcia et al., 2000). Neonatal T cells are considered 'immature' for a number of reasons including decreased proliferation and IL-2 production when stimulated (Hassan and Reen, 1997) and an inability to fully activate B cells (Splawski et al., 1991). As with neonatal innate cells, T cells are also skewed toward a Th2, rather than Th1 response. This has been demonstrated through transfer of neonatal $CD4^+$ lymph node cells into adult mice to exclude the possibility that other cell types in neonates were creating a Th2 skewing environment. These studies revealed that neonatal $CD4^+$ cells were greatly deficient in the ability to mount Th1 responses to antigen stimulation when adoptively transferred into an adult host (Adkins et al., 2002). In addition to a Th2 skewing, neonatal $CD4^+$ cells have also been shown to differentiate into $FOXP3^+CD4^+$ cells in response to TCR stimulation and *in vivo* injection of anti-CD3, which did not occur in adult $CD4^+$ T cells (Wang et al., 2010a). However, a recent publication by Gibbons *et. al*. (2014) has challenged the idea that neonatal T cells are skewed solely towards Th2 and regulatory functions due to the discovery of an IL-8 producing T cell population in newborn pre-term babies. Interestingly, no equivalent population was identified in neonatal mice. Both CD4 and CD8 cells were identified as producing significantly more IL-8 than equivalent adult populations; a cytokine known to activate antimicrobial neutrophils and γδT cells. The group hypothesised that this newly identified cytokine secretion from T cells may be responsible for discrimination between pathogens and commensal bacteria of the newly emerging neonatal microbiome and described the cytokine as a pro-inflammatory immunoprotective cytokine (Gibbons et al., 2014).

1.2.4 Development of B cells

As with innate and T cells, B cells are also phenotypically and functionally different in neonates and adults (Marshall-Clarke et al., 2000). Immature IgM^+IgD^- B cells from both adults and neonates undergo apoptosis upon ligation of the B cell receptor (BCR). However in neonatal mature IgM^+IgD^+ B cells, ligation of the BCR also causes apoptosis while in adult cells this leads to proliferation of the mature B cells (Norvell and Monroe, 1996). Antigen processing and presentation by neonatal B cells has also been implicated in the Th2/tolorogenic responses of neonates as these cells have a reduced capacity to up-regulate MHCII after ligation of the BCR (Tasker and Marshall-Clarke, 1997). Neonatal B cells also present reduced or no co-stimulatory molecules, such as CD86, meaning T cells are likely to become unresponsive to the presented antigen (Marshall-Clarke et al., 2000).

The antibody repertoire differs greatly between neonates and adults and this has been highlighted through the difficulty in establishing effective, long term immunisations for young children (Schallert et al., 2002). Foetal derived IgA and IgG can be detected in the serum after 30 weeks of gestation and IgE in the liver after 11 weeks whereas at birth, children have a predominant population of IgM^+ B cells in cord blood. However adult levels of Ig in the serum are not present until age 2 for IgM, 6 for IgG and puberty for IgA (Miller et al., 1973; Schroeder et al., 1995). In contrast, the highest level of serum IgE is observed in school aged children and declines towards adulthood (Wittig et al., 1980). During early life, transfer of maternal IgG across the placenta and both IgG and IgA through breast milk also provides protection against pathogens to which the mother has been exposed (Macchiaverni et al., 2011). In addition to a reduced repertoire of antibodies in neonates, the avidity of neonatal antibodies for antigenic epitopes is also a fraction of that of adults. In adults, high affinity antibodies are generated through somatic hypermutation, antigen driven selection and affinity maturation (Schallert et al., 2002). In children the ability of B cells isolated from cord blood to cause somatic hypermutation of genes is negligible, however this increases up to 6 months of age

where evidence of the selection of high affinity antibodies has been observed (Van Es et al., 1992; Ridings et al., 1998).

1.2.5 Symbiosis of the colonising microbiome and the developing immune system

In addition to the Th2/regulatory phenotype of the neonatal immune system preventing inflammation to innocuous stimuli, this environment is also known to permit the development of a colonising microbiome. The new-born gut is colonised with bacteria almost immediately after birth with Gammaproteobacteria known to be the first early colonisers (Deshmukh et al., 2014). A number of studies have highlighted the symbiotic existence of the newly colonising bacteria in both the lung and gut with the neonatal immune environment; including neutrophils, T cells and ILCs. Inhibition of the microbiome, through antibiotic administration, was shown to prevent the normal influx of neutrophils into the periphery and subsequent re-colonisation of these neonates induced the production of IL-17 by ILC3s in the gut (Deshmukh et al., 2014). In the lung, the colonisation by the microbiome has been found to be responsible for the generation of induced regulatory Helios$^-$ T cells that dampen the neonatal immune response to HDM exposure (Gollwitzer et al., 2014). The generation of IL-8 observed in T cells isolated from preterm babies had also been hypothesised to have a role in discriminating microbiome bacteria from potentially pathogenic bacteria (Gibbons et al., 2014).

1.2.6 The development of atopy and allergy in early life

Despite the numerous control mechanisms that exist in early life to maintain a homeostatic environment and prevent the onset of inappropriate immune responses, many individuals develop atopy and allergies at this age. A number of factors are thought to contribute toward this loss of control; including genetics, infection and exposure to pollution (Kusel et al., 2007; Latzin, 2013).

Infection with certain viruses during early in life has been shown to increase the risk of developing asthma including rhinovirus and respiratory syncytial virus (RSV) (Jackson et al., 2012; Kusel et al., 2007). One theory as to why viruses are associated with the development of asthma is that they trigger symptoms that would have occurred in predisposed individuals (Fuchs and von Mutius, 2013). Another hypothesis is that viruses, including RSV, cause an increased expression of TLR4 receptors on the epithelial cell surface, therefore enhancing the response of the epithelium to allergen exposure such as HDM (Monick et al., 2003).

The hygiene hypothesis was originally proposed in 1989 to account for the growing incidence of atopy and asthma in developed countries linking a high level of cleanliness to an increased risk of disease (Strachan, 1989). Essentially, this has been supported by experiments showing early exposure to pathogens and animal products boost the Th1 response over the Th2 response, therefore preventing the development of Th2 associated allergies and asthma (Platts-Mills et al., 2001; Von Ehrenstein et al., 2000). However, more recently a 'counter-regulatory' model has been proposed to also take into account the rising incidence of autoimmune disease along with allergy in the developed world. This modernised model hypothesises that an immune system challenged regularly with pathogens produces a natural up-regulation of IL-10 that acts to suppress immunological responses to allergens (Wills-Karp et al., 2001).

A large bank of evidence also supports the theory that certain individuals are predisposed to asthma from birth and may therefore be more susceptible to environmental influences. Detection of IgE in cord blood has been significantly linked to the development of food allergies at 12 months, a common condition predisposing to asthma (Kaan et al., 2000). An increased production of IL-13 in $CD4^+$ T cells isolated from cord blood was also identified as a determining factor for the development of allergic disease later in life (Ohshima et al., 2002).

1.2.7 Mouse models of AAD during early life

Due to the numerous differences between adult and neonatal responses to allergen exposure and the high incidence of allergy development in childhood, a number of neonatal mouse models of allergy have been developed. To investigate the effects of allergen exposure on a maturing immune system, a neonatal model of inhaled HDM exposure commencing at day 3 of life was developed by Saglani *et. al*. (2009). HDM exposure led to robust allergic airways disease (AAD) characterised by increased airway resistance, BAL and lung inflammation and the development of antigen specific IgE. However, age at onset of allergen challenge appears to be critical in determining the response to allergen. In a recent study investigating the effect of influenza A exposure in early life mice were exposed to HDM from day 14 of life and the authors showed that these neonatal mice did not develop AAD unless prior infection with Influenza occurred at day 3 of life (Al-Garawi et al., 2011). The lack of response to HDM was attributed to an increased baseline level of the regulatory cytokines L-10 and TGFβ in the lungs of day 14 mice, which was overcome by early infection with influenza at day 3 of life. The effects of conveying allergen tolerance to neonates through transfer of allergen derived peptides in breast milk has also been investigated (Krishnamoorthy et al., 2012; Verhasselt et al., 2008). Verhasslet *et. al*. (2008) demonstrated that OVA exposed mothers peptide, rather than specific antibodies, to nursing neonates, which resulted in antigen specific oral tolerance, generated towards OVA. When these mice were challenged with OVA in adulthood they developed reduced AAD compared to mice breastfed by non-exposed mothers. The effect of early viral infection on the oral tolerance generated through breast-milk transfer has been investigated by Krishnamoorthly *et. al*. (2012). Female mice were exposed to OVA while pups remained naïve and antigens were transferred through breast milk, resulting in the generation of OVA specific regulatory T cells (Tregs) in a TGFβ dependent manner. However, this protection was lost if neonates were infected with RSV at the time of weaning (day 21) as OVA specific Tregs were skewed to a Th2 phenotype and began to produce IL-13 resulting in the loss of tolerance. The delicate balance of the

maturing immune system and the constant exposure to new environmental allergens as well as colonisation by the microbiome has recently been described by Gollwitzer *et. al*. (2014), in collaboration with our lab. The emergence of microbiome-induced Helios$^-$ Tregs around day 14 of life explained the discrepancies described between Saglani *et. al*. (2009) and Al-Garawi *et. al*. (2011) as HDM exposure resulted in AAD before the emergence of these cells but as the Helios$^-$ Treg population began to expand; neonatal mice underwent a stage of non-responsiveness to allergen exposure between days 14 and 21. The introduction of a virus at this time-point, as in Krishnamoorthy *et. al*. (2012) would likely disrupt the development of these Tregs.

1.3 The role of IL-33 and ST2 in allergic asthma

1.3.1 Genome wide association studies

In addition to environmental factors influencing the onset of asthma, strong heritability through families shows genetic factors also contribute to the onset of disease (Duffy et al., 1990). A number of genome wide association studies (GWAS) have been carried out to identify causal genes of asthma and therefore potential therapeutic targets. A number of single nucleotide polymorphisms (SNPs) have been associated with asthmatic populations, comprising genes known to be associated with inflammation as well as genes not typically associated with allergic disease. These include SNPs in the region of ORMDL3, RORA and SLC22A5, which have previously been associated with Crohn's disease. Two SNPs in the region of genes known to be associated with inflammation were IL-33 and the IL-33 receptor ST2 (Moffatt et al., 2010; Ramasamy et al., 2012; Torgerson et al., 2011).

1.3.2 IL-33

IL-33 is a member of the IL-1 family of inflammatory cytokines, which exists as both an intranuclear and cytosolic protein. IL-33 is constitutively expressed in cells at environmental barriers, such as epithelial cells, fibroblasts and keratinocytes, and as a nuclear cytokine primed for release as an alarmin in the event of cellular damage (Carriere et al., 2007; Küchler et al., 2008). In contrast to the constitutive expression of nuclear IL-33, expression of the cytokine is actively induced in stimulated immune cells where expression is cytosolic to allow active secretion (Nile et al., 2010). mRNA analysis of murine and human tissue show differential expression profiles of IL-33 with murine IL-33 expressed heavily in the central nervous system as well as skin, lung and stomach, while human IL-33 expression is concentrated to fibroblasts, smooth muscle and bronchial epithelial cells (Schmitz et al., 2005). Despite differential expression, mouse and human IL-33 share 55% amino acid homology (Liew et al., 2010). IL-33 was originally described as a ligand for the orphan receptor ST2 (Schmitz et

al., 2005), a discovery which was made via computational analysis to identify possible interactors with the receptor as traditional receptor pull down methods had failed to isolate a ligand that resulted in signalling through ST2 (Gayle et al., 1996; Kumar et al., 1995). As IL-33 is a member of the IL-1 family of cytokines it was initially proposed that caspase-1 processing would be required to derive an active form of the cytokine (Schmitz et al., 2005). However, subsequent analysis of cytokine processing revealed caspase-1 resulted in inactivation of the cytokine and IL-33 was active in its full-length, uncleaved form (Cayrol and Girard, 2009). In addition to being active as an uncleaved cytokine, processing of IL-33 by neutrophil elastase and cathepsin G has been shown to generate mature forms of IL-33; IL-$33_{95\text{-}170,}$ IL-$33_{99\text{-}270}$ and IL-$33_{109\text{-}270}$, identified as having more potent biological activity than the full length cytokine (Lefrançais et al., 2012). It was proposed that this allows multiple roles for IL-33 with an alarmin function – when IL-33 is released in an unprocessed form in response to cellular damage - and a ten-fold more potent action under conditions of inflammation when the protein is cleaved. This hypothesis was assessed by administering adenoviral constructs encoding either full-length or processed IL-33 (IL-$33_{109\text{-}266}$) to the lungs of mice via the intra-tracheal route (Luzina et al., 2012). Both full length and cleaved IL-33 resulted in lymphocyte accumulation in the lung and this effect was greatest in mice expressing the cleaved IL-33. In addition to greater overall inflammation, only expression of processed IL-33 resulted in the recruitment of eosinophils to the lung, goblet cell hyperplasia and an increase in Th2 cytokines.

As a secreted cytokine IL-33 binds to its receptor ST2, causing intracellular activation of NF-κβ and downstream, cell specific activation. IL-33 signalling through ST2 leads to the polarisation of T cells towards a Th2 phenotype (Schmitz et al., 2005) and results in the activation of mast cells (Ho et al., 2007), natural killer cells (Smithgall et al., 2008), eosinophils and basophils (Pecaric-Petkovic et al., 2009). In addition to numerous effects of the secreted form of IL-33, the cytokine has also been shown to act as a chromatin-associated nuclear factor involved in transcriptional regulation (Carriere et al., 2007). Subsequent investigation of this function determined that IL-33 binds to the p65 unit of

NK-κβ in the nucleus of epithelial cells leading to gene transcription and cell activation (Choi et al., 2012). Nuclear IL-33 activity caused the up-regulation of the adhesion molecules intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on the surface of endothelial cells therefore potentially contributing to a number of inflammatory diseases through the induction of inflammation. IL-33 has the potential to contribute greatly towards the asthmatic phenotype as, in addition to endothelial cells, a number of immune cells also produce IL-33 including macrophages (Chang et al., 2011), dendritic cells (Yanagawa et al., 2011), mast cells (Hsu et al., 2010) and neutrophils (Lefrançais et al., 2012). Release of IL-33 from all of these potential sources will lead to the rapid accumulation of ILC2s (Spits and Di Santo, 2011).

1.3.3 ST2

ST2 was originally identified in 1989 as an orphan receptor proposed to have roles in growth signal transduction (Tominaga, 1989). As with IL-33, ST2 is also expressed by both structural and immune cells including fibroblasts, epithelial cells, mast cells, ILCs, T cells and macrophages (Demyanets et al., 2013). ST2 has since been recognised as a cellular marker to differentiate between Th1 and Th2 cells and results in the production of Th2 cytokines when stimulated (Meisel et al., 2001). ST2 is a member of the Toll-like/IL-1-receptor superfamily. Two different isoforms of the receptor exist, a membrane bound receptor, known as ST2L, and a secreted form, known as soluble ST2 (sST2) (Yanagisawa et al., 1993). ST2L signalling in response to IL-33 is dependent on the formation of a complex with the interleukin-1 accessory protein (IL-1RAcP) (Palmer et al., 2008), this complex recruits MyD88 and dimerization of the two leads to the recruitment of interleukin-1 receptor-associated kinase (IRAK). The downstream stimulation of TNF receptor-associated factor 6 (TRAF 6) then causes the activation of the inhibitor of nuclear factor-κβ (IKK) to release NF-κβ, leading to expression of the inflammatory cytokines IL-4, IL-5 and IL-13 (Kakkar and Lee, 2008; Schmitz et al., 2005). This inflammatory signalling pathway is controlled at a number of levels both when IL-33 is

bound to the ST2 receptor and circulating. Upon binding of IL-33 to ST2, single Ig IL-IR-related molecule (SIGIRR) can form a complex with ST2 to directly inhibit signalling (Bulek et al., 2009). Conversely, sST2 acts to prevent the activation of immune cells in response to IL-33 by 'mopping up' excess unbound cytokine therefore preventing the binding of IL-33 to ST2L (Hayakawa et al., 2007). Investigation of sST2 levels in asthmatic patients has shown that serum levels of the soluble receptor are elevated during an acute asthma attack, therefore actively limiting excess damage that could be caused by large quantities of released IL-33 (Oshikawa et al., 2001). The interaction between ST2 and IL-33 is summarised in figure 2:

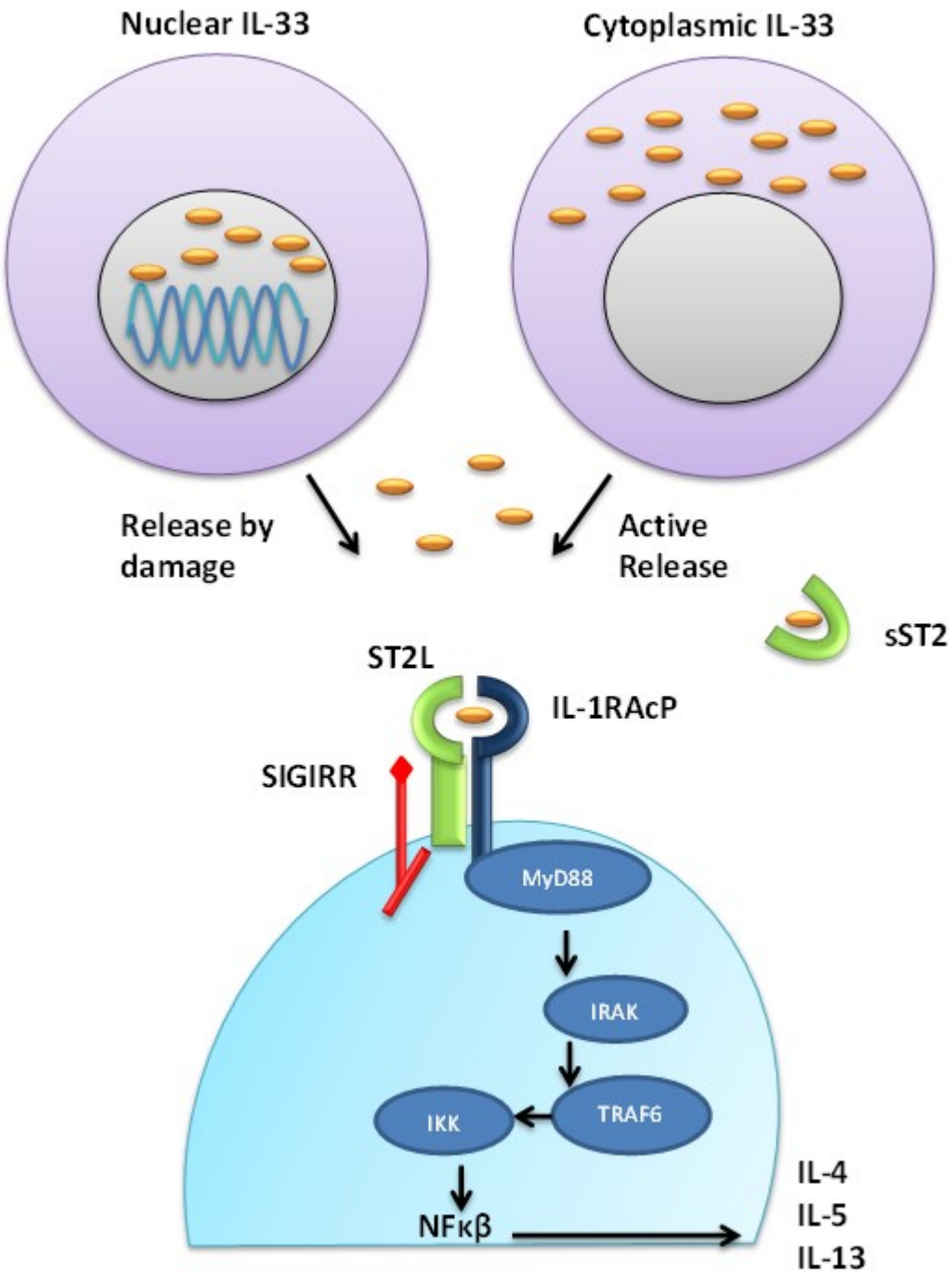

Figure 1.2 IL-33 and ST2 signalling.

The interaction of IL-33 with both soluble ST2 (sST2) and ST2 ligand (ST2L). IL-33 is stored in cells in either the nucleus or cytoplasm. The cytokine is released by either cellular damage (nuclear) or by active release (cytoplasmic). IL-33 then binds to either the decoy receptor sST2, to inhibit signalling, or ST2L where the receptor acts in concert with the IL-1 receptor accessory protein (IL-1RAcP) to recruit MYD88. Signalling via interleukin-1 receptor-associated kinase (IRAK), TNF receptor-associated factor 6 (TRAF6) and inhibitor of nuclear factor-κβ (IKK) then release NFκβ to produce the inflammatory cytokines IL-4, IL-5 and IL-13. ST2 signalling is regulated by the action of single Ig IL-IR-related molecule (SIGIRR).

1.3.4 IL-33 and ST2 in asthmatic patients

SNPs have been identified in the gene regions of both ST2 and IL-33 which are associated with asthma, however the influence of the genetic difference on the expression levels of the protein have not been identified (Moffatt et al., 2010; Ramasamy et al., 2012; Torgerson et al., 2011). Differences in total levels of both IL-33 and ST2 have been identified in patients with allergic and asthmatic disease. Examination of lung biopsies from adult asthmatic and control patients reveal that the expression of IL-33 in airway smooth muscle cells (ASMC) in asthmatics was higher than control patients (Préfontaine et al., 2009). Increased IL-33 expression has also been identified in the bronchial epithelium (Préfontaine et al., 2010), subepithelial cells (Saglani et al., 2013) and recently in serum (Guo et al., 2014a) of asthmatic patients.

In addition to increased IL-33, increased expression of ST2 has also been identified in inflammatory conditions. In patients with allergic rhinitis the nasal epithelium has elevated levels of both IL-33 and ST2L (Kamekura et al., 2012). Unpublished data from our lab show elevated levels of ST2L in bronchial epithelial cells cultured from paediatric patients with severe therapy resistant asthma (STRA) when compared to cultured control cells.

1.3.5 Animal models of IL-33/ST2 targeting

Due to increased levels of IL-33 and ST2 in patients with inflammatory disease, both the cytokine and receptor have been suggested as therapeutic targets for disease and a number of animal models have been investigated to determine the effect of blocking the pathway. Blocking of IL-33 or ST2 has been studied in a number of disease models including rheumatoid arthritis (Palmer et al., 2009), allergic asthma (Kim et al., 2012a; Saglani et al., 2013) and cardiac disease and blocking was shown to be beneficial in these inflammatory conditions. Early therapeutics designed to block ST2 showed Th2 cells in culture, as well as OVA specific Th2 cells adoptively transferred to mice, produced

significantly lower levels of Th2 cytokines when ST2 was blocked before cell stimulation and fewer eosinophils were recruited to the lungs of treated OVA exposed mice (Coyle et al., 1999). Treatment of mice with α-ST2 during the resolution phase of OVA induced AAD resulted in a significant reduction of airway resistance and inflammation one week after the final OVA exposure compared to an isotype control (Kearley et al., 2009). Increasing the level of sST2 to prevent IL-33 binding to ST2L has been achieved by the administration of an adenovirus encoding sST2 prior to OVA sensitisation (Yin et al., 2012). This resulted in reduced IgE production, eosinophil infiltration and reduced Th2 cytokines in the BAL. Similar results have been observed using of an anti-IL-33 blocking antibody in a model of OVA AAD. α-IL-33 treatment throughout OVA sensitisation in these experiments resulted in reduced eosinophils, OVA specific IgE and Th2 cytokine production (Kim et al., 2012b; Liu et al., 2009).

In addition to antibody blocking of both the cytokine and receptor both IL-33$^{-/-}$ and ST2$^{-/-}$ mouse models have been generated and utilised with varying results describing the effect of targeting the pathway for the prevention of allergic disease. Experiments with IL-33 knockout mice showed a reduced influx of eosinophils to the lung, less pulmonary inflammation and reduced airway resistance upon inhaled OVA challenge when compared to IL-33 $^{+/+}$ mice (Oboki et al., 2010). The use of ST2$^{-/-}$ mice in a model of OVA induced AAD has also been carried out and the loss of ST2 caused a dramatic reduction in airway resistance compared to sensitised WT mice and also resulted in a significantly lower population of ILC2s trafficked to the lung (Barlow et al., 2013). As OVA induced AAD involves systemic sensitisation with OVA and adjuvant prior to airway exposure it is not considered an accurate representation of how asthma develops through mucosal sensitisation in humans. ST2$^{-/-}$ mice exposed to 10 consecutive days of intranasal HDM had dramatically reduced Th2 cytokines, reduced eosinophils and reduced total IgE (Chu et al., 2013). Similar results were found in a paediatric ST2$^{-/-}$ model of HDM induced AAD which caused ablation of AHR, reduced pulmonary inflammation and, in contrast to the adult model of OVA exposure, no generation IgE (Saglani et al., 2013). IL-33$^{-/-}$ mice have also been exposed to intranasal HDM and papain and a similar pattern of

reduced BAL inflammation and eosinophils has been documented, although no information on the generation of IgE was provided (Oboki et al., 2010).

1.3.6 Allergen induction of IL-33

The production of IL-33 by the murine pulmonary epithelium has been investigated in response to a number of pathogens and allergens. Infection of mice with Influenza resulted in increased expression of IL-33 in the lung as well as secretion into the BAL (Le Goffic et al., 2011) and infection of mice with RSV led to increased IL-33 protein expression in the lung (Stier et al., 2014). Experiments investigating the expression of IL-33 in response to papain, a protease associated with occupational asthma, utilised an IL-33 reporter mouse model to show the protease activity induced the expression of IL-33 in pulmonary alveoli (Pichery et al., 2012). HDM induced IL-33 production by epithelial cells has been investigated both *in vivo* and *in vitro* utilising air-liquid interface (ALI) cell cultures. Exposure of the epithelium to high dose HDM caused the production of IL-33 in a TLR4 dependent manner involving IL-1α signalling (Willart et al., 2012) while exposure to low dose HDM caused IL-33 production in a GM-CSF dependent manner (Llop-Guevara et al., 2014a). The fungal allergen *Alternaria alternata* (Alt) has been associated with a more severe asthmatic phenotype in both adults and children (Blatter et al., 2014; Halonen et al., 1997; O'Driscoll et al., 2009; Zureik et al., 2002). Alt is a common airborne allergen, which makes limiting exposure difficult. Recently, the association of Alt with severe asthma was investigated in an adult mouse model and found to be due to direct release of IL-33 from the alveolar epithelium in response to serine protease activity of the fungal allergen (Snelgrove et al., 2014). The enhanced IL-33 production and secretion in response to Alt over HDM may be responsible for the association of the allergen with a severe asthma phenotype as IL-33 has been associated with steroid resistance in both human patients and mouse models (Saglani et al., 2013).

1.4 Current and future therapeutics for allergic asthma

Very early treatments developed for asthma focussed on dilating constricted airways with substances including herbs and salts with the active components being refined to generate the now commonly used β_2 agonist bronchodilators (Holgate, 2012). Leukotriene inhibitors are also used in a subset of patients to mediate smooth muscle contraction, mucus secretion and vasodilation (Lynch et al., 1999). However, to date, no other therapy has proved as effective as the anti-inflammatory effects of steroids (Cerasoli, 2006). The majority of patients with asthma respond well to steroid treatment, either inhaled or oral, along with long acting β_2 agonists making symptom management possible. Steroids act by directly interacting with cytoplasmic glucocorticoid receptors (GRs) which transport to the promoter elements and interact with glucocorticoid response elements (GREs) upon steroid ligation (Adcock and Caramori, 2001). Steroids promote the expression of anti-inflammatory mediators such as IL-10 and the inhibitor of NF-κβ I-κBα in addition to suppressing inflammatory mediators such as cytokines, chemokines and adhesion molecules (van der Velden, 1998). Steroids also have the capacity to increase the level of pulmonary Tregs in paediatric patients (Hartl et al., 2007). Despite this broad range of action to decrease inflammation, a small proportion of asthmatics are non-responsive to inhaled, oral and systemic corticosteroids; these patients are classified as steroid resistant asthmatics (SRA) (Barnes and Woolcock, 1998).

1.4.1 Severe asthma and steroid resistance

Severe asthma is defined as involving the use of daily inhaled steroids, periodic use of oral steroids, a history of hospital admissions and a forced expiratory volume (FEV_1) of <80% from predicted baseline (Chung et al., 2014). Although approximately only 5% of asthmatics are defined as having severe asthma they account for almost 50% of the healthcare costs related to asthma (Serra-Batlles et al., 1998). Determining the mechanism of steroid resistance in asthma is an active area of

research in both adult and paediatric disease (Keenan et al. 2012 ; Yim and Koumbourlis 2011). Cells isolated from the BAL of steroid resistant asthmatics showed an increase in the expression of inflammatory cytokines IL-2, IL-4 and IL-5 when compared to cells from steroid sensitive patients (Leung et al., 1995). A decreased level of histone deacetylase (HADC) enzymes – enzymes responsible for the modulation of histone acetylation - have also been identified in lung biopsies from patients with steroid resistant asthma (Hew et al., 2006; Ito et al., 2002). Initial trials of HADC inhibitors in models of mouse allergic airway disease show these drugs have the potential to inhibit airway inflammation (Choi et al., 2005). Due to the extensive effects of corticosteroids, it is unlikely that one abnormal cell type or inflammatory mediator is responsible for resistance. A number of cellular and molecular mechanisms have been suggested to contribute towards steroid resistance including increased collagen in asthmatic airways interfering with steroid signalling (Hakonarson et al., 2005), an increase NO gas causing nitrosylation of the glucocorticoid receptor (Barnes and Adcock, 2009), reduced/defective GRs or an increase in inflammatory transcription factors that compete for binding to the GR (Dong et al., 1988; Leung and Bloom, 2003). Observations from our laboratory indicate that IL-33 is implicated in steroid resistance in paediatric patients (Saglani et al., 2013). A number of treatment options are available for patients with severe therapy resistant asthma and further targets have been proposed as future therapeutics, as summarised below.

1.4.2 Anti-IgE

Omalizumab is an anti-IgE antibody that was developed for use in the therapy of allergic asthma as an addition to current therapies. The antibody targets FCεR3 on IgE and therefore prevents the binding of IgE to both FCεRI and FCεRII (Di Domenico et al., 2011). Omalizumab reduces free circulating IgE as well as mast cell associated IgE to abrogate the acute phase response to allergen exposure (Djukanović et al., 2004). In addition to affecting the acute phase allergic responses, Omalizumab also targets the late phase allergic response through impairing the IgE-dependent

uptake of allergens by mature DCs (Schroeder et al., 2010). Despite the broad effects of the drug and the critical role of IgE in allergic asthma, only a subset of patients respond to treatment despite no obvious differences in the phenotype of disease between responders and non-responders (Bousquet et al., 2007; Humbert et al., 2009).

1.4.3 Targeting IL-4 and IL-13

A number of cytokine and cytokine receptor therapies have been designed and trialled to target the Th2 component of allergic asthma.

IL-4 and IL-13 are structurally similar and signal through a shared receptor (IL-4Rα) to mediate a number of features of asthma (Wills-Karp et al., 1998b). Both IL-13 and IL-4Rα have been targeted as potential therapies for asthma with variable outcomes. An antibody designed to block IL-4α signalling demonstrated no overall improvement in asthmatic symptoms except in a small subset of patients that had higher initial baseline scores in an Asthma Control Questionnaire (ACQ) (Corren et al., 2010). In contrast, an IL-4Rα antibody used by Wenzel *et. al*. (2013) resulted in a significant improvement in lung function parameters, reduced asthma exacerbations and reduced markers of Th2 inflammation in a trial conducted in asthmatics pre-selected for high sputum eosinophil levels (Wenzel et al., 2013). The most recent trials of α-IL-13 antibody have focussed on targeting the cytokine in a subset of patients with severe asthma. A study of GSK679586, a humanised IgG1 mAb, in severe asthmatics demonstrated that the antibody was not responsible for unacceptable adverse events, however, it did not result in clinically significant improvement in treated patients (De Boever et al., 2014). Another human mAb designed to bind to and neutralise IL-13 – Tralokinumab – has also recently been trialled and included a yearlong assessment of exacerbation rates. This trial identified a subgroup of patients which responded to treatment with reduced asthma exacerbations over a year, examination of this subgroup identified patients had baseline high serum levels of periostin or

DP44, both of which are upregulated by IL-13 (Brightling et al., 2014; Takayama et al., 2006; Yong Zhang et al., 2014). Results from this trial further highlight the need for detailed phenotyping of asthmatic patients to identify patients likely to benefit from therapeutic treatment.

1.4.4 Anti-IL-5

IL-5 is also an attractive target for therapeutic intervention as IL-5 production is associated with the proliferation and differentiation of eosinophils, a cell type associated with poor control of allergic asthma (Lopez et al., 1988; Romagnoli et al., 2002). Eosinophils are strongly associated with severe exacerbations of asthma as up to 50% of patients suffering exacerbations were found to have high sputum eosinophil levels (Jayaram et al., 2006). Antibodies against IL-5 have been effective in reducing asthmatic exacerbations and improving lung function in a subset of patients with severe, eosinophilic asthma (Castro et al., 2011; Flood-Page et al., 2007; Nair et al., 2009; Pavord et al., 2012).

1.4.5 Targeting epithelial derived cytokines

As targeting IL-4, IL-5 and IL-13 proved to only be partially successful in improving symptoms of allergic asthma it has been suggested that generating therapies against upstream mediators of disease, such as those secreted by the epithelium, may be more effective as they would prevent further development of allergic inflammation (Lloyd and Saglani, 2010). Cytokines produced by the lung epithelium in response to allergen exposure include IL-25, IL-33 and Thymic stromal lymphopoietin (TSLP) (Hallstrand et al., 2014). To date, therapeutic targeting of IL-33 or ST2 has not been carried out in human asthmatics, although mouse models targeting this pathway indicate such treatments will be beneficial to disease (Kearley et al., 2009; Lee et al., 2014; Yin et al., 2012). As

with IL-33, treatment directed at the IL-25 signalling pathway has also not been trialled in humans despite IL-25 having a role in the remodelling of murine lungs in a HDM model of AAD (Gregory et al., 2013). The only therapy directed against epithelial cytokines in human trials is AMG 157, a human monoclonal antibody that binds TSLP and prevents it from interacting with the TSLP receptor (Gauvreau et al., 2014). This trial assessed the effect of α-TSLP in mild, stable allergic asthma and caused a reduction in eosinophils and reduced bronchoconstriction in response to allergen challenge. However, due to the mild asthma phenotype of the subjects, no improvement in baseline FEV_1 was measured and further analysis is therefore required to assess the effectiveness of the therapy in a cohort of patients with difficult to manage asthma.

1.4.6 Allergen specific immunotherapy

A further option for the treatment of allergic asthma is the targeting of allergen specific immune cells through allergen immunotherapy (AIT). AIT allows the underlying mechanism of disease to be targeted with the aim of inducing anergy or cell death in immune cells responsible for the pathogenesis of disease (Viswanathan and Busse, 2012). AIT has been recognised as a treatment for allergies for over 100 years and recent advances in understanding the underlying mechanisms of action allow for safer and more effective treatment (Gibeon and Menzies-Gow, 2013). Traditionally, increasing concentrations of allergen were introduced to the patient over a period of 3-5 years, however, this carried the risk of both local and systemic hypersensitivity reactions (Abramson et al., 2010). The recent introduction of synthetic peptides to replace whole allergen or allergen fragments has eliminated the possibility of IgE cross linking that led to adverse responses in earlier trials, and has also revealed promising initial results in cat allergic patients via the induction of IL-10 secreting cells (Moldaver and Larché, 2011).

1.5 Hypothesis

The overall hypothesis for this thesis was that both environmental and genetic factors have a significant role in determining the development and severity of allergic airways disease. We hypothesised that the age of first exposure to allergen was a significant determinant, along with the type of allergen to which mice were exposed and that the genetic background would affect disease outcome.

1.5.1 Aims

1. To ascertain the effect of a maturing immune system on the development of allergic airways disease.
2. To identify the differences between HDM and fungal sensitisation and therefore determine the reason for the association of fungal sensitisation with severe, steroid resistant disease.
3. To determine the effect of genetic predisposition to disease by investigating two genes associated with altered SNPs in allergic asthma - IL-33 and ST2.
4. To investigate two potential therapeutics for the treatment of severe asthma – α-ST2 and α-IL-13.

Chapter 2. Materials and methods

2.1 Mice

Balb/c mice were originally purchased from Charles River (Oxford, UK), two breeding pairs of severe combined immune deficient (Beige-SCID) were purchased from Harlan (Bicester, UK). Two breeding pairs of ST2 knockout ($ST2^{-/-}$) mice were obtained through collaboration (Dr Andrew McKenzie, Cambridge, United Kingdom), IL-$33^{-/-}$ (M10011) and a WT control (Balb/CJ) were purchased from Medimmune (Cambridge, UK). All mice were housed at Imperial college animal facility in specific pathogen–free conditions and given food and water *ad libitum.* All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986. All genotypes were maintained by in-house breeding; this was carried out at a ratio of two females to one male. Pregnant female mice were housed separately and once born, remained with litters until they were weaned at day 21 of life.

2.2 Induction of allergic airways disease

2.2.1 HDM preparation and dosing

House dust mite extract (Greer Laboratories, Lenoir, USA) was reconstituted in sterile PBS (Sigma, Poole, UK) to a concentration of 1mg/ml for experiments in which mice received 15µg HDM and 2mg/ml for mice receiving 25µg of protein. Mice were first exposed to HDM from a variety of ages including day 3, 7, 14, 21, 28 or 6-8 weeks and exposure was continued for three weeks, three times per week. Up to day 14, neonatal mice received intranasal HDM (10µg or 20µg in 10µl PBS) or PBS without anaesthesia. After day 14 of life, mice were dosed by intranasal administration of HDM (15µg in 15 µl PBS or 25µg in 12.5µl PBS) or equivalent volumes of PBS using recovery anaesthesia of inhaled isoflurane. These volumes were chosen to closely mimic the volumes administered to mice in a study conducted by Saglani *et. al* (2009) for low dose experiments and Al-Garawi *et. al*. (2011) for high dose experiments. Neonatal experiments were conducted on a mix of male and female mice

while adult experiments were conducted on female mice only. Outputs for HDM induce AAD were assessed 4 hours after the final allergen exposure.

2.2.2 *Alternaria alternata* preparation and dosing

Lyophilised *Alternaria alternata* (Greer) was reconstituted in sterile PBS to a concentration of 1mg/ml and stored in aliquots at -20°C, the allergen was then diluted further with sterile PBS immediately prior to dosing. Exposure of neonates began at day 3 of life with mice receiving 5µg protein suspended in 10µl PBS i.n. After day 14 of life and throughout adult exposure; mice received 10µg protein suspended in 25µl PBS i.n, or equivalent volumes of PBS, under recovery anaesthesia of inhaled isoflurane three times per week. During short term dosing protocols (Chapter 6) mice received either 20µg or 25µg Alt, both suspended in 25µl PBS. This volume of allergen was selected due to preliminary Alt exposure experiments conducted in the lab that determined exposure of the lower airways to Alt was vital for sensitisation to occur (Data not shown). Neonatal experiments were conducted on a mix of male and female mice while adult experiments were conducted on female mice only. Outputs for Alt induced AAD were assessed 18 hours after the final allergen exposure.

2.2.3 Ovalbumin preparation and dosing

Mice were sensitised using OVA (Sigma) as previously described (McMillan et al., 2002). Mice were injected i.p with OVA/Alum at days 0 and 12 and control mice received PBS/Alum. OVA/Alum was prepared by generating a 0.1mg/ml OVA solution in sterile PBS and mixing this at a ratio of 1:2 with alum (Au-Gel-S, Serva Electrophoresis). This solution was mixed for 45 minutes at room temperature before injecting a volume of 300µl per mouse i.p. Both OVA/alum and PBS/alum mice were then exposed daily to 5% aerosolised OVA for 20 minutes between days 19 and 24. Parameters were

assessed 24 hours or 7 days after the final exposure to OVA. Female Balb/c mice aged 6-8 weeks were used for all OVA sensitisation experiments.

2.2.4 Recombinant IL-33 dosing

Recombinant mouse IL-33 (rIL-33) (eBioscience, San Diego, USA) was administered to 14 day old mice either concurrently with HDM exposure (Balb/c mice) or alone (Balb/c and SCID mice). rIL-33 was dosed i.n at a concentration of 50ng/g in a volume of 15µl PBS; mice were dosed under recovery anaesthesia of inhaled isoflurane three times per week for one or two weeks. When administered alongside HDM exposure, rIL-33 dosing was conducted at least 6 hours prior to HDM exposure to avoid a high volume of fluid in the lungs of young mice. When administered individually, inflammatory parameters were assessed 18 hours after the final dose.

2.3 Preventative and therapeutic interventions

2.3.1 Budesonide

Budesonide (Bud) Respules (Breath Ltd. UK) (0.25 mg/mL) were used for intranasal administration at a concentration of 0.6 mg/kg. Neonatal mice received Bud five times per week without anaesthesia up to day 14 of life and under recovery anaesthesia of inhaled isoflurane after day 14 of life. On days in which mice received Bud and HDM, Bud was administered at least 6 hours prior to HDM to avoid large volumes of fluid being administered.

2.3.2 Anti-IL-13

α-IL-13 antibody (UCB CA154_582), a gift from UCB Celltech, was administered i.p (10 mg/kg) twice weekly throughout Alt exposure of Balb/c mice. Relevant isotype-matched antibodies were used as controls for injection. For preventative experiment protocols, α-IL-13 was administered prior to the first exposure to Alt.

2.3.3 Anti-ST2

During OVA exposure of Balb/c mice α-ST2 was administered as previously described (Kearley et al., 2009) or administered throughout OVA exposure. Mice were injected i.p three times with either 2mg/kg or 10mg/kg α-ST2 (CNTO3914) or 10mg/kg isotype control (CNTO5516) during or after OVA exposure. In short-term Alt exposure protocols, α-ST2/isotype control was injected either i.p or subcutaneously (sub-cut) prior to exposure to Alt.

2.4 Measurement of lung function by flexivent

Airways resistance was measured using the FlexiVent small-animal ventilator (SCIREQ, Montreal, Canada) according to our established protocols (Saglani et al., 2009). Mice were anesthetized with pentobarbital sodium (intraperitoneal at 50mg/kg) and ketamine (intramuscular at 200mg/kg). After testing for a loss of pain response by pinching the mouse's paws, mice were tracheotomised and connected to the flexiVent ventilator via a blunt-ended needle; 21-gauge needle for 3 week old mice and 19-gauge from 4 weeks old onwards. 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 H_2O. Measurements of resistance were determined from a user-defined protocol by using the snapshot-150 perturbation, which is a single-frequency sinusoidal wave at a

frequency equivalent to the ventilation rate (2.5 Hz). Resultant data were fitted by using multiple linear regressions to the single-compartment model in the following form:

$$Pressure = (Resistance * Flow) + (Elastance * Volume) + Fittingconstant$$

Changes in resistance to increasing concentrations of nebulized methacholine (3-100 mg/ml, Sigma) were measured from snapshot perturbation measurements taken by using the forced oscillation technique.

2.5 Isolation of tissues, cells and serum

2.5.1 BAL collection and preparation

After flexivent analysis, bronchoalveolar lavage (BAL) was collected via the tracheal cannula. For mice aged three weeks, 3x300 µL PBS was used and from 4 weeks onwards, 3 × 400µL PBS was used, recovered volume was recorded and cell counts were adjusted accordingly to give a number per ml recovered BAL. Lavage fluid was centrifuged at 200*g* for 5 minutes at 4°C. Cell pellets were re-suspended in 0.5 mL of complete medium (RPMI + 10% FCS, 2 mmol/L l-glutamine, and 100 U/mL penicillin/streptomycin) and total yield of BAL cells were quantified using a haemocytometer. BAL supernatant was collected and stored at -80°C for subsequent analysis of cytokines.

2.5.2 Lung collection and preparation

After lavage, the lungs were removed intact and lobes were separated. The superior lobe was isolated and fixed in 10% normal buffered formalin for 24 hours. Tissue was then processed in house (Lorraine Lawrence, Leukocyte biology histology service, Imperial College) by embedding in paraffin and mechanically slicing to 4µm onto a glass slide before staining with haematoxylin and eosin (H&E) (Lorraine Lawrence) or using for immunohistochemistry (IHC). The middle and inferior lobe was snap

frozen in liquid nitrogen and stored at -80°C prior to processing for examination of cytokines by ELISA. The post-cavical lobe was submerged in RNAlater® (Lifetechnologies, Carlsbad, USA) for 24 hours at 4°C before removal of the RNAlater® and storage of the lobe at -80°C prior to extracting RNA. The left lung lobe was mechanically chopped and incubated at 37°C for 1 hour in complete media containing 0.15 mg/mL collagenase (Type D; Roche Diagnostics, Mannheim, Germany) and 25 mg/mL DNAse (Type 1, Roche Diagnostics). Cells were recovered by means of filtration through a 70µm nylon sieve (Falcon, BD Biosciences, Oxford, UK). Red blood cells were lysed (with RBC lysis buffer prepared in house - 8.29g NH_4Cl, 1.0g $KHCO_3$, 37.2mg Na_2EDTA to 800ml dH_20, adjusting to pH 7.2-7.4 and bringing to a volume of 1L) in 5ml buffer for 3 minutes, washed twice and re-suspended in 1 mL of complete medium. The total cell yield was quantified by using a haemocytometer.

2.5.3 Blood collection and preparation

At the termination of a protocol, cardiac puncture was performed under terminal anaesthesia. Blood was transferred to a microtainer with serum separation gel (BD, Fischer scientific) and centrifuged at 14000RPM for 5 minutes to separate serum. Tubes were then stored at -20°C prior to analysis of cytokines and immunoglobulins.

For tail bleed experiments, mice were housed individually in a hot box at 37°C before restraining in a restraint tube and puncturing a tail vein with a needle. Blood was collected from the tail vein by capillary collection and allowed to clot overnight at 4°C. Sequential tail bleeds were performed every 4 days over a three week period during the Alt exposure protocol with a maximum 40µl collected at each time point. The samples were then centrifuged at 14000RPM and serum transferred to a small Eppendorf tube for storage at -20°C.

2.5.4 Spleen collection and preparation

Splenic cells were used for compensation staining during flow cytometry experiments. The spleen was filtered through a 70µm filter and centrifuged at 1200RPM for 8 minutes at 4°C. The cell pellet was then re-suspended in RBC lysis buffer before washing twice. The RBC lysed pellet was then re-suspended in 5ml RPMI.

2.6 Cytospins and differential counts

BAL and lung cells were diluted to a concentration of $5x10^5$ per ml and 100µl was spun onto a glass slides by cytocentrifugation at 400RPM for 4 minutes (Cytospin 4, ThermoShandon, UK). Slides were then air dried and cells fixed in 100% methanol. Staining with Wright-Geimsa (Sigma) was carried out before differential counts were performed to determine the proportions of macrophages, eosinophils, neutrophils and lympho-mononuclear cells. A minimum of 400 cells were counted for each sample. All cells were performed blind by the same observer.

2.7 Flow cytometry

2.7.1 Extracellular staining

After preparation and counting of pulmonary, BAL and splenic cells, cells were plated on round-bottom 96 well plate at 500,000 cells per well. Cells were stained in PBS/1% FCS/0.1% sodium azide. To prevent non-specific binding, cells were incubated with rabbit serum (Sigma-Aldrich) for 15 min before staining with extracellular antibodies (Table 2.1) or a fluorescent minus one (FMO) control for 20 min at 4°C in filtered facs buffer. Stained cells were washed twice and fixed in fixation buffer overnight (eBioscience).

2.7.2 Intracellular and intranuclear staining

For intracellular cytokine staining, cells were stimulated with PMA (20ng/ml) (Sigma-Aldrich) Ionomycin (1µg/ml) (Emdchemicals Inc, San Diego, CA, USA) in the presence of Brefeldin A (10µg/ml) (Sigma-Aldrich) for 3 hours prior to extracellular staining. Thereafter, cells were permeabilised using permeabilisation buffer (eBioscience) before staining for intracellular cytokines or FMO controls (Table 2.1). Cells were then washed once in permeablisation buffer and twice in filtered facs buffer before being re-suspended in 200µl facs buffer for analysis. Intranuclear staining for transcription factors was carried out in intranuclear fixation and permeabalisation buffer (ebioscience).

2.7.3 *In vitro* stimulation with allergen

Prior to PMA/Ionomycin stimulation, some mixed lung cell samples were incubated *ex vivo* with *Alternatia alternata* (Alt). Cells were plated at 500,000 cells per well in 96 well plates in complete media. All samples were stimulated with 2µg Alt per well for 48 hours before stimulating and staining for facs analysis. Half of the supernatant was removed after 24 hours and stored at -80°C for downstream cytokine analysis. This was replaced with fresh media and Alt for a further 24 hours before being removed and stored for analysis.

2.7.4 Flow cytometric analysis

All stained samples were analysed using a LSR Fortessa (BD) and data was analysed using FlowJo software (Tree star, version 7.6.5).

Target	Conjugated Dye	Supplier	Isotype	Dilution
CD3	PB	eBioscience	Rat IgG2b	1:100
CD3	APC	BD Biosciences	Rat IgG1	1:100
CD4	PerCP-CY5.5	eBioscience	Rat IgG2a	1:100
CD45	Percp	eBioscience	Rat IgG2b	1:100
ICOS	PeCY7	Biolegend	Hamster IgG1	1:100
T1ST2	Fitc	Morwell Diagnostics	Rat IgG1	1:100
Lineage cocktail (CD3, CD45R, CD11b, TER-119. and LY-G6)	PB	eBioscience	Rat IgG1	1:50
CD25	APC	eBioscience	Rat IgG1	1:100
CD11b	PB	eBioscience	Rat IgG2b	1:100
CD11c	PerCP-CY5.5	eBioscience	Hamster IgG	1:100
SiglecF	PE	BD Biosciences	IgG2a	1:100
GR1	APC	eBioscience	Rat IgG2b	1:100
CD68	FITC	Biolegend	Rat IgG2a	1:100
IL-13	PE	eBioscience	Rat IgG1	1:100
IL-17	APC	BD Biosciences	Rat IgG1	1:100
FoxP3	PE	eBioscience	Rat IgG2a	1:100
Helios	FITC	Biolegend	Hamster IgG	1:100
KI67	PeCY7	eBioscience	Rat IgG2a	1:100

Table 2.1 Antibodies for extracellular, intracellular and intranuclear staining.

A list of antibodies detailing antibodies used for cell staining. Dye abbreviations; FITC - Fluorescein isothiocyanate, PE -R– Phycoerythrin, PE-Cy7 - Tandem dye-R-Phycoerythrin with Cy7, PerCP-Cy5.5 - Tandem dye-Peridinin chlorphyll protein with Cy5.5, PB – Pacific Blue eFluor 450, APC - allophycocyanin.

2.7.5 Innate cell gating strategy

Eosinophils, neutrophils and macrophages were identified by gating for live cells and gating out the lymphocyte population. Eosinophils were gated as Siglec F*CD11c^{-}, neutrophils as GR1^{high}CD11b^{+} and macrophages as CD68^{+}CD11c^{+}.

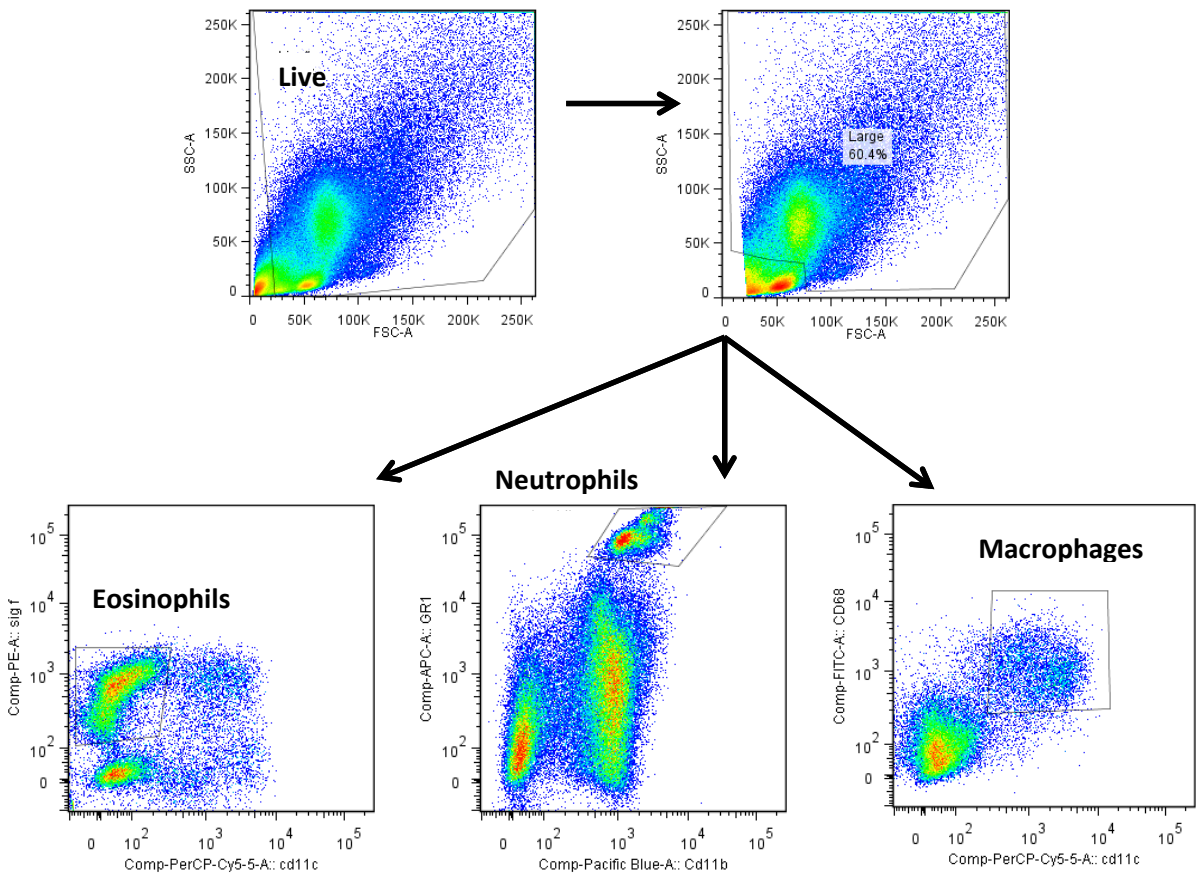

Figure 2.1 Innate cell gating strategy.

Live cells were selected and the lymphocyte population excluded by selecting all cells except low SSC and low FSC. Eosinophils were gated as SiglecF^{+}CD11c^{-}, neutrophils as GR1^{high}CD11b^{+} and macrophages as CD68^{+}CD11c^{+}. Figure shows representative flow cytometry plots.

2.7.6 Innate lymphoid cell gating strategy

Innate lymphoid cells were gated as lineage negative, CD45 and ICOS positive ($Lin^{-}CD45^{+}ICOS^{+}$). FMO controls were then used to enable the gating of IL-13^{+} ILCs. Due to the large population of $ST2^{-}$IL-13^{+} ILCs, it was decided not to include ST2 in the gating strategy.

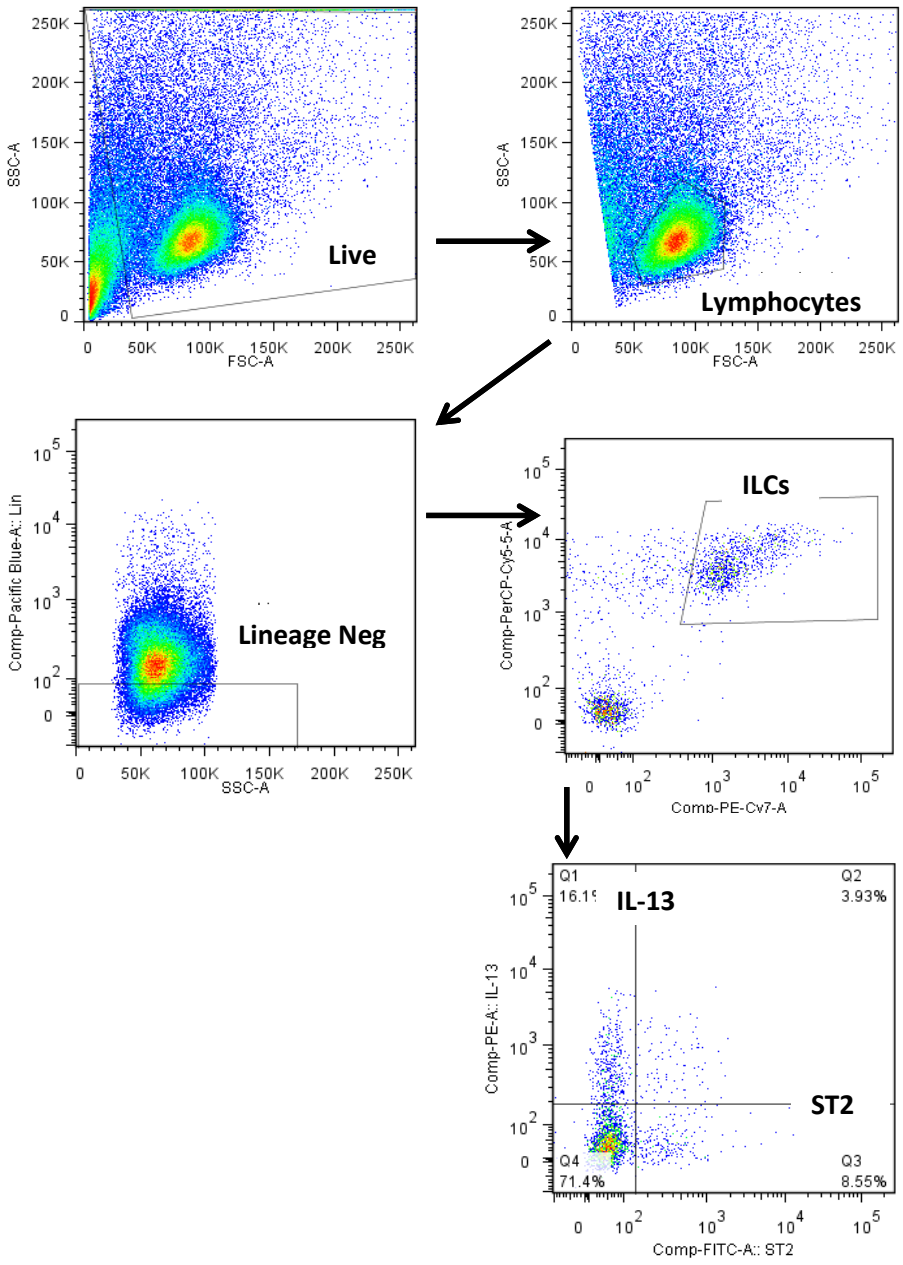

Figure 2.2 Gating of ILCs

Live cells were selected and lymphocytes selected by gating SSC and FSC low. Lineage negative cells were isolated and a population of CD45+ICOS+ cells represent ILCs. ILCs were investigated for IL-13 and ST2. Figure shows representative flow cytometry plots.

2.7.7 T cell gating strategy

T cells were gated as $CD3^{+}CD4^{+}$ lymphocytes. T cells were then gated for both $IL\text{-}13^{+}$ subsets and in *in vitro* experiments, $Ki67^{+}$ T cells. T cells were also analysed for CD25, FoxP3 and Helios to identify regulatory T cells.

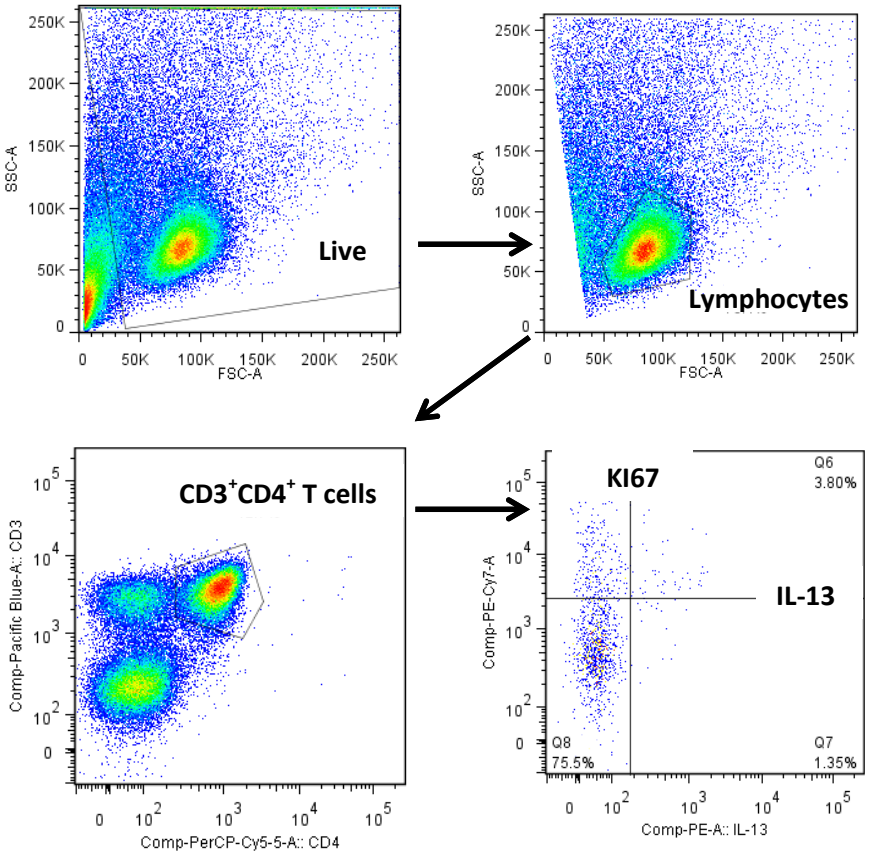

Figure 2.3 T cell gating strategy

Live cells were selected and the lymphocyte population gated by selecting SSC and FSC low cells. CD3+CD4+ cells were then gated and these were investigated for both KI67 and IL-13 production. Figure shows representative flow cytometry plots.

2.8 Immunoglobulin and cytokine measurements

2.8.1 ELISA protocol

A generalised ELISA protocol was used for most assays, with the exception of pre-coated kits. 96 well enhanced protein-binding plates were coated with capture antibody in an appropriate coating buffer overnight at 4°C. Plates were then washed and blocked before diluted samples were added in an appropriate dilution buffer. Simultaneously, a serially diluted standard was plated to allow the generation of a standard curve for quantification of protein, along with blank wells with no standard as a negative control. Samples were incubated overnight at 4°C before plates were washed and biotinylated antibodies were added. Plates were then washed again and streptavidin-HRP added and plates incubated at room temperature for 30 minutes. Plates were washed and K-blue substrate (Neogen, Lansing, USA) added until the lower end of the standard curve developed to a light blue colour. The reaction was then terminated by adding 0.19M H_2SO_4 and the optical density was measured using a spectrophotometer (Tecan, Switzerland) set to detect 450 nm. Prism (GraphPad 5) was used to analyse protein concentration by plotting non-linear regression of standard curves allowing the calculation of unknown values.

2.8.2 Serum immunoglobulin ELISAs

IgE, IgG1 and IgG2a were measured in collected serum using paired antibodies (BD Biosciences) as per manufacturer's instructions. HDM, Alt and OVA specific immunoglobulins were also quantified in the serum. Enhanced protein binding plates were coated with allergens; 50µg/ml for HDM and Alt, 100µg/ml for OVA specific assays, overnight at 4°C. Plates were then washed and blocked before Ig levels were detected using biotinylated secondary antibodies (BD bioscience).

2.8.3 Cytokine ELISA

BAL supernatant, serum and snap frozen lung tissue were all analysed for cytokines. Lung tissue was homogenized at 50 mg/ml in HBSS (Gibco, Life Technologies) containing protease inhibitor tablets (Roche Diagnostics) and centrifuged at 1600RPM for 20 min, and supernatants were collected. Paired antibodies for IL-4, IL-5, TGFβ and IFNγ were used in a sandwich ELISA (BD Biosciences). IL-33, MCPT-1 and IL-10 were measured using R&D duoset kits (R&D systems, Abingdon, UK). IL-13, IL-25 and TSLP were measured using Ready-SET-Go® ELISA kits (eBiosciences) with the exception of chapter 3, in which IL-13 was measured using a precoated IL-13 Quantikine™ kit (R&D systems). All ELISA kits were used as per manufacturer's instructions.

2.9 RNA isolation and PCR

2.9.1 RNA isolation

For RNAseq analysis and further investigation of results by qPCR, RNA was extracted from lungs isolated from allergen naïve mice of varying ages – day 3, day 7, day 14 and day 28 in addition to day 14 mice that had been exposed to HDM from day 3 of life. For maximal RNA yield, whole lung was collected and RNA extraction performed after 24 hours in RNAlater. RNA was extracted using the RNeasy mini kit (Qiagen, Crawley, UK) with the addition of an on column DNA digestion step with DNA-free DNase (Ambion, Lifetechnologies). Concentration and an absence of DNA contamination was assessed using a spectrophotometer (Nanodrop, Thermo Scientific).

2.9.2 RNAseq analysis

For RNAseq analysis, isolated RNA was shipped to the Wellcome Trust Centre for Human Genetics (WTCHG) where analysis was undertaken. On two distinct chips, two technical replicates of each

sample were analysed via a paired-end sequencing to a read depth of 50bp using the Illumina HiSeq 2000 platform. TopHat 2 was utilised to align sequences to the Mouse37 reference genome and produce Binary Alignment/Map (BAM) files. BAM files were then made available for downstream analysis by our lab in addition to a list of the top 100 differentially expressed genes between our selected age comparisons – day 14 vs. day 3, day 28 vs. day 3 and day 14 vs. day 3. Downstream analysis in our lab was then conducted through import of BAM files to Partek Genome Suite (PGS). Principle component analysis of technical replicates showed all replicates were sufficiently clustered to allow merging of the data. Gene expression levels were then analysed against Ensemble *mus muscularis* reference transcriptome (release 75) and abundant transcripts were identified as those with $2^{9.5}$ mapped fragments in at least one sample. Hierarchical clustering was then used to determine relative expression between day 3 (D3), day 14 (D14) and day 28 (D28) samples. Clusters in which expression was $D3^{high}$+$D28^{high}$+$D14^{low}$ or $D3^{low}$+$D28^{low}$+$D14^{high}$ were then selected and further refined by excluding transcripts for which differential expression between the D3+D28 cluster and the D14 cluster was statistically insignificant. The final transcript list contained 101 genes which, combined with a literature review, was used to generate a list of 6 candidate transcripts for validation by qPCR.

2.9.3 Validation of RNAseq results by qPCR

qPCR was used to quantify expression of the 6 candidate genes identified by analysis of RNAseq results. RNA was extracted as described above (2.9.1) using RNeasy plus mini kits (Qiagen). 1µg of RNA was then reversed transcribed using a high capacity reverse transcription kit (Applied Biosystems, Warrington, UK). TaqMan probes and TaqMan advanced mastermix was used (Applied Biosystems) to identify selected cDNA. 6µl real-time PCR reactions were then performed on ViiA7 system (Applied Biosystems).

2.10 GFP AAV library screening

A Library of GFP expressing adeno-associated virus (AAV) serotypes were provided by the University of Pennsylvania Vector Core Facility (Philadelphia, PA, USA). A number of serotypes were administered i.n or i.v, by injection of the superficial temporal vein, to investigate the most appropriate candidate and route for alveolar pulmonary overexpression of genes. For i.n administration, 1 day old neonates received $4x10^{11}$ genome copies of the AAV serotypes suspended in 10µl sterile PBS without anaesthesia. For injection of the superior temporal vein a protocol described by Dr. Simon Waddington was utilised (Rahim et al., 2011). Briefly, 1 day old neonates were subjected to hypothermic anaesthesia before injection of $4x10^{11}$ genome copies of AAV, suspended in 40µl sterile PBS, was injected into the superior temporal vein using a 33-gauge needle. Neonates were then allowed to warm up before returning them to their mothers. Both i.n and i.v injected mice were assessed after 6 weeks for pulmonary expression of GFP. The serotypes investigated are displayed in table 2:

Serotype	**Route of administration**
AAV2	Intranasal and intravascular
AAV2/9	Intranasal and intravascular
AAV2/6.2	Intranasal and intravascular
AAV9	intranasal and intravascular
AAV2/8	intranasal and intravascular
AAV2/5	intranasal and intravascular

Table 2.2 AAV serotypes screened for GFP expression

A table of AAV serotypes containing GFP that were screened and route of administration.

2.10.1 GFP Immunohistochemistry

Lung sections from mice infected with GFP-expressing AAV constructs were investigated for GFP expression via immunohistochemistry. Sections were de-waxed by incubating in Histoclear (Fisher Scientific) three times for 5 minutes. Slides were then rehydrated in 100%, 90%, 75% alcohol and water for 30 seconds each. Antigen retrieval was achieved through microwaving slides in 10mM sodium citrate buffer for 9 minutes before washing twice in PBS (2 x 5 minutes). Sections were then blocked by incubating in 10% donkey serum (Sigma) for 30 minutes at room temperature. After blocking, sections were incubated with Anti-GFP (Abcam ab290; 1:1000) in 1% mouse serum (Sigma) at 4°C overnight. Slides were then washed in PBS and incubated with a flourochrome conjugated anti-goat antibody (Alexa 594 or Alexa 488) (Life Technologies) at a concentration of 1:250 in 1% mouse serum for an hour at room temperature. Slides were washed again with PBS before mounting a coverslip with pro-long anti-fade mounting media with DAPI (Life Technologies).

2.11 Plasmid generation and expansion

2.11.1 Expansion of Trueclone plasmid

A pCMV6-Kan/Neon plasmid containing a full length mus muscularis IL-33 (cDNA clone MGC:6426) was purchased from TrueClone (OriGene Technologies, Rockvville, USA). The plasmid insert corresponded to the Genebank sequence for full length murine IL-33 – NM_133775.2, with the exception of a single nucleotide at position 595. This plasmid was transformed into α select chemically competent E.coli cells (Bioline, UK) as per manufacturer's instructions. Transformed cells were selected via Kanamycin resistance by plating E.coli on agar plates containing 50µg/ml kanamycin (Life Technologies). Individual colonies were then selected and expanded in 5ml LB broth containing 25µg/ml Kanamycin overnight. Eternal cultures were generated using a mix of glycerol

and cultured E.coli before the plasmid was extracted from the culture using a Miniprep kit (Qiagen) as per manufacturer's instructions. The plasmid insert was then sequenced (Eurofin Scientific, Luxembourg) before being used for downstream applications.

2.11.2 Transfection of macrophages

Bone marrow derived macrophages were generated from Balb/c bone marrow. Femurs were flushed with sterile RPMI to collect bone marrow cells. Cells were then pelleted by centrifugation at 1500RPM for 5 minutes and re-suspended in media containing M-CSF (Peprotech, New Jersey, USA), L-Glutamine and β-mercaptoethanol to a concentration of $5x10^5$ cells per ml. Cells were cultured in 10cm petri dishes for 8 days at 37°C. Cells were harvested with EDTA and plated on a 24 well plate in fresh media at a concentration of 500,000 cells per well. Cells were incubated overnight before transfection with the IL-33 containing plasmid using X-tremeGENE HP DNA transfection reagent (Roche) as per manufacturer's instructions. Transfected cells were incubated for 24 hours before addition of PBS, HDM (5µg/ml), LPS (10µg/ml) or Alt (5µg/ml) to the culture supernatant. Cells and supernatant were then collected 1, 2, 4 and 24 hours after stimulation for assessment of IL-33 production and release.

2.11.3 Correction of single nucleotide

In order to correct a single nucleotide at position 595, 5'-phosphorylated forward and reverse primers were generated as shown in table 2.3; the forward primer contained the correct nucleotide (highlighted in yellow). 'Inverse PCR' was performed, as described by Reikofski *et. al*. (1992), to allow correction of the single nucleotide in the TrueClone plasmid (Reikofski and Tao, 1992). *pfu* DNA polymerase (Thermo Scientific) was used to ensure minimal errors and cause the required blunt ends to allow the linearised PCR product to re-circularise and generate a plasmid with the desired

correction. The PCR product was then re-sequenced to validate the corrected nucleotide before continuing with insertion into the AAV backbone plasmid – pZac2.1.

2.11.4 Insertion of genes into AAV backbone plasmid

To allow generation of an AAV9 virus containing full length IL-33 the DNA sequence was inserted into pZac2.1 (Penn Vector Core, Pennsylvania, USA). Primers were designed to allow the isolation of IL-33 from the base pair corrected Trueclone plasmid with the addition of restriction sites. The forward primer was designed to contain an Xho1 'sticky end' and the reverse primer provided a Not1 addition (Table 2.3). PCR was performed to expand the insert with the addition of restriction sites using *pfu* polymerase (Termo Scientific) to minimise error, and restriction digest was performed to ligate the insert and pZac2.1 using Xho1 and Not1 restriction enzymes (Invitrogen) as per manufacturer's instructions. Due to inverted terminal repeats (ITR) in the backbone plasmid, recombinase minus E.coli – Sure2 supercompetent cells (Aglient, santa Clara, USA) – were used for plasmid transfection as per manufacturer's instructions. The resulting plasmid (Fig2.4 A) was then expanded in LB broth with the addition of Ampicillin (Sigma) and an endotoxin free Megaprep (Qiagen) was used to isolate expanded plasmid. The plasmid components were verified by restriction digest with XhoI, Not1, PVUII, BGLII, HindIII, ClaI and PVUI (Invitrogen) (Fig 2.4 B) before 1.2mg was sent to the Vector Core facility (University of Pennsylvania, USA) for insertion into AAV9.

Primer	Sequence	Melting temperature (°C)
IL-33 forward	Phospho - TGGATGGGAAGAAGCTGATGGTGAAC	69
IL-33 reverse	Phospho - CACCGTCGCCTGATTGACTTGCAG	71
IL-33 forward + xho1	ATATCTCGAGATGAGACCTAGAATGAATTATTCCAACTAA	65.5
IL-33 Reverse + Not1	ATATGCGGCCGCCGTTTTACTGCATTAGATTTTCGAG	64.5

Table 2.3 Primer sequences for nucleotide correction and addition of restriction enzyme sites

Primer sequences used for the correction of a single nucleotide using phosphorylated forward and reverse primers – base pair correction is highlighted in yellow. Also, primer sequences for the addition of restriction enzyme sites Xho1 and Not1 to the IL-33 sequence – the xho1 restriction site is highlighted in blue and the Not1 site in green.

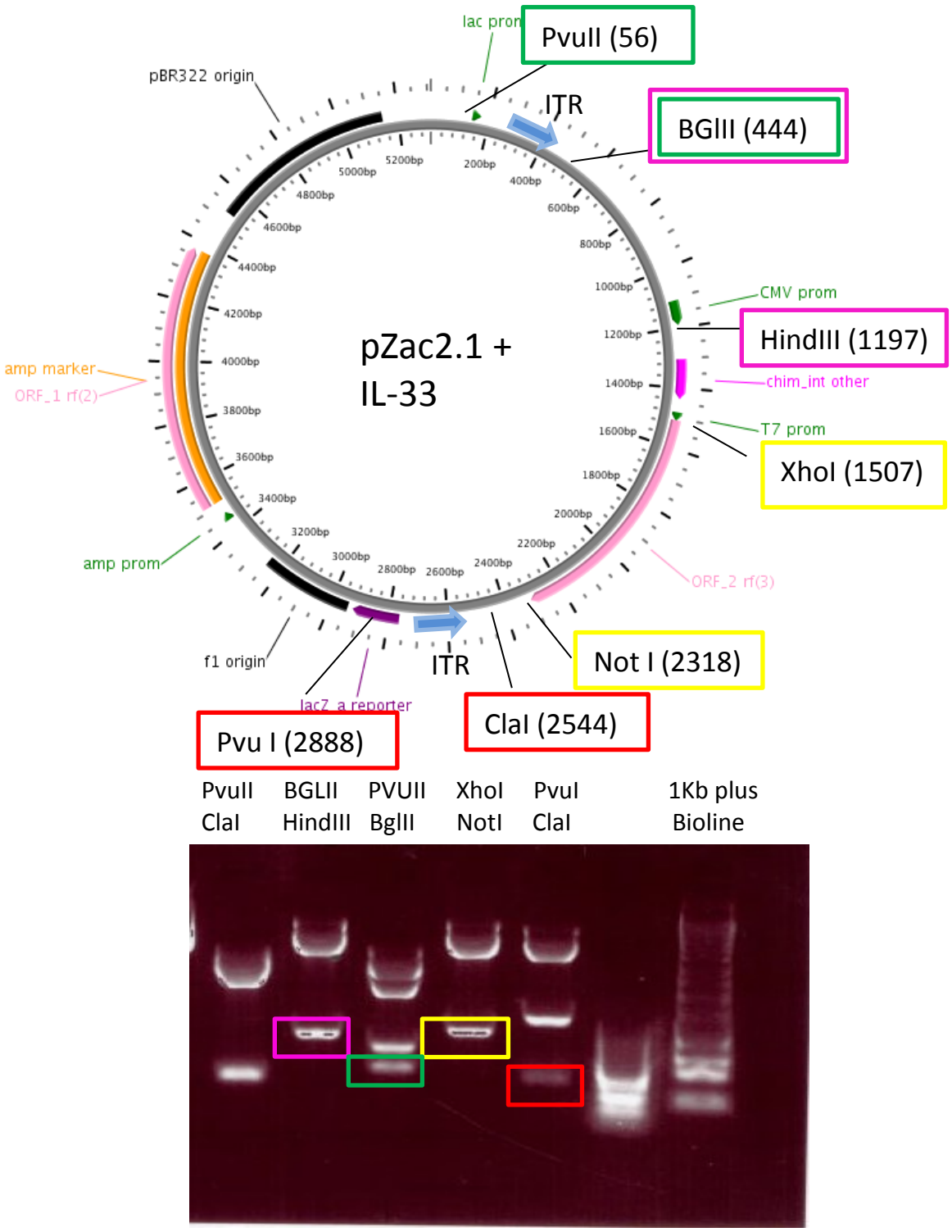

Figure 2.4 Plasmid composition for insertion into AAV9

Verification of the plasmid construct. Plasmid map showing the key components to be verified by restriction digest **(A)** and an agarose gel image of restriction digest products; inverted terminal repeats are depicted in red and green, the CMV promoter in pink and the IL-33 insert in yellow **(B)**.

2.12 Statistical analyses

Data were analysed using GraphPad Prism 5 software (GraphPad Software). Non-parametric tests were used to detect differences between groups and statistical significance accepted when $P < 0.05$. Mann–Whitney U was used to detect differences between two groups and Kruskal-Wallis with a Dunns post-test was used to assess differences between multiple groups. Power calculations to determine the number of mice required per experiment were originally carried out as required by the Home Office for project licence application and determined to be four control and six treated mice per experiment. Power calculations were performed to calculate a sample size necessary per treatment group to be 90% confident to correctly detect differences of at least 30% and therefore use the minimum number of animals to determine significance. Any experiments utilising fewer mice were therefore preliminary.

Chapter 3. Age at first allergen exposure determines the magnitude of allergic response

3.1 Introduction

Our lab have previously published that chronic exposure to inhaled HDM initiated either from day 3 of life in neonatal mice (Saglani et al., 2009) or at 6-8 weeks of life in adult mice (Gregory et al., 2009) resulted in robust AAD with increased airway hyperresponsiveness (AHR), pulmonary inflammation, Th2 cytokines and HDM specific IgE. However, several recent publications have shown that young mice exposed to inhaled HDM from 14 days of age did not develop AAD without a previous stimulation, such as Influenza infection in the first week of life (Al-Garawi et al., 2011). It was proposed that young mice were hypo-responsive to HDM exposure because they had elevated levels of IL-10 and TGFβ compared to adult mice. The development of the postnatal immune system occurs in the presence of constant exposure to previously unseen antigens at mucosal surfaces including both innocuous particles and the developing microbiome. To prevent unnecessary inflammation in early life the postnatal immune system is skewed towards developing both Th2 and regulatory responses to allow the development of tolorogenic responses to encountered antigens (Adkins et al., 2003; Al-Garawi et al., 2011; Krishnamoorthy et al., 2012). Two recent studies have identified cell populations in early life thought to allow the colonisation of the microbiome in the absence of inflammatory responses. In a murine model, the emergence of Helios$^-$ Tregs has been shown as vital for tolerance of the colonising pulmonary microbiome. In human infants, a recent publication has identified a population of IL-8 producing T cells in preterm infants with comparable levels shown in cord blood from babies born at term (Gibbons et al., 2014). It was hypothesised that this newly discovered cell subset is responsible for the influx of neutrophils to the blood in the first days of life and that in turn; these neutrophils are primed to distinguish pathogenic bacteria from the developing microbiome. The authors suggested the production of IL-8 was a pro-inflammatory immunoprotective response of neonatal T cells. This goes against the commonly held idea that the postnatal immune system is strictly focused towards Th2 or regulatory responses and highlights the requirement for further study of the developing immune system during early life.

The variable data generated from murine studies that have examined pulmonary immune responses to infection and allergens to date have highlighted how critical it is to consider both the maturing immune system and lung development when investigating mechanisms underlying disease in early life (Amy et al., 1977; Burri, 1984). In addition, the effect of maternally transferred products; such as antibodies via the placenta or antigens through breast milk, also need to be considered as they influence the response of the neonatal immune system to allergen exposure (Polte et al., 2008; Verhasselt et al., 2008). The development of the human immune system throughout gestation has been described, however, little is known about postnatal immune development over the timecourse from birth through to adulthood (Holt and Jones, 2000).

Due to the contradictory results showing the effects of HDM exposure when allergen administration was initiated in early life in murine models (Al-Garawi et al., 2011; Saglani et al., 2009), and limited knowledge relating to early immune development we investigated the effect of HDM exposure when initiated at different ages throughout early life.

The aim was to determine whether age at first exposure to allergen affected the development of AAD. Balb/c mice were exposed to intra-nasal HDM commencing on day 3, 7, 14, 21 or 28 of life before assessment of lung function, inflammation and generation of allergen specific IgE, the hallmark features of AAD. Naïve were then assessed at the same ages for baseline pulmonary populations of inflammatory innate lymphoid cells (ILCs) and T cells to assess the capacity of resident cells in the airways to respond to allergen challenge. Finally, whole lung RNA from naïve mice aged 3, 14 and 28 days, and day 14 mice exposed to HDM from day 3 of life, was assessed by RNAseq to determine differences in gene expression throughout development.

3.1.1 Hypothesis

Age at first exposure to allergen influences the magnitude of AAD and is determined by the composition of resident cell populations in the lung at first exposure to allergen.

3.1.2 Aims

1. To characterise HDM induced AAD in mice when first exposure begins from day three of life through to early adulthood at day 42.
2. To determine if the differences in response to HDM are due to resident pulmonary immune populations and if these can be manipulated to overcome the effects of age.
3. To determine which genes are responsible for differing responses to HDM exposure at different ages.

3.2 Methods

In protocol A, neonatal mice were exposed to intranasal (i.n) HDM three times a week for three weeks. The first exposure to HDM was initiated at either day 3, 7, 14, 21 or 28. Neonatal mice were administered 20µg HDM in 10µl PBS without anaesthesia until 14 days old. After day 14 of life mice were dosed with 25µg HDM in 12.5µl PBS using recovery anaesthesia of inhaled isoflurane. Control mice were given an equivalent volume of PBS.

In protocol B, all mice were treated from day 14 of life. For the first week, mice received 2µg i.n IL-33 in the morning and 25µg HDM in the evening, for the final two weeks mice were exposed to HDM only. A dose of 2µg per mouse of rIL-33 was chosen as it has previously been shown to exacerbate allergen induced allergic airways disease (Kurowska-Stolarska et al., 2008).

In protocol C, 14 day old SCID and Balb/c mice were given 2µg i.n rIL-33 for two weeks.

Endpoint analysis for protocols A and B was carried out 4 hours after the last dose of HDM and for protocol C, 18 hours post last dose of rIL-33.

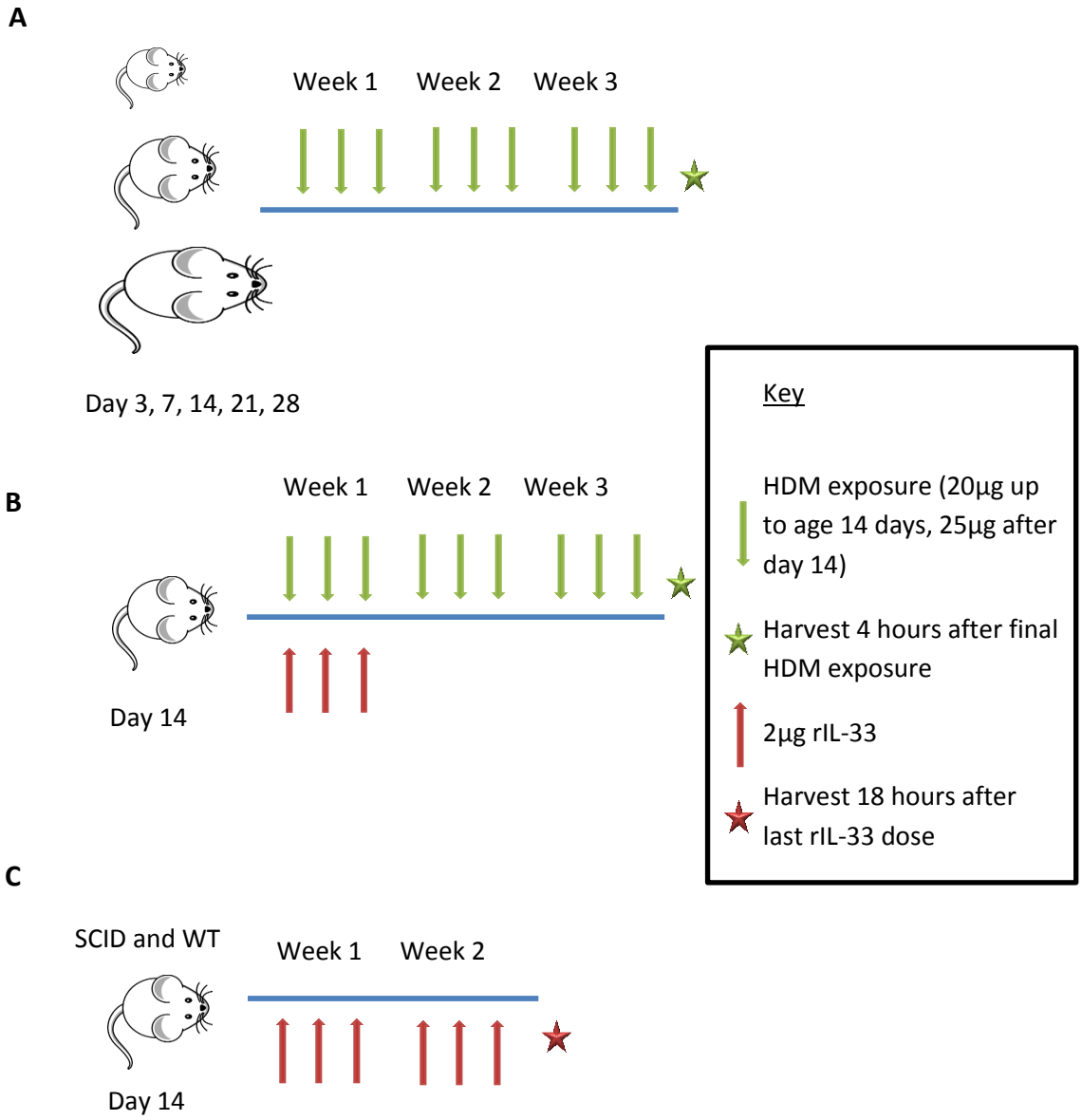

Figure 3.1 Dosing protocols for age of onset analysis.

Balb/c mice aged 3, 7, 14, 21 or 28 days were dosed for three weeks with 25µg intranasal HDM or PBS three times per week before analysis or AHR by flexivent and collection of BAL, lung tissue and blood **(A)**. Balb/c mice aged 14 days were dosed intranasal with 2µg rIL-33 and 25µg HDM or PBS three times per week for one week, followed by two weeks of HDM/PBS alone before analysis or AHR by flexivent and collection of BAL, lung tissue and blood **(B)**. 14 day old Balb/c and severe combined immune deficient (SCID) mice were dosed for two weeks with intranasal 2µg rIL-33 or PBS before analysis or AHR by flexivent and collection of BAL, lung tissue and blood **(C)**.

3.3 Results

3.3.1 Neonatal mice exposed to HDM from day 3 or 7 of life develop robust AHR.

Neonatal mice exposed to HDM from day three of life (Fig 3.1 A) had increasing airway resistance to methacholine (MCh), as measured by Flexivent (Fig 3.2 A). Resistance at 100mg/ml MCh was significantly higher than PBS control (Fig 3.2 B). Compliance was significantly lower in HDM exposed mice (Fig 3.2 C). First exposure to HDM at day 7 of life also resulted in significantly increased airway resistance and reduced compliance including reduced compliance at baseline (Fig 3.2 D-F). As compliance is an inverse measure of lung elastance a significantly reduced compliance at baseline signifies stiffer lungs and therefore is a determinant of pulmonary remodelling and inflammation.

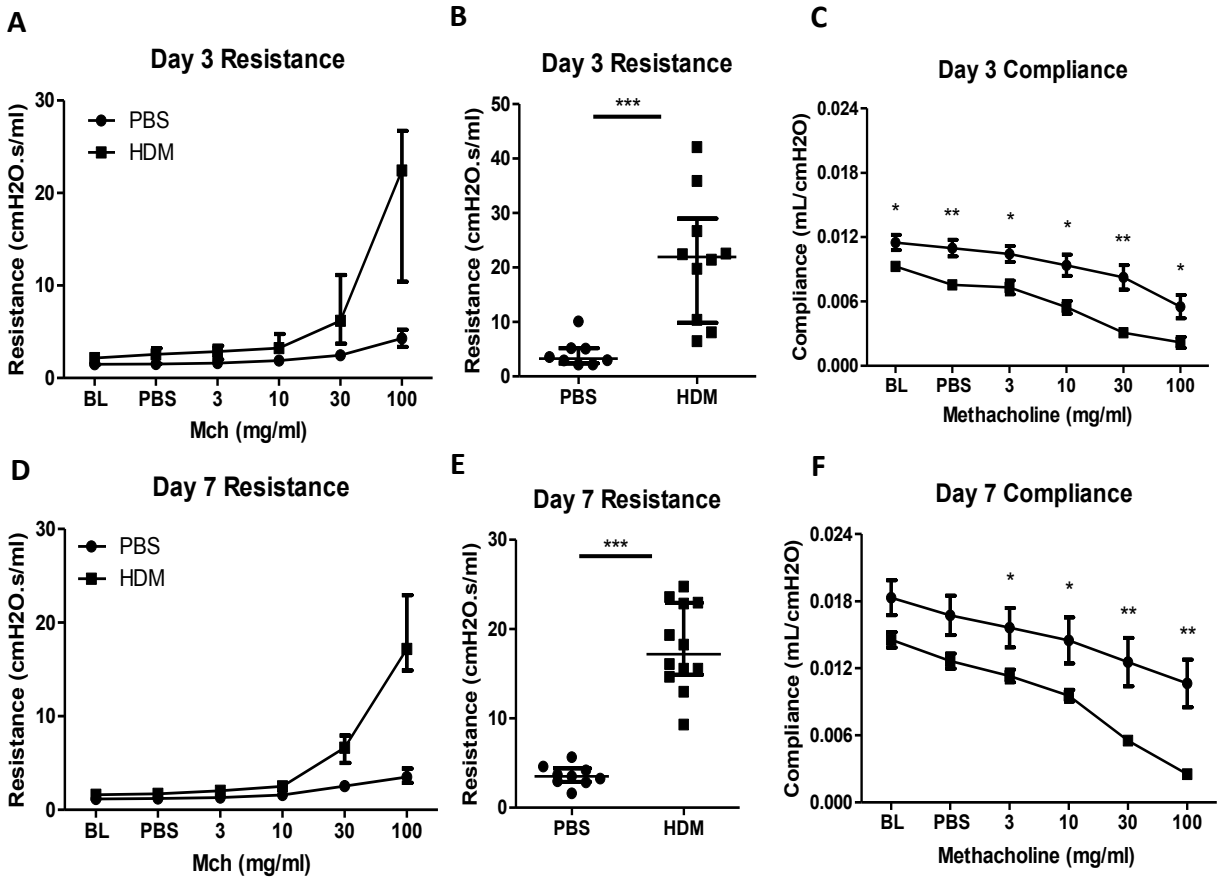

Figure 3.2 Neonatal HDM induced airway resistance.

AHR measured by flexivent. Airway resistance with increasing concentrations of methacholine **(A)**, at 100mg/ml MCh **(B)** and airway compliance **(C)** in mice dosed for three weeks with HDM commencing at day 3 of life. Airway resistance with increasing concentrations of methacholine **(D)**, at 100mg/ml MCh **(E)** and airway compliance **(F)** in mice dosed for three weeks with HDM commencing at day 7 of life. n= 8-12 mice per group, results from two separate experiments. Data displayed as median and interquartile range. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test.

3.3.2 Mice exposed to HDM from day 14 or 21 did not develop AHR.

In contrast to the robust airway resistance induced by HDM when dosing began at day 3 or 7, first exposure to HDM at day 14 (Fig 3.3 A, B) or day 21 of life (Fig 3.3 D, E) did not result in increased airway resistance. Airway compliance was also unaffected in response to HDM when dosing began at day 14 (Fig 3.3 C) or 21 (Fig 3.3 F), indicating a lack of remodelling in mice exposed to HDM from day 14 and 28. However if first exposure to HDM occurred in early adulthood, when the mice were 28 days old, AHR was restored after three weeks of HDM exposure (Fig 3.3 G-I). Overall, when mice were first exposed to HDM from day 3 or 7 of life airway resistance to 100mg/ml MCh was 5 fold higher than PBS controls. When first exposure began at day 14 or 21 of life there was no significant change in lung function from PBS and mice exposed to HDM from day 28 had increased AHR, but the fold change was not as high as when exposure began in the first week; on average 2.5 times above PBS control (Fig 3.3 J).

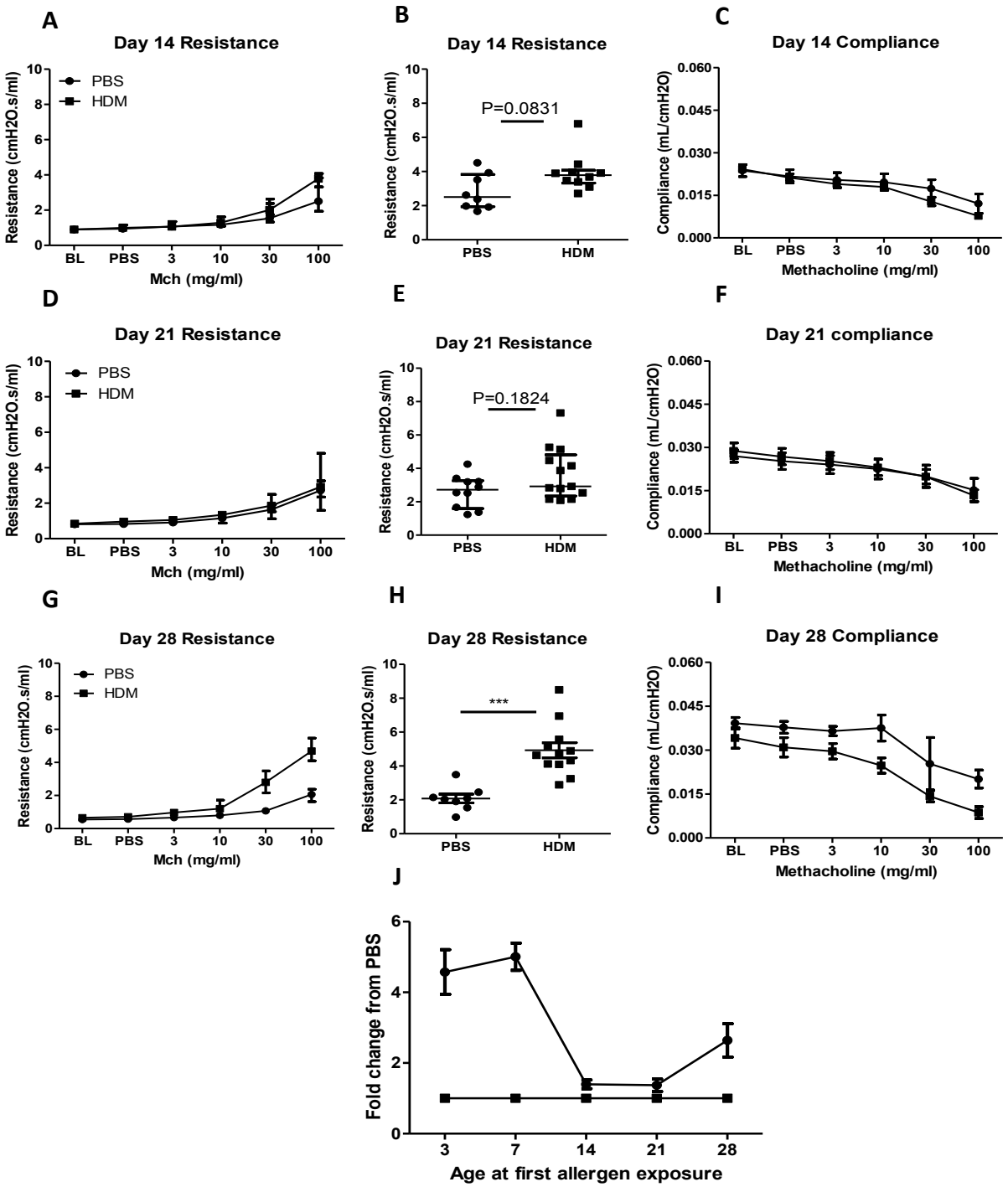

Figure 3.3 Day 14, 21 and 28 HDM induced airway resistance.

AHR measured by flexivent. Airway resistance with increasing concentrations of MCh **(A)**, Resistance at 100mg/ml MCh **(B)** and compliance **(C)** in mice dosed with HDM commencing at day 14 of life. Airway resistance with increasing concentrations of MCh **(D)**, Resistance at 100mg/ml MCh **(E)** and compliance **(F)** in mice dosed with HDM commencing at day 21 of life. Airway resistance with increasing concentrations of MCh **(G)**, Resistance at 100mg/ml MCh **(H)** and compliance **(I)** in mice dosed with HDM commencing at day 28 of life. A graph of fold change from PBS of airway resistance **(J)**. n= 8-12 mice per group, results from two separate experiments. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. Data displayed as median and interquartile range.

3.3.3 Inflammation and immunoglobulin levels follow a similar pattern to AHR.

Change in inflammation in the bronchalveolar lavage (BAL) when allergen exposure was started at different ages was similar to the pattern of observed AHR (Fig 3.4 A). Although there was increased BAL inflammation in mice that had been exposed to HDM from day 14 and 21 it was significantly lower than in mice exposed at day 3, 7 or 28. Eosinophils were present in the BAL of mice exposed to HDM at all ages although again a substantially lower percentage were present in mice exposed to HDM from day 14 and 21 (Fig 3.4 B). Importantly, both total serum and HDM specific IgE, which are associated with allergic sensitisation, were absent in mice when HDM exposure began at day 14 and 21 but significantly increased from PBS levels in mice exposed from day 3, 7 and 28 of life (Fig 3.4 C, D). Total IgG1 was significantly elevated from PBS levels in mice exposed from day 21 and this correlated with a significant elevation in HDM specific IgG1 (Fig 3.4 E, F). Total serum IgG2a was detectable in mice exposed to HDM at all ages, however this was not reflected in the HDM specific IgG2a which was only increased in mice that were exposed to HDM from day 3 and 7 (Fig 3.4 G, H).

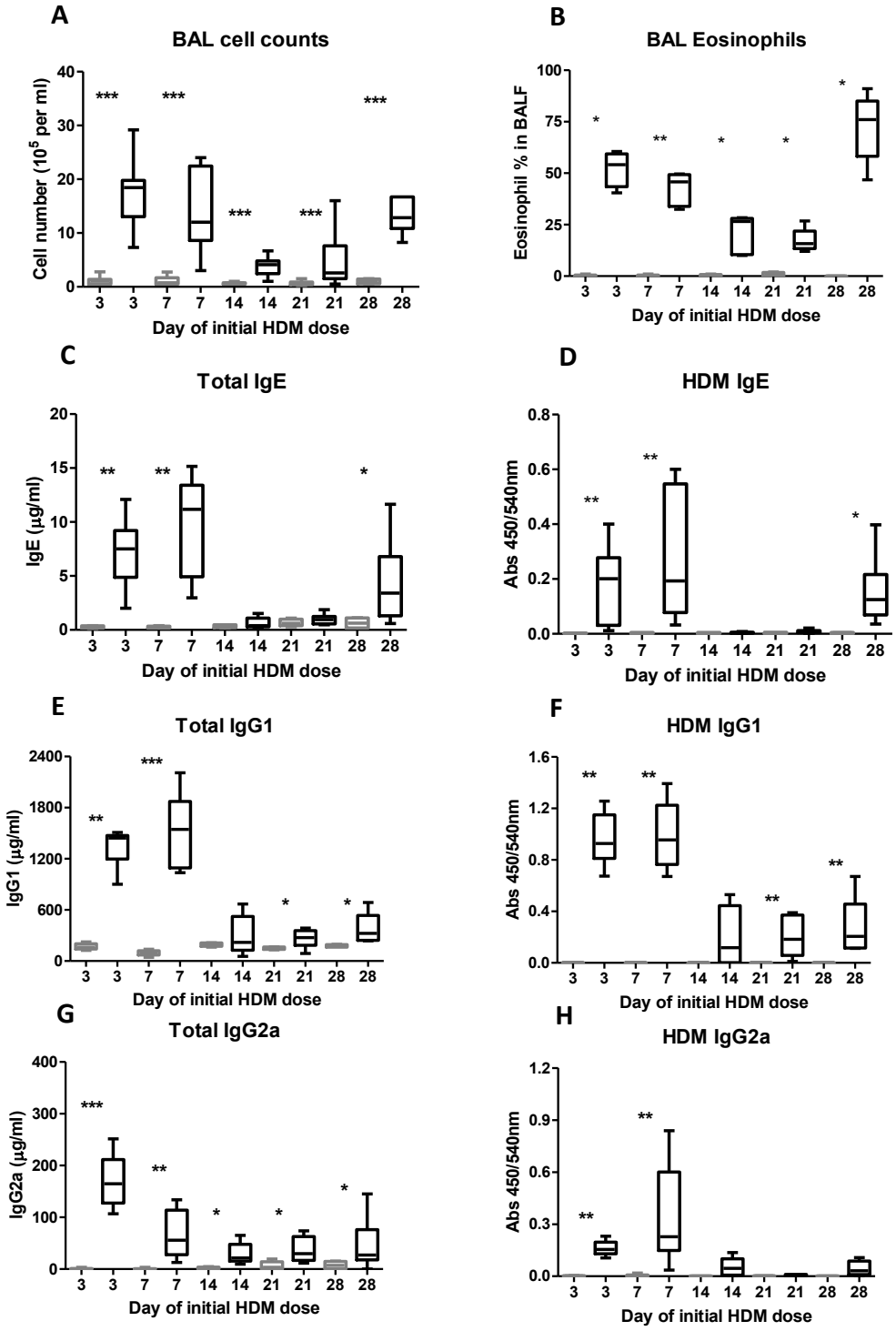

Figure 3.4 Inflammation and immunoglobulin levels after HDM exposure in mice of different ages.

BAL and blood were collected after three weeks of intranasal HDM commencing at varying ages. Cell count per ml of BAL recovered **(A)**. Eosinophils as percentage of cells in BAL **(B)**. Serum total **(C)** and HDM specific IgE **(D)**. Serum total **(E)** and HDM specific IgG1 **(F)**. Serum total **(G)** and HDM specific IgG2a **(H)**. PBS values in grey, HDM in black n= 8-12 mice per group, results from two separate experiments. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. Data displayed as box and whiskers depicting interquartile range and minimum and maximum values.

3.3.4 Th2 cytokines were down regulated at day 14, while IL-33 remained elevated.

In order to investigate the contribution of both pro- and anti-inflammatory cytokines on the development of AHR total lung homogenate was analysed by ELISA. IL-4 levels followed a similar pattern to airway resistance and IgE with no increase in mice exposed to HDM from 14 or 21 days and significantly increased levels at all other ages (Fig 3.5 A). IL-5 was also elevated in groups that responded with airway resistance, although not significantly at day 28 (Fig 3.5 B). Similarly, IL-13 was undetectable in mice exposed to HDM starting from day 14 or 21 and was significantly elevated from PBS at all other time points. IL-13 levels were highest in mice exposed to allergen from day 3 of life (Fig 3.5 C). Interestingly, IL-33 levels did not mirror the pattern of airway resistance and similar levels were detectable in mice exposed to HDM commencing at all ages (fig 3.5 D). In order to determine whether increased anti-inflammatory cytokines were responsible for the reduced airway resistance and inflammation seen when sensitisation commenced at day 14 and 21 IL-10 and TGF-β were measured. However, both IL-10 and TGF-β were significantly lower than control levels in mice exposed to HDM from day 14, and levels were similar to controls on day 21 (Fig 3.5 E, F).

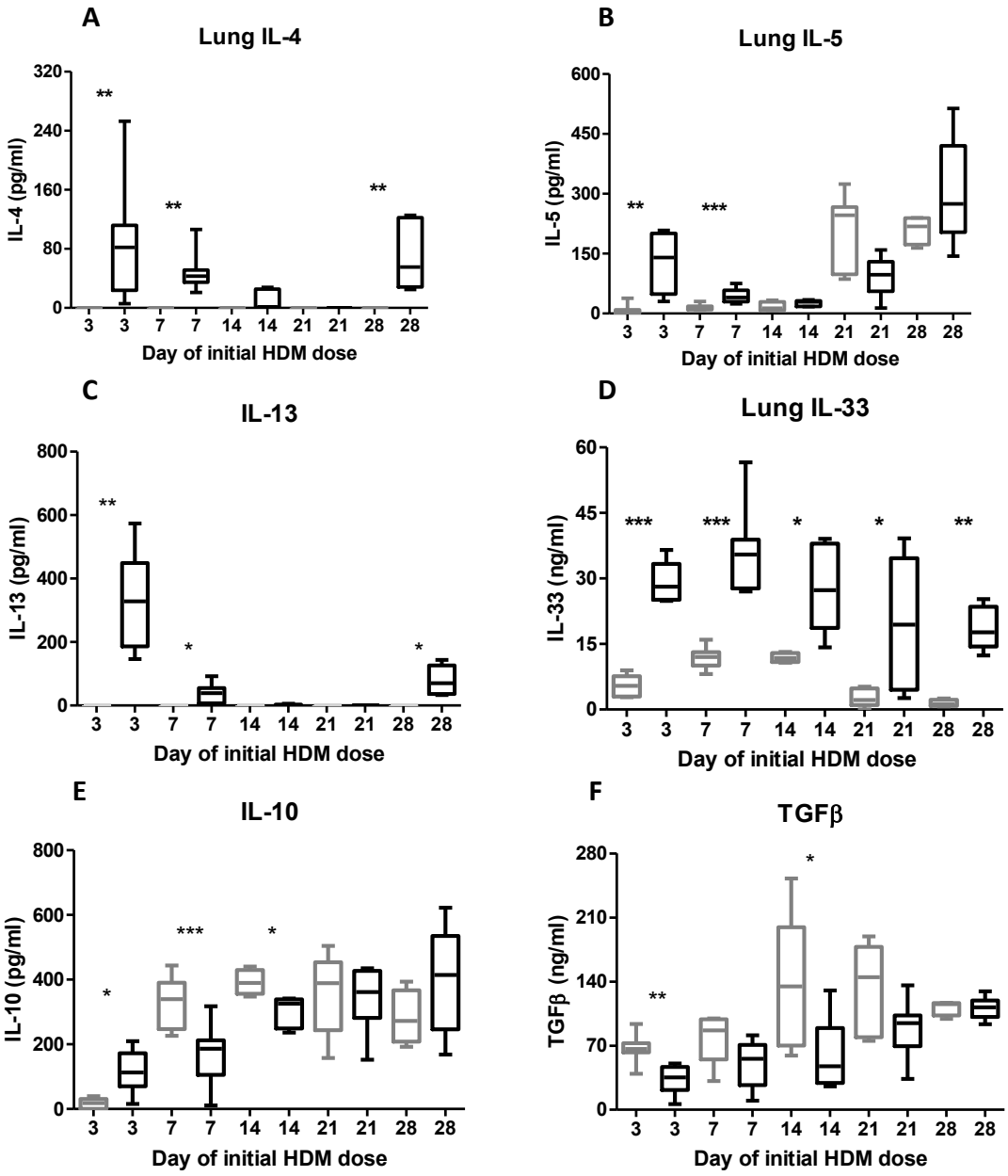

Figure 3.5 Pro- and anti-inflammatory cytokines in response to HDM exposure.

Lung was collected and homogenised after three weeks of intranasal HDM commencing at varying ages. IL-4 **(A)**, IL-5 **(B)**, IL-13 **(C)**, IL-33 **(D)**, IL-10 **(E)** and TGFβ **(F)** as concentration per ml lung homogenate (50mg/ml). PBS values in grey, HDM in black n= 8-12 mice per group, results from two separate experiments. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. Data displayed as box and whiskers depicting interquartile range and minimum and maximum values.

3.3.5 Pulmonary populations of IL-13$^+$ T cells and IL-13$^+$ ILCs were lower in naïve mice between day 14 and day 21

Our results indicated that airway resistance does not develop in the absence of IL-13. We therefore aimed to characterise the populations of resident pulmonary cells with the capacity to generate IL-13 in response to allergen challenge at various ages. Naïve mice aged 3, 7, 14, 21 and 28 days were culled and lung immune cells isolated for analysis of IL-13$^+$ ILCs (Fig 3.6 A) and IL-13$^+$ T cells (Fig 3.6 B) by flow cytometry. Lungs from non-allergic, untreated 3 day old mice contained approximately double the number of IL-13$^+$ CD3$^+$CD4$^+$ in comparison to IL-13$^+$ ILCs. This population of IL-13$^+$ CD3$^+$CD4$^+$ cells declined over time with the lowest number of cells present in the lung at day 14 to day 28 of life. The smallest population of resident IL-13$^+$ ILCs was also found at day 14 before populations increased with age. In contrast to 3 day old mice, adult mice had a higher proportion of resident pulmonary IL-13$^+$ ILCs than IL-13$^+$ CD3$^+$CD4$^+$ cells (Fig 3.6 C).

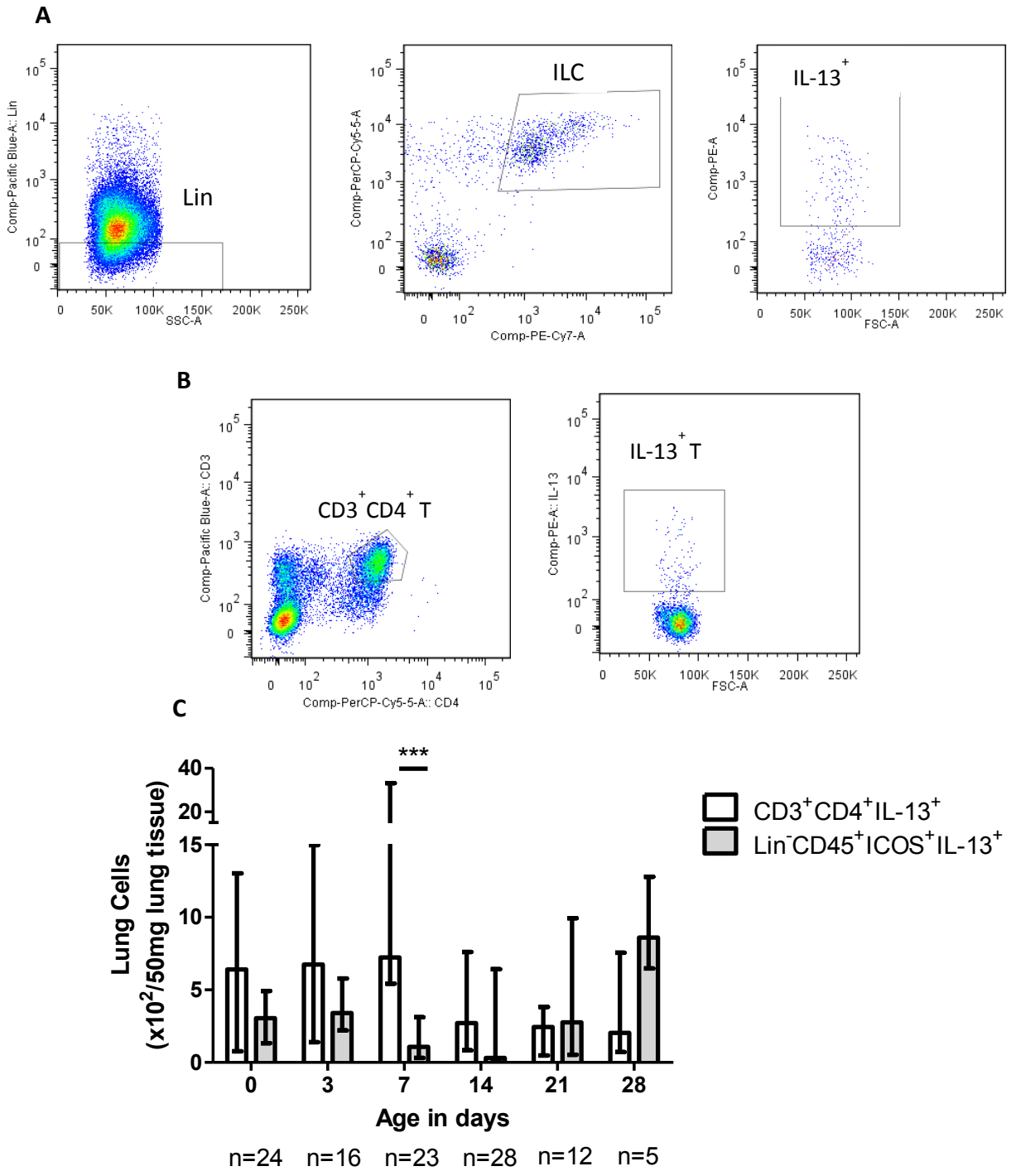

Figure 3.6 IL-13$^+$ ILCs and T cell populations in naïve mice

Lungs were collected from naïve mice aged 3, 7, 14, 21 and 28 days old. Cells were isolated and analysed by flow cytometry for populations of IL-13$^+$ ILCs (Lin$^-$CD45$^+$ICOS$^+$) **(A)** and IL-13$^+$ T cells (CD3$^+$CD4$^+$) **(B)**. Comparison of IL-13$^+$ ILC and T cell populations over time **(C)**. Data represented as median ± interquartile range. ***p<0.001 by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range. n= 5-28 per group.

3.3.6 Altering the immune environment with rIL-33 overcomes the natural tolerance phase

IL-33 released from the pulmonary epithelium upon contact with allergen attracts IL-13^{+} ILCs to the lung to initiate AAD (Kim et al., 2012a). In order to determine if the reduced population of pulmonary resident IL-13^{+} ILCs and IL-13^{+} CD3^{+}CD4^{+} cells documented at day 14 and day 21 (Fig 3.6 C) reflected the lack of an allergic response to HDM we aimed to alter the resident population by dosing mice with rIL-33 at the initiation of HDM exposure in 14 day old mice (Fig 3.7 A). The addition of rIL-33 to the first week of HDM exposure, initiated when mice were 14 days old, resulted in significantly increased airway resistance when compared to mice exposed to HDM alone at both baseline and 100mg/ml MCh (Fig 3.7 B-D). rIL-33 and HDM in combination resulted in significantly increased BAL inflammation in comparison to mice only exposed to HDM (Fig 3.7 D). Lung tissue inflammation and eosinophil numbers reflected the pattern of inflammation in the BAL (Fig 3.7 E, F).

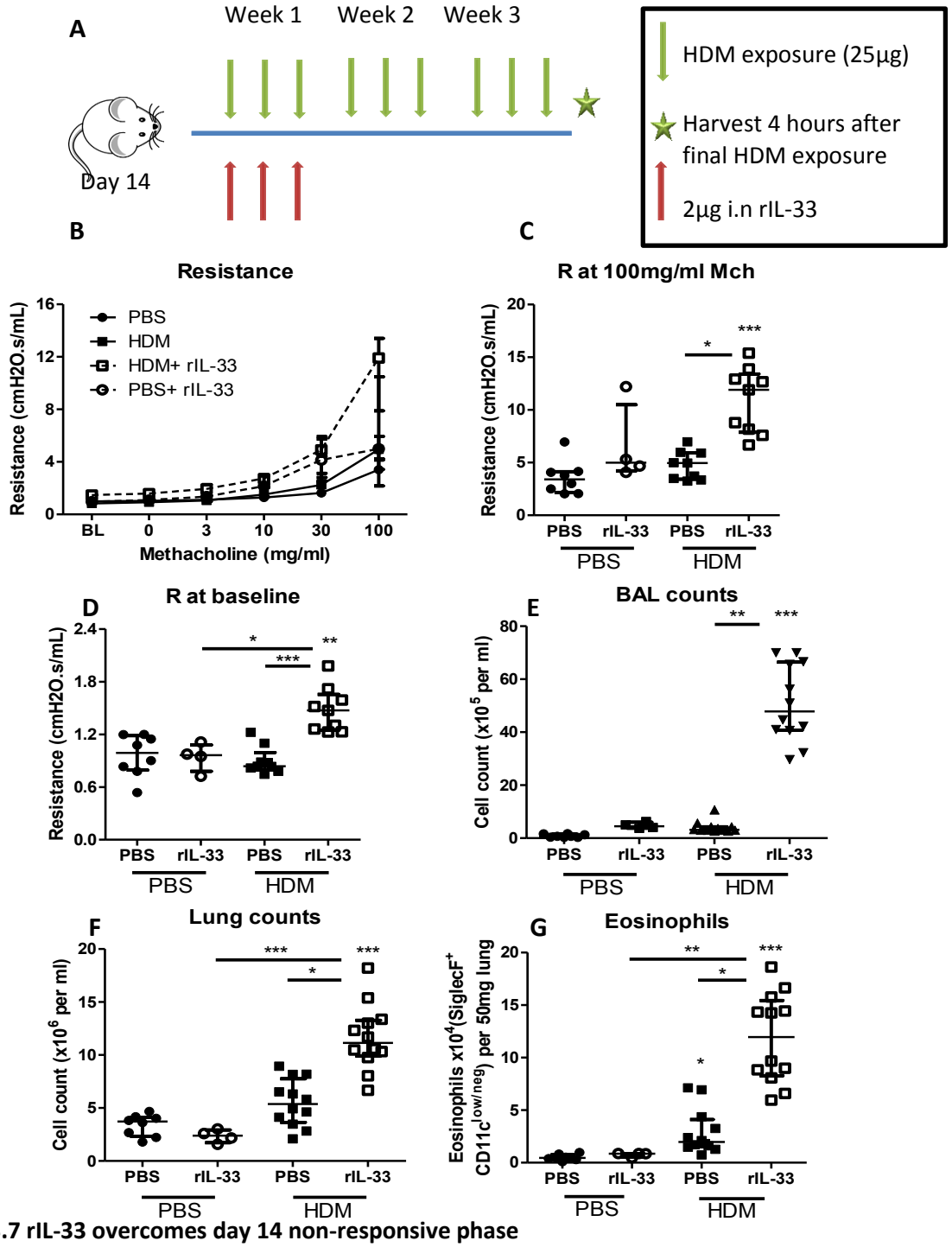

Figure 3.7 rIL-33 overcomes day 14 non-responsive phase

Experiment protocol showing three weeks of HDM exposure commencing at day 14 with the addition of rIL-33 in the first week **(A)**. Airway resistance over increasing concentrations of methacholine **(B)**. Resistance at 100mg/ml Methacholine **(C)** and baseline **(D)**. Cell count per ml of BAL fluid **(E)**, lung cell count per ml **(F)** and eosinophils (SiglecF^{+}CD11c^{-}) per 50mg lung tissue **(G)**. rIL-33 treated mice displayed as hollow symbols, PBS treated as solid symbols. n= 4-12 mice per group, results from two separate experiments. * p<0.05 ** p<0.01 and *** p<0.001 from PBS control or as indicated by bars.by Kruskal-Wallis with Dunn's multiple comparison test Data displayed as median and interquartile range.

3.3.7 rIL-33 in combination with HDM exposure from day 14 causes the induction of inflammatory cytokines and cells in the lung.

The addition of rIL-33 to the first week of HDM exposure commencing at day 14 of life induced pulmonary IL-13 and IL-4 as well as serum total IgE (Fig 3.8 A-C). Pulmonary IL-33 was increased from PBS control levels in mice that received rIL-33 during the first week with no further exposure for two subsequent weeks. In addition to the IL-33 induced by HDM alone, levels were significantly increased by dosing mice with rIL-33 during the first week of HDM exposure (fig 3.8 D).

As well as enhancing endogenous IL-33, ectopic delivery of rIL-33 also caused an increase in the populations of total and IL-13^+ ILCs which were still present in the lung two weeks after the final dose of rIL-33 (fig 3.9 A, B). HDM alone also caused an increase in these populations; however, the combination of both allergen and cytokine significantly enhanced the influx of these cells into the lung (fig 3.9 A, B). Total T cells ($CD3^+CD4^+$) and IL-13^+ T cells were increased in mice exposed to both HDM and rIL-33; IL-13^+ T cells were induced by HDM alone (Fig 3.9 E, F).

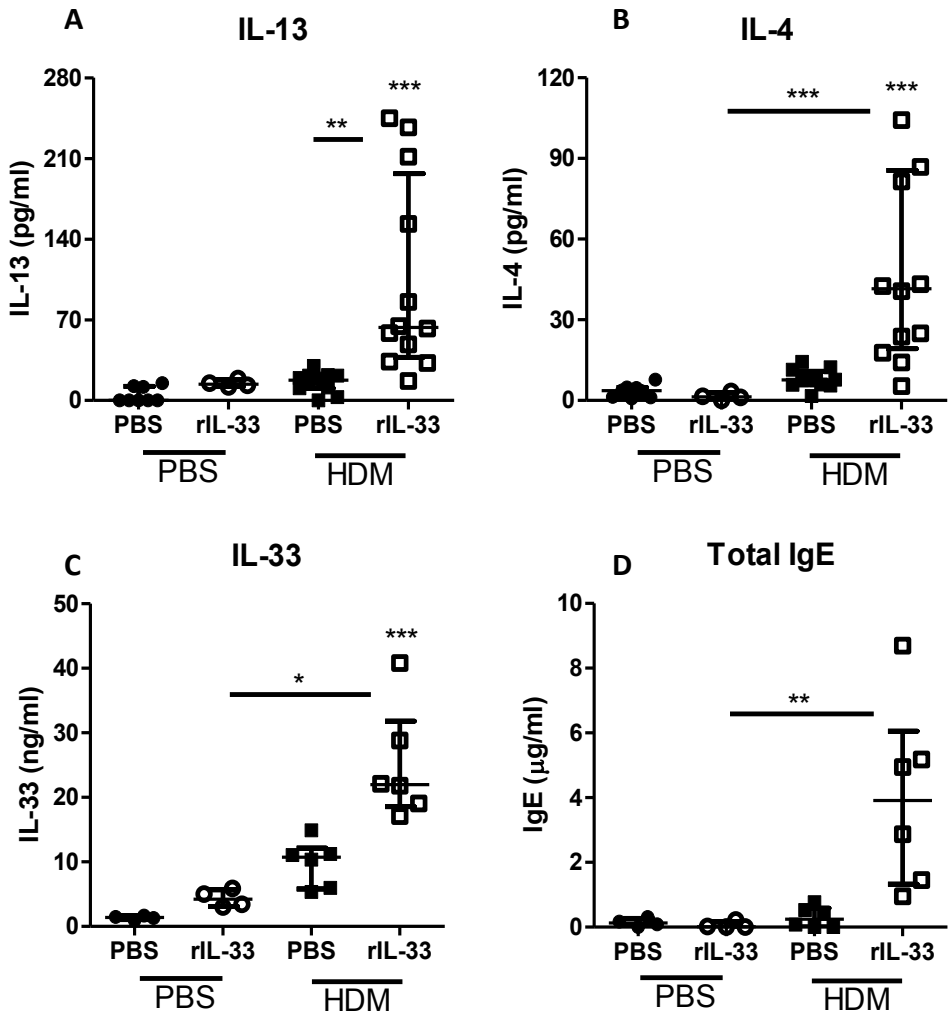

Figure 3.8 Ectopic delivery of rIL-33 in combination with HDM exposure from day 14 of life promotes IL-4, IL-13 and IgE

Lung and blood were collected and homogenised after three weeks of intranasal HDM/PBS and one week of rIL-33/PBS commencing at day 14 of age. IL-13 **(A)**, IL-4 **(B)** and IL-33 **(C)** as concentration per ml lung homogenate (50mg/ml). Total IgE **(D)** as µg/ml serum. rIL-33 treated mice displayed as hollow symbols, PBS treated as solid symbols. n= 4-12 mice per group, results from two separate experiments. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

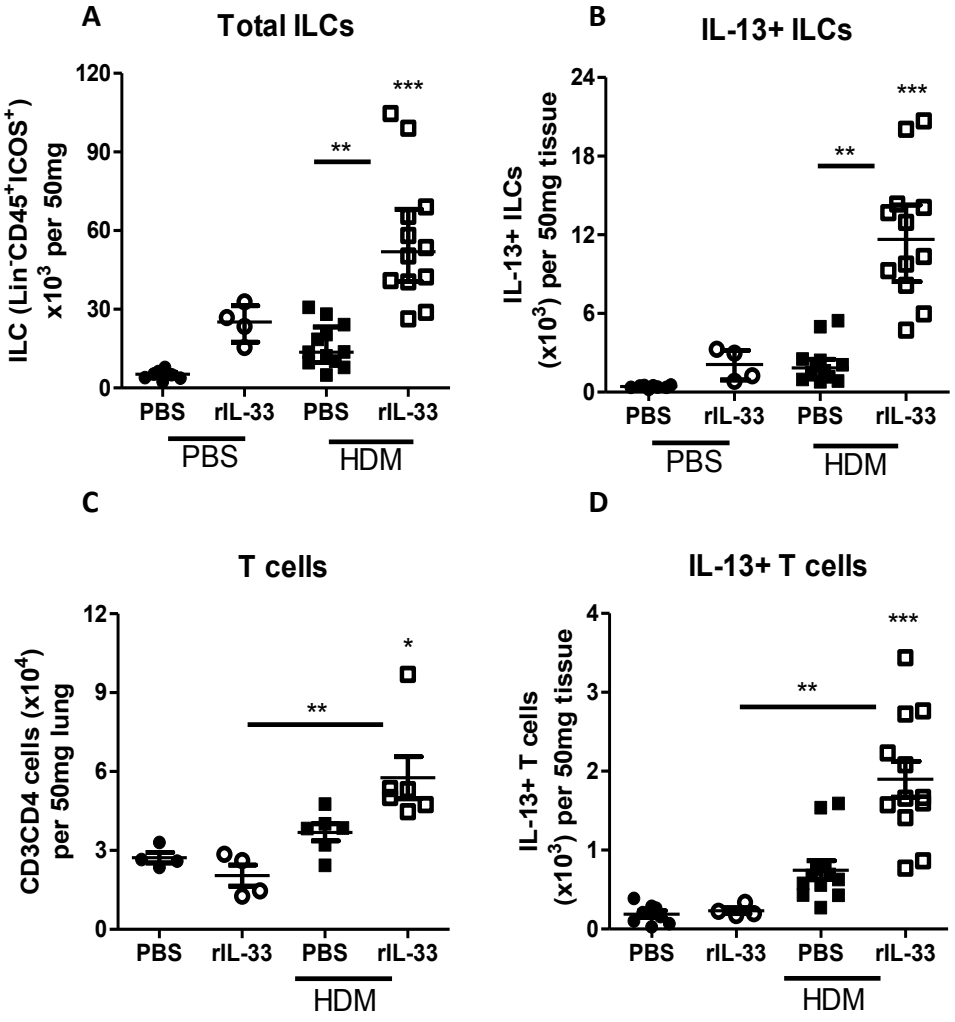

Figure 3.9 Ectopic delivery of rIL-33 in combination with HDM exposure from day 14 of life promotes ILCs and T cells

Lungs were collected cells isolated for flow cytometry analysis after three weeks of intranasal HDM/PBS and one week of rIL-33/PBS commencing at day 14 of age. Total ILCs (Lin-CD45+ICOS+) **(A)** IL-13+ ILCs **(B)** total T cells (CD3+CD4+) **(C)** and IL-13+ T cell **(D)** populations were determined by flow cytometry. rIL-33 treated mice displayed as hollow symbols, PBS treated as solid symbols. n= 4-12 mice per group, results from two separate experiments. ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

3.3.8 rIL-33, unlike HDM exposure, causes the generation of AHR when exposure begins at day 14 in a T cell independent manner.

One observation from the above experiments was the striking difference in the proportion of IL-13$^+$ ILCs (Fig 3.9 B) and IL-13$^+$ T cells (Fig 3.9 F) generated in response to exposure of mice to a combination of HDM and rIL-33. The population of IL-13$^+$ ILCs present in the lungs after both rIL-33 and HDM exposure was 6 fold higher than the corresponding population of IL-13$^+$ T cells. As one of the clinical features of patients with severe asthma is increased expression of IL-33 in pulmonary cells (Préfontaine et al., 2010; Saglani et al., 2009) we aimed to determine the effect of this cytokine alone in the generation of AHR from day 14 of life. In addition, due to the observed skewing towards an ILC response we aimed to determine if T cell responses contribute a vital role in the generation of cytokine induced AHR using a severe combined immune deficient (SCID) mouse model as these mice do not have the ability to generate T cells.

WT and SCID mice were exposed to rIL-33 from day 14 of life for two weeks before assessment of AHR (Fig 3.10 A). Both genotypes responded to increasing concentrations of MCh in a similar manner, with equivalent airway resistance and compliance (Fig 3.10 A-D). Despite the comparable AHR, BAL and lung inflammation differed between WT and SCID mice. SCID mice did have increased BAL inflammation compared to their PBS control; however, it was lower than the BAL inflammation in WT mice. In contrast, lung tissue inflammation was not increased above control in SCID mice and WT mice responded to rIL-33 with significantly elevated pulmonary inflammation compared to SCID mice (Fig 3.10 E, F).

No IL-4 was generated in the lungs of SCID mice in response to rIL-33 (Fig 3.11 A). However, IL-5 and IL-33 were generated to the same level in SCID mice as WT mice (Fig 3.11 B, C). IL-13 levels in the lung and BAL were also not significantly different between the two strains of mice, however, lung IL-13 was higher in PBS control SCID mice compared to WT mice and additional numbers will be required to determine if this is a true feature of SCID mice (Fig 3.11 D, E).

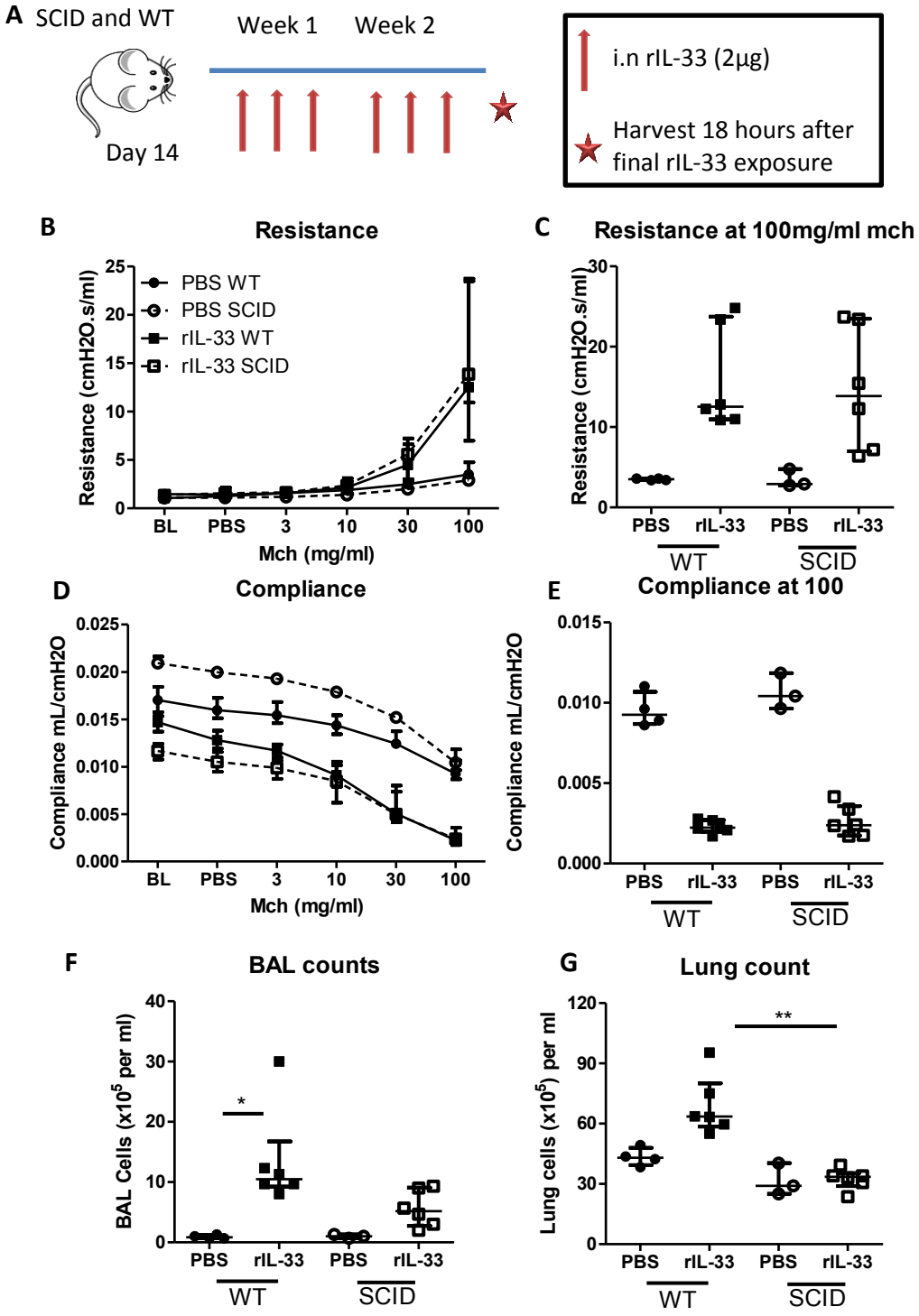

Figure 3.10 rIL-33 exposure from day 14 of life causes AHR in a non-T cell dependent manner.

WT and severe combined immune deficient (SCID) mice dosed for two weeks with rIL-33 commencing at day 14 of life **(A)**. Airway resistance over increasing concentrations of methacholine **(B)**. Resistance at 100mg/ml Methacholine **(C)** Compliance over increasing concentrations of methacholine **(D)**. Compliance at 100mg/ml methacholine **(E)** Cell count per ml of BAL fluid **(F)** and lung cell count per ml **(G)**. SCID mice displayed as hollow symbols, WT mice as solid symbols. n= 3-6 mice per group. * $p<0.05$ and ** $p<0.01$ by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

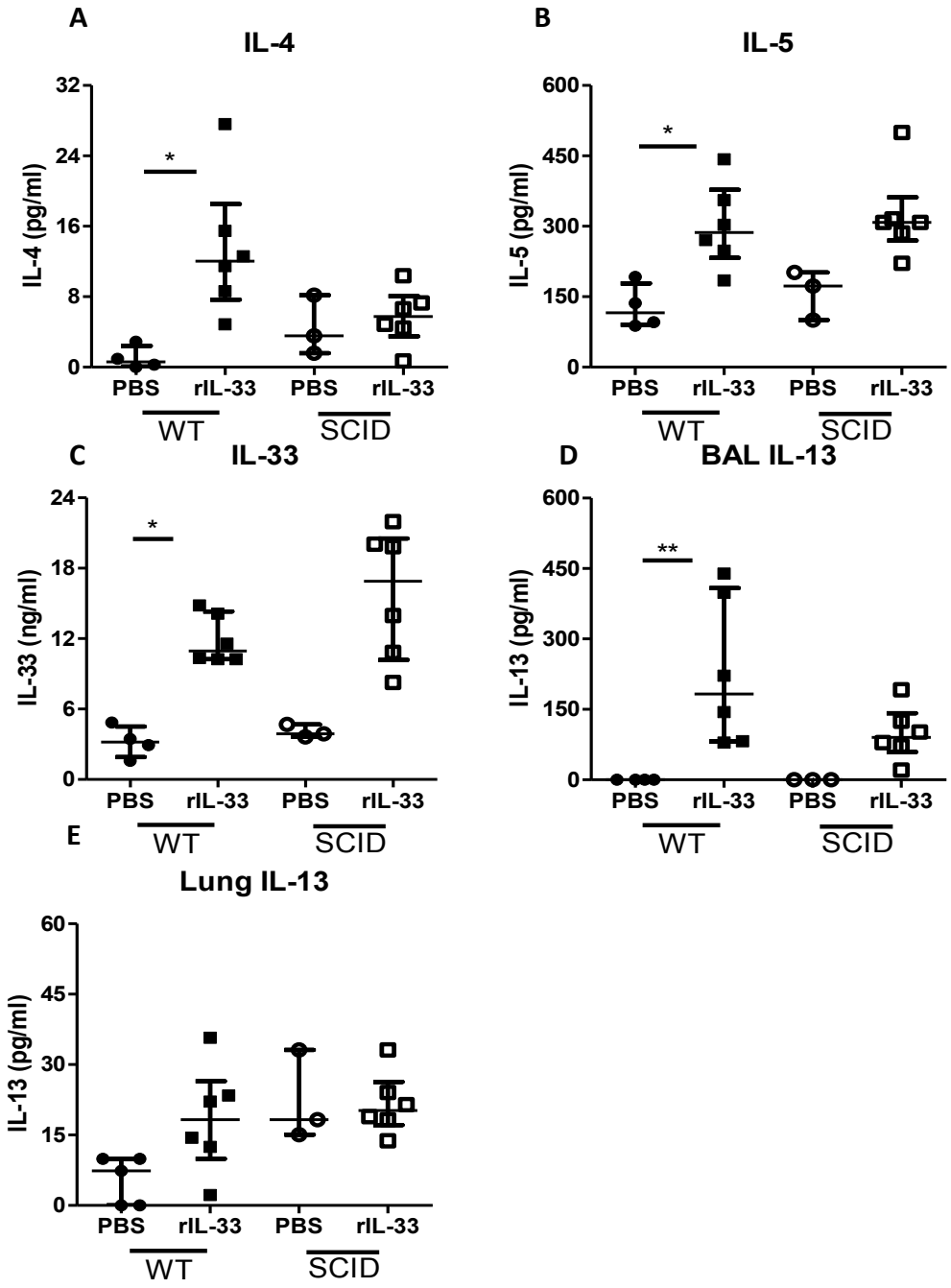

Figure 3.11 Inflammatory cytokines in WT and SCID mice exposed to rIL-33

Lung and BAL were collected and lungs homogenised after two weeks of intranasal rIL-33/PBS commencing at day 14 of age. IL-4 **(A)**, IL-5 **(B)** and IL-33 **(C)** as concentration per ml lung homogenate (50mg/ml). BAL IL-13 **(D)** as pg/ml BAL supernatant, and lung IL-13 **(E)**. SCID mice displayed as hollow symbols, WT mice as solid symbols. n= 3-6 mice per group. * $p<0.05$ and ** $p<0.01$ by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

3.3.9 Despite lower total ILC populations in SCID mice, similar IL-13+ ILCs were generated in response to rIL-33 in WT and SCID mice

WT mice had a larger population of lung eosinophils in response to rIL-33 than SCID mice and no significant difference in the number of neutrophils was observed between strains (Fig 3.12 A, B). A population of IL-13^{+} T cells was induced in the lungs of WT mice exposed to rIL-33 which was absent in SCID mice (Fig 3.12 C). ILCs were induced by rIL-33 exposure in both strains of mice although to a lesser extent in SCID mice (Fig 3.12 D). Despite the lower population of total ILCs in SCID mice, there was no significant difference in the number of IL-13^{+} ILCs between strains and a larger percentage of ILCs in rIL-33 exposed SCID mice were producing IL-13 in comparison to WT ILCs (Fig 3.12 E, F).

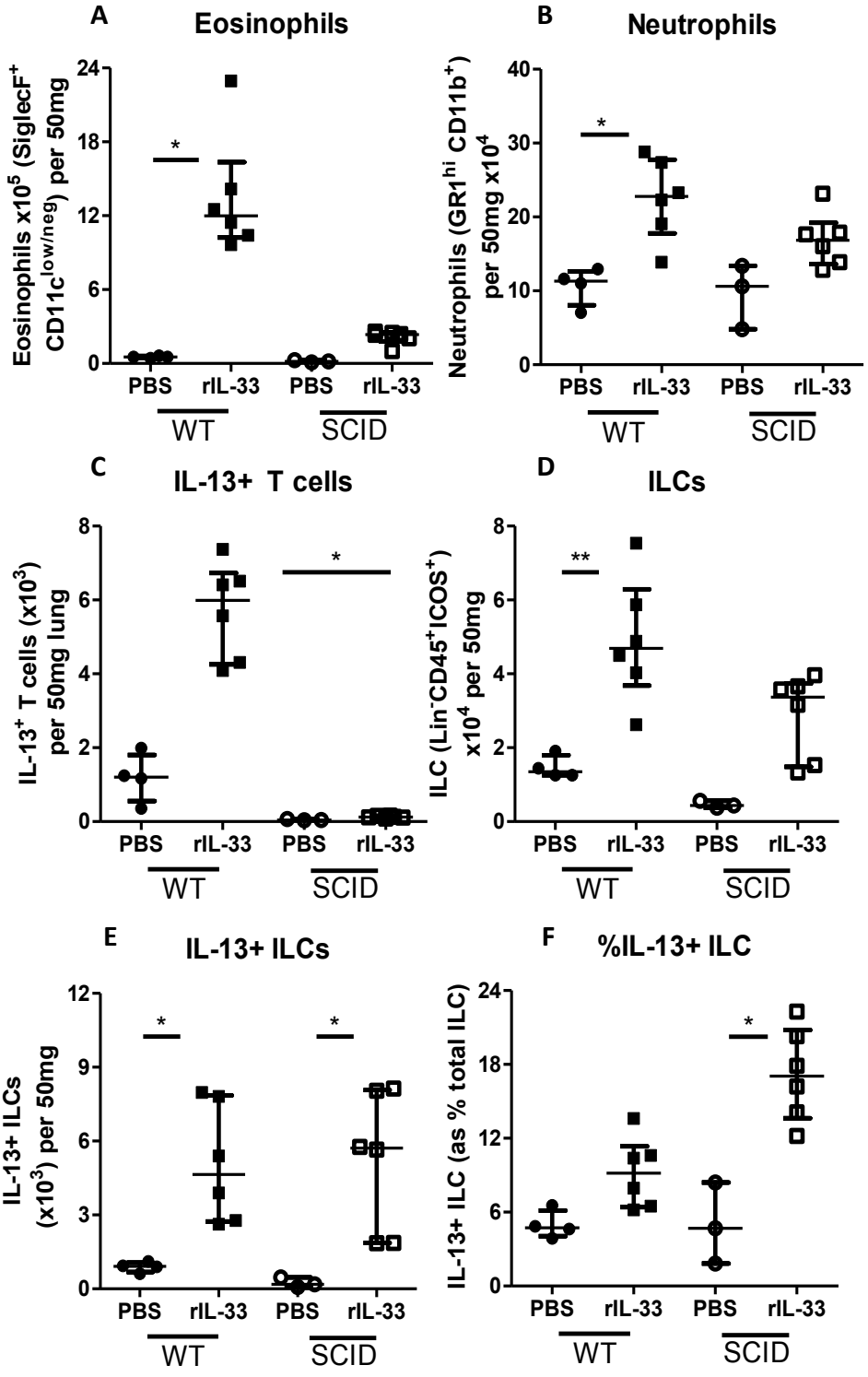

Figure 3.12 Inflammatory cells in SCID and WT mice exposed to rIL-33

Lungs were collected cells isolated for flow cytometry analysis after two weeks of intranasal rIL-33/PBS commencing at day 14 of age. Eosinophils **(A)** neutrophils **(B)** IL-13$^+$ T cells (CD3$^+$CD4$^+$) **(C)** Total ILCs (Lin$^-$CD45$^+$ICOS$^+$) **(D)**. IL-13$^+$ ILCs **(E)** and % of ILCs producing IL-13 **(F)** populations were determined by flow cytometry. SCID mice displayed as hollow symbols, WT mice as solid symbols. n= 3-6 mice per group. * $p<0.05$ and ** $p<0.01$ by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

3.3.10 RNAseq analysis of naïve whole lung RNA

We have demonstrated that resident immune cell populations in the lungs of neonatal mice change throughout development (Fig 3.6) and that the immune environment at the commencement of allergen exposure dictates the magnitude of AAD. As both human (Burri, 1984) and murine (Amy et al., 1977) neonatal lungs continue to develop postnatally we aimed to define genetic changes that could be responsible for the altered immune environment within the lung or highlight additional aspects of development that potentially contribute towards the reduced period of allergen responsiveness between days 14-21 of life.

Lungs from naïve mice aged 3, 14 and 28 days, as well as from 14 day old mice exposed to HDM from day 3 of life, were collected and RNA was extracted. RNA was sent to the Wellcome Trust Centre for Human Genetics (WTCHG) for analysis by RNAseq. Principle component analysis (PCA) showed both technical replicates for each sample clustered sufficiently to allow samples to be merged before analysis (Fig 3.13).

In order to identify genes that may be responsible for the change in AHR observed at different ages we focussed our investigations on three main comparisons – day 3 vs. day 14, day 14 vs. day 28 and day 3 vs. day 28. The top ten genes identified to be differentially expressed – as rated by P value – for each comparison are shown in tables 3.1, 3.2 and 3.3.

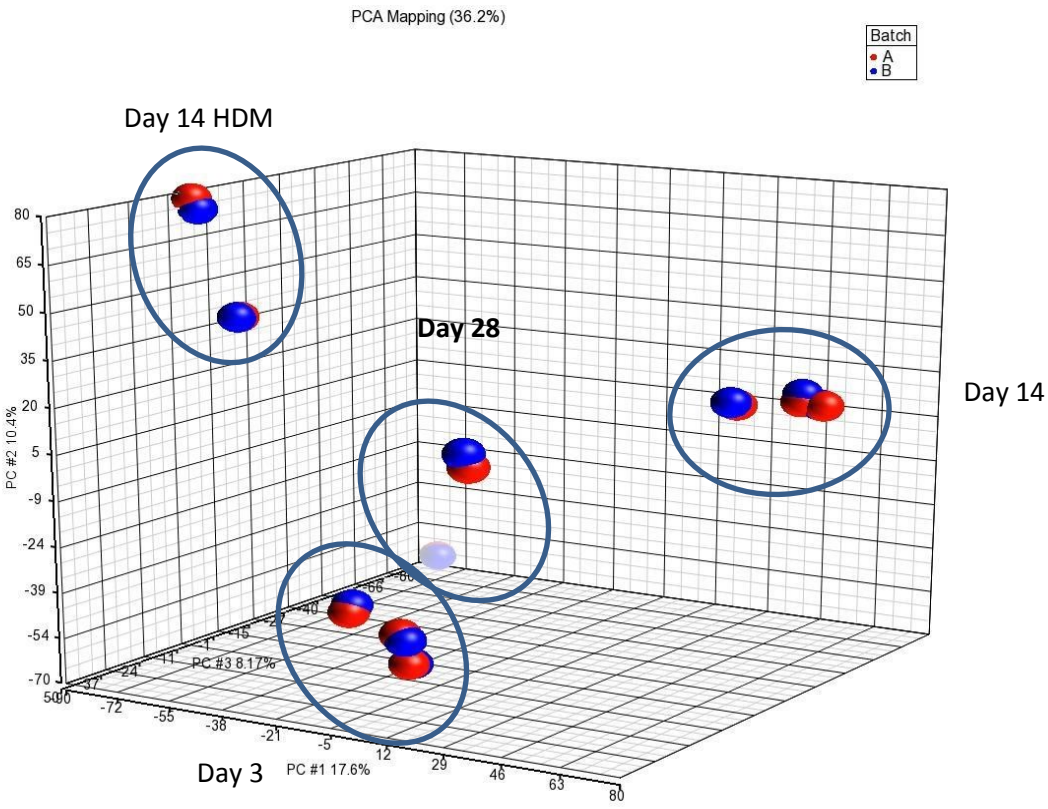

Figure 3.13 Principle component analysis

A principle component analysis of technical replicates. RNAseq results from whole lung RNA extracted from mice aged 3, 14, 28 days and mice aged 14 days treated with HDM from day 14 of life. Axes display principal components #1, #2 and #3. Batch is indicated by colour, and replicate pairs are connected by lines, which are hidden by the proximity of the points in each pair. Each sample is closer in Cartesian distance to its technical replicate than to any other sample. This allows replicates to be merged before differential expression analysis.

Gene description	Gene name	P value	LogCPM
mesoderm specific transcript	Mest	1.02E-85	7.211792
insulin-like growth factor 2	Igf2	2.06E-55	7.353991
indolethylamine N-methyltransferase	Inmt	1.17E-46	10.10275
matrix metallopeptidase 3	Mmp3	1.45E-46	3.556217
cDNA sequence BC100530	BC100530	3.75E-46	3.018132
ADAMTS-like 2	Adamtsl2	1.24E-44	7.03812
MAM domain containing 2	Mamdc2	1.45E-44	6.575771
RIKEN cDNA 2610203C20 gene	2610203C20Rik	6.63E-43	5.901648
stefin A3	Stfa3	2.16E-42	2.139631
fibrillin 2	Fbn2	1.68E-40	5.627277

Table 3.1 Day 14 vs. day 3.

Top ten differentially expressed genes between day 14 and day 3 lung RNA as determined by RNAseq analysis. LogCPM and P value as determined by edgeR (Bioconductor 3.0) analysis.

Gene description	Gene name	P value	LogCPM
granzyme A	Gzma	3.23E-43	2.641041
CDC42 binding protein kinase gamma (DMPK-like)	Cdc42bpg	7.74E-38	7.426844
C1q and tumor necrosis factor related protein 6	C1qtnf6	8.79E-34	5.657464
secreted phosphoprotein 1	Spp1	5.24E-33	3.529507
disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 17	Adamts17	4.78E-32	5.8904
inter alpha-trypsin inhibitor, heavy chain 4	Itih4	5.79E-32	4.455176
thrombospondin 2	Thbs2	2.49E-30	5.412655
KDELendoplasmic reticulum protein retention receptor 3	Kdelr3	9.56E-29	5.1715
fibrillin 2	Fbn2	1.41E-28	5.627277
H19 fetal liver mRNA	H19	3.28E-26	8.252975

Table 3.2 Day 28 vs. Day 14

Top ten differentially expressed genes between day 28 and day 14 lung RNA as determined by RNAseq analysis. LogCPM and P value as determined by edgeR (Bioconductor 3.0) analysis.

Gene description	Gene name	P value	LogCPM
flavin containing monooxygenase 3	Fmo3	1.55E-71	6.037883
inter alpha-trypsin inhibitor, heavy chain 4	Itih4	8.12E-67	4.455176
insulin-like growth factor 2	Igf2	2.20E-61	7.353991
mesoderm specific transcript	Mest	9.49E-61	7.211792
fibrillin 2	Fbn2	5.84E-60	5.627277
insulin-like growth factor binding protein 6	Igfbp6	1.72E-56	7.251023
BAI1-associated protein 2-like 1	Baiap2l1	6.21E-56	7.340725
matrix metallopeptidase 3	Mmp3	3.86E-55	3.556217
CDC42 binding protein kinase gamma (DMPK-like)	Cdc42bpg	2.76E-51	7.426844
H19 fetal liver mRNA	H19	6.75E-51	8.252975

Table 3.3 Day 28 vs. Day 3

Top ten differentially expressed genes between day 28 and day 3 lung RNA as determined by RNAseq analysis. LogCPM and P value as determined by edgeR (Bioconductor 3.0) analysis.

3.3.11 Top genes differing between day 3/28 and day 14

In order to identify individual genes that have altered expression levels throughout development the transcripts for which the expression levels at day 3 were more similar to day 28 than day 14 were used to generate a curated list. Clusters in which expression was $D3^{high}$+$D28^{high}$+$D14^{low}$ or $D3^{low}$+$D28^{low}$+$D14^{high}$ and in which expression levels differed by at least a fold difference of 1.5 were selected. A fold change of 1.5 was selected to allow the detection of subtle genetic changes occurring throughout early development in allergen naïve mice. This list was further refined by excluding transcripts for which differential expression between the D3+D28 cluster and the D14 cluster was statistically insignificant as calculated by GSA. The final transcript list contained 101 genes, represented as a heat map in figure 3.14.

The top 6 genes of interest were selected from this list and by performing a literature search of function. qPCR was carried out to verify the changes observed in the RNAseq analysis (Fig 3.15). From the 6 genes investigated, two were confirmed to follow the pattern of increased expression at day 14 compared to day 3 and day 14 identified by RNAseq, but this requires confirmation with larger sample numbers. CD302 and Angpt2 will be targeted in future studies.

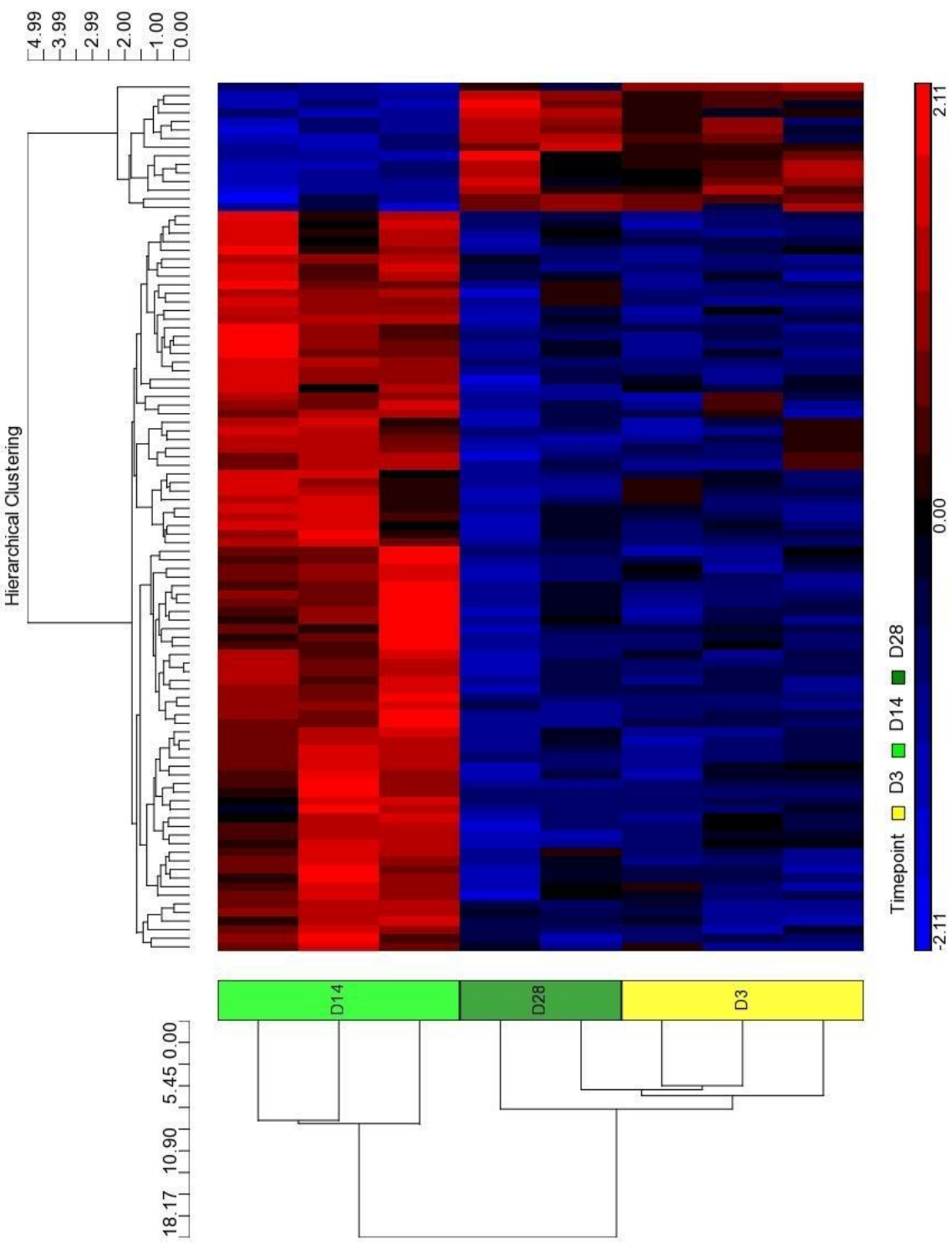

Figure 3.14 Hierarchical clustering map

Hierarchical clustering map of 101 differentially expressed transcripts between day 3, day 14 and day 28. Each column is a transcript and each row is a sample. Relative expression is indicated by colour and time point group is shown by the dendrogram on the left axis.

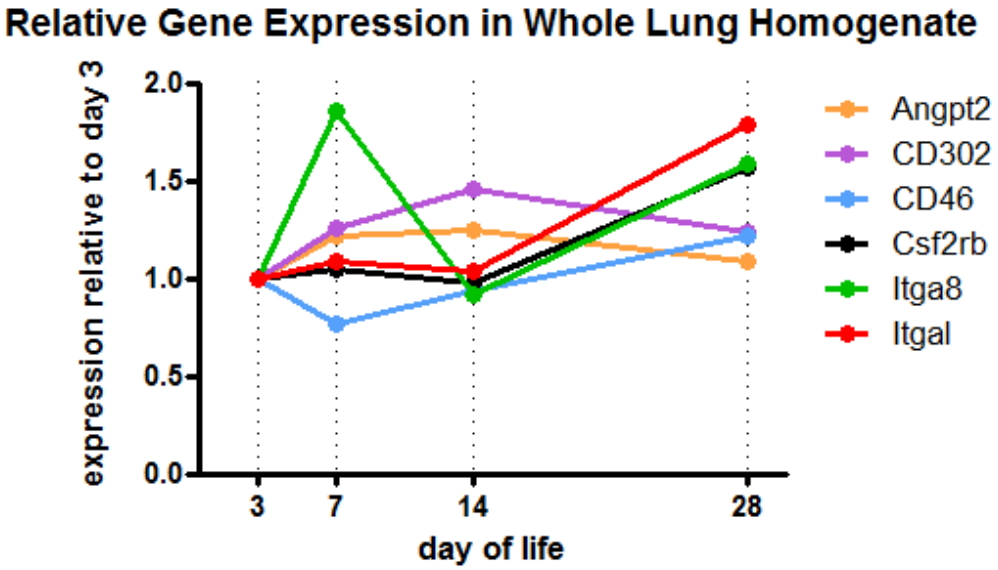

Figure 3.15 candidate genes

qPCR results from the top 6 selected genes differing between day 3+28 and day 14 by at least 1.5 fold. Whole lung RNA isolated from mice aged day 3, 7, 14 and 28 was analysed by qPCR. Data displayed as relative expression to day 3 and represents median values. n= 4-9 per group.

3.4 Discussion

The overall aim of this chapter was to investigate the relationship between age at first allergen exposure and the development of AAD. Further analysis aimed to determine if alteration of the immune cell composition of the lung negated any effects of age on the development of disease.

Initial experiments investigated the relationship between age of first exposure to HDM and the development of AHR. The requirement for these experiments stemmed from contradictory results published showing the onset of robust AAD in neonates when allergen exposure began at day 3 of life (Saglani et al., 2009) and the absence of AAD when allergen exposure began at day 14 (Al-Garawi et al., 2011). Key differences in experimental protocols existed between the publications including quantity of HDM and time-point of AHR analysis after final allergen exposure. These variables could have accounted for the contradictory outcomes and a protocol was therefore developed to eliminate these factors and fully assess the contribution of age to the development of AHR. Interestingly, the current study showed both published results were reproducible under identical experimental conditions and revealed an age dependent development of AHR in response to HDM exposure. When HDM exposure began at day 3 or 7 airway resistance was increased approximately 5 fold form PBS control and when exposure began in adulthood resistance was increased approximately 2 fold. In contrast, mice were non-responsive to allergen exposure beginning at days 14-21 only. Differences in compliance were also not observed when allergen exposure commenced at day 14 or 21, signifying these mice did not have HDM induced airway remodelling. To minimise the chance of type I and II statistical errors each flexivent analysis was performed at least twice with HDM exposure commencing at day 14 repeated an additional two times as a control in figure 3.7. In addition to the elimination of AHR at day 14-21; no IgE, IL-13 or IL-4 were induced by HDM, indicating the lack of AHR at this age was due to immunological rather than structural development in the neonatal lung. Interestingly, IL-33 was consistently increased in response to HDM despite the age of first exposure to allergen and IL-13 levels were substantially higher in mice exposed to HDM

from day 3 of life than mice first exposed in adulthood. IL-33 is released from the epithelium in response to HDM in a TLR4 dependent manner (Willart et al., 2012) and attracts cells such as T cells and ILCs to initiate inflammatory lung disease through the secretion of downstream inflammatory cytokines such as IL-13 (Barlow et al., 2012).

In allergen naïve mice, a natural nadir in the populations of T cells and ILCs expressing IL-13, induced in response to PMA and ionomycin stimulation - at day 14 to 21 indicated reduced populations of inflammatory resident cells abrogated the response to HDM exposure in neonates. In addition to both IL-13^+ cells being reduced in pre-weanling (day 14) mice, a shift in the predominant IL-13 producing cell type occurs throughout development from principally T cells at day 3 of life to ILCs in adult mice.

In contrast to previously published studies, no increase in the level of the regulatory cytokines IL-10 or TGF-β (Al-Garawi et al., 2011) was observed in 14 day mice compared to adult mice, although this study measured levels after three weeks of allergen exposure. Investigations conducted in parallel to the current study focussed on the involvement of the maturing lung microbiome in the development of allergic responses in neonates, pre-weanling and adult mice (Gollwitzer et al., 2014). The emergence of a regulatory $CD4^+CD25^+Foxp3^+Helios^-$ T cell population, termed microbial-induced Tregs, was dependent on interaction with a colonising microbiome in the lung through the expression of PD-1 on DCs presenting microbial components. In collaboration, we were able to determine that the timeline of the development of this regulatory cell subset coincided with an absence of AHR in response to HDM exposure at day 14 of life. In the study presented here, both IL-13^+ T cells and ILCs are present at day 3 of life in mice, paralleling studies of the human immune system that have revealed the existence of both T cells and ILC2 cells at birth (Garcia et al., 2000; Gibbons et al., 2014; Mjösberg et al., 2011). Investigation into the potential role of the interaction of T cells and ILC2 cells with the colonising microbiome will therefore be of great interest.

The influence of early life exposure to allergens on the development of allergic asthma in humans is well documented and may therefore be mechanistically similar to the early life (day 3-14 of life) generation of murine disease observed in these studies. For example, 95% of asthmatics develop symptoms by age 6 (Masoli et al., 2004). In studies of immigrating populations to the UK, if a child migrates to the UK before age 5 they adopt the asthma prevalence in the UK, however, if they migrate after age 5 the prevalence of the home country remains - this illustrates the close relationship between early life exposure and the development of disease (Kuehni et al., 2007). Although the similarities between early life human and murine studies appear attractive, the stages of development in mice and humans are hard to compare as mice are weaned at week 3 of life but then reach sexual maturity at week 4-5. Further studies into the development of the human immune system in early life are therefore required to assess the translatability of observations in the mouse models. Interference with the developing immune system has been implicated in the pathogenesis of paediatric allergic disease in a variety of forms; including a lack of stimulation of Th1 cells (Platts-Mills et al., 2001; Von Ehrenstein et al., 2000), early viral infections causing dysregulation of a naturally occurring regulatory environment (Jackson et al., 2012; Kusel et al., 2007) and the prevention of Helios$^-$ Treg development through blocking of PD-1 (Gollwitzer et al., 2014). Results from the current study show altering the pulmonary cellular environment, through the addition of rIL-33, during early life was sufficient to overcome the naturally occurring tolerance associated with early life.

In addition to the involvement of environmental factors, a strong genetic component exists in the pathogenesis of allergic asthma (Duffy et al., 1990). GWAS of paediatric asthma patients has identified SNPs in the gene region of IL-33 as a possible contributor to disease (Moffatt et al., 2010) and increased levels of IL-33 have been observed in both the lungs and serum of asthmatic patients (Guo et al., 2014a; Préfontaine et al., 2010; Saglani et al., 2013). In the current study, addition of rIL-33 to the allergen during the window of non-responsiveness was used to mimic a situation in which genetically predisposed individuals may secrete increased levels of IL-33 in response to allergen

exposure. When first allergen exposure commenced at day 14 along with the addition of rIL-33, the hypo-responsive effect of age was eliminated and mice developed AHR, HDM specific IgE and IL-13. rIL-33 also caused a substantial increase in the populations of IL-13^{+} T cells and IL-13^{+} ILCs above HDM exposure alone from day 14 of life. Interestingly, ILC populations were approximately 6 fold higher than T cells, leading to an investigation into the importance of T cells in overcoming the hypo-responsive time-point in development.

The use of SCID mice, that have no functional T or B cells, revealed that the absence of T cells did not affect the development of rIL-33 induced AHR. IL-33 released from the epithelium upon allergen exposure attracts ILC2s to the lung and leads to rapid expansion of the population (Neill et al., 2010). Recently, one study has revealed RSV infection also causes the release of pulmonary IL-33, leading to increased ILC2 populations during infection (Saravia et al., 2014). One hypothesis to explain the development of asthma during childhood is therefore an increased population of ILCs, either in genetically predisposed individuals or through the involvement of a virus, which interferes with the natural nadir of ILC2s in early life and therefore overcomes the natural hypo-responsive immune period, leading to the development of allergic asthma. The effect of early life viral infection on the phenotype of Tregs has been described (Krishnamoorthy et al., 2012); viral infection caused the up-regulation of GATA3 expression in Tregs and the subsequent production of Th2 cytokines from these cells. A parallel study of the effect of early infection on the phenotype of ILCs would be of great interest.

Immune cells isolated from adults and neonates respond differently to ligation of surface receptors *in vitro* with neonatal cells producing more Th2 and regulatory mediators and proliferating less than adult cells; including T cells (Hassan and Reen, 1997; Splawski et al., 1991), B cells (Marshall-Clarke et al., 2000) and dendritic cells (Belderbos et al., 2009; Eger et al., 2006). In addition to determining the total cells and inflammatory mediators involved in the hypo-responsive period during early life, this study also aimed to identify changes in gene expression that occur throughout early life and may be

responsible for reduced response to allergen exposure. Originally, the aim was to isolate RNA from facs sorted IL-13^{+} ILCs and T cells, however, due to the low numbers of these cells in naïve mice and the difficulty of isolating good quality RNA from cells isolated via an intracellular stain, whole lung RNA was analysed by RNAseq to identify genes of interest. Potential candidate genes were identified by seeking targets that were either increased or decreased in day 3 and 28 mice when compared to day 14 by a magnitude of 1.5 fold. Two initial genes of interest have been highlighted from preliminary analysis of the data – CD302 and Angpt2 – although further, in-depth analysis of the RNAseq results will be required to identify all potential genes associated with immune development. Future work will focus on refining the techniques for isolation of high quality RNA from sorted IL-13^{+} cell populations as well as more in depth analysis of results generated by RNAseq.

CD302 encodes the protein DCL-1 which is a C-type lectin receptor expressed on the surface of monocytes, macrophages, granulocytes and dendritic cells (Kato et al., 2007). The receptor has been identified as a phagocytic receptor and hence has a potential role in generating anti-microbial responses (Hoving et al., 2014). CD302 is therefore of great interest as its raised gene expression at day 14 has the potential for being associated with the sampling of the developing lung microbiome and the subsequent development of the essential Helios^{-} Tregs associated with the hyporesponsive phase to allergen exposure in early life (Gollwitzer et al., 2014). Further examination of this potential relationship will be an important next step in this investigation.

Angpt2 also has increased expression at day 14 compared to day 3 and day 28. Angpt2 encodes angiopoientin 2, a protein associated with inhibiting angiogenesis. Administered *in vivo* in a mouse model of paw inflammation caused oedema and the trafficking of lymphocytes (Roviezzo et al., 2005). Interestingly, increased levels of angiopoientin 2 have recently been documented in the sputum of asthmatic patients on comparison to healthy controls, this level was further increased in smoking subjects (Petta et al., 2014). In the context of this study, angiopoietin 2 may be required for the influx of immune cells to the lung in response to microbial colonisation.

3.4.1 Conclusion

This chapter described the relationship between the age at first allergen exposure and the development of AAD. A hypo-responsive window during development was described at age 14-21 days where mice did not respond to three weeks of HDM exposure with AHR, IgE or IL-13. This time point coincided with a natural reduction in the populations of resident T cells and ILCs with the capacity to produce IL-13, under *ex vivo* stimulation, and the development of Helios$^-$ Tregs, as documented by Gollwitzer *et. al*. (2014). The administration of rIL-33 in addition to HDM overcame the hypo-responsive period in a manner that was not dependent on T cells. A number of potential candidate genes were also identified by RNAseq analysis of whole lung with the potential to contribute towards the hypo-responsive period.

Due to the higher AHR and IL-13 observed in mice that were exposed to allergen from day 3 of life compared to adults, the next chapter will go on to investigate the differences between adult and neonatal immune responses to two different allergens; HDM and the fungal allergen *Alternaria alternata* (Alt). This is important as patients with asthma are often poly-sensitised to several allergens and sensitisation to fungal allergens is specifically associated with severe, steroid resistant asthma and the mechanisms underlying severe asthma with fungal sensitisation (SAFS) are unexplored.

Chapter 4 – Allergen type determines the magnitude of AAD in both adults and neonates

4.1 Introduction

The previous chapter described a period during development when pre-weanling mice, aged between 14 and 21 days, were non-responsive to HDM stimulation. However, neonatal mice, when exposed to HDM from 3 days of age, had significantly higher airway resistance and IL-13 compared to all other age groups analysed.

Childhood onset asthma is the most common chronic disease in children, with approximately 7 million children below the age of 17 in the USA affected by the disease (Freeman G, 2011). Asthma in early life is difficult to diagnose as patients often present with a transient wheeze which does not necessarily develop into asthma (Martinez et al., 1995). Studies have also suggested that healthy children have naturally hyper-responsive airways when compared to adults (Tepper, 1987), a feature which is thought to be dependent on maturation of the airways (Hopp et al., 1985). Infections and allergen exposure in this time of airway maturation have the potential to interfere with normal airway development and predispose to the generation of asthma in early life.

Childhood asthma is most commonly associated with atopic disease and known to be strongly IgE mediated (Guilbert et al., 2004). In contrast, the majority of adult onset asthma is non-atopic and instead commonly results from industrial exposure to chemicals, stress or overuse of drugs such as aspirin or paracetamol throughout life (Nijs et al., 2013). Despite the majority of adult onset asthma being non-atopic, a large proportion of adults with severe asthma suffer from atopic disease, often with sensitisation to multiple allergens (Moore et al., 2010). The definition of severe asthma is variable between research organisations but involves the use of high-dose inhaled steroids, periodic or regular use of oral steroids, an FEV1 of <80% from predicted baseline and a history of hospital admissions (Chung et al., 2014).

Another feature of severe allergic asthma in both adults and children is sensitisation to fungal allergens, in particular *Alternaria alternata* (Alt) (Blatter et al., 2014; Halonen et al., 1997; O'Driscoll

et al., 2009; Zureik et al., 2002). Alternaria is an airborne allergen making limiting exposure difficult. Recently, the association of Alt with asthma exacerbations was investigated in an adult mouse model and found to be due to direct release of IL-33 from the epithelium in response to serine protease activity of the fungal allergen (Snelgrove et al., 2014). The enhanced IL-33 production and secretion documented by this study may be responsible for the association of fungal allergens with severe asthma since IL-33 has been associated with increased asthma severity and steroid resistance in both human patients and mouse models (Guo et al., 2014b; Saglani et al., 2013).

The majority of patients with asthma respond to inhaled steroid treatment making symptom management possible. Steroids act by directly interacting with promoter elements on genes by either suppressing inflammatory mediators such as cytokines, chemokines and adhesion molecules, or promoting the expression of anti-inflammatory mediators (van der Velden, 1998). The use of corticosteroids in paediatric asthmatics has been associated with an increased level of pulmonary regulatory T cells (Tregs) (Hartl et al., 2007). Despite this broad range of action to decrease inflammation, a small proportion of asthmatics are non-responsive to high-dose inhaled and systemic corticosteroids; these patients are classified as steroid resistant asthmatics (SRA) (Barnes and Woolcock, 1998). The small proportion of asthmatics that are SRA account for almost 50% of the healthcare costs related to asthma (Serra-Batlles et al., 1998).

The aim of this chapter was to investigate the severity of AAD induced by either HDM or Alt in neonatal as well as adult mice. We also aimed to investigate the relationship between AAD generated by these allergens and steroid resistance by treating neonatal mice with both preventative and therapeutic steroid regimens during allergen exposure.

4.1.1 Hypothesis

Neonates develop an exaggerated response to allergen exposure in comparison to adult mice.

The fungal allergen Alt induces a more severe allergic phenotype, which is associated with steroid resistance, in comparison to HDM in both adults and neonates.

4.1.2 Aims

1. To compare three weeks of HDM and Alt exposure in neonatal and adult mice.
2. To determine if Alt sensitisation is linked to steroid resistance in a neonatal model of preventative budesonide treatment.
3. To investigate the effects of preventative versus therapeutic steroid intervention in a model of HDM exposure in neonatal mice.

4.2 Methods

In protocol A, neonatal (day 3) Balb/c or Balb/CJ mice were administered intranasal Alt or HDM for three weeks. For the first two weeks, neonatal mice were given 5µg Alt or 20µg HDM with no anaesthesia three times per week. For the final week, mice received 10µg Alt or 25µg HDM using recovery anaesthesia of inhaled isoflurane. Mice were culled 4 hours after the last dose of HDM and 18 hours post last Alt dose. 18 hours after final Alt exposure was chosen as a time point as flexivent analysis of mice examined 4 hours post Alt exposure showed extremely variable airway resistance results between mice (data not shown) while HDM exposed mice had reproducible resistance values between mice after 4 hours.

In protocol B, adult (6-8 weeks) Balb/c or Balb/CJ mice were given 10µg Alt or 25µg HDM three times per week for three weeks using recovery anaesthesia of inhaled isoflurane. Control mice received an equivalent volume of PBS. Mice were culled 4 hours after the last dose of HDM and 18 hours after the last Alt dose.

In protocol C, neonatal Balb/c mice were dosed with 10µg HDM or 5µg Alt intranasally three times weekly for the first two weeks of life. Mice were then dosed with 15µg HDM or 10µg Alt HDM using recovery anaesthesia of inhaled isoflurane. After the first week of allergen exposure mice were dosed 5x per week with 0.6mg/kg intranasal budesonide. Mice were culled 4 hours post last dose HDM and 18 hours post last dose Alt. Previous work in the lab had established HDM induced AHR in adult mice was ablated using three weekly doses of 1mg/kg budesonide (unpublished data). For neonatal mice, the volume of solution was too large for 1mg/kg to be administered i.n so an equivalent weekly quantity of budesonide was administered 5 times per week with 0.6mg/kg/dose.

In protocol D, neonatal mice were exposed to HDM for three weeks, after AAD was established at this time, mice were dosed with 0.6mg/kg/day i.n budesonide 5 days per week and HDM exposure was continued 3x weekly. Mice were culled 4 hours after the last dose of HDM.

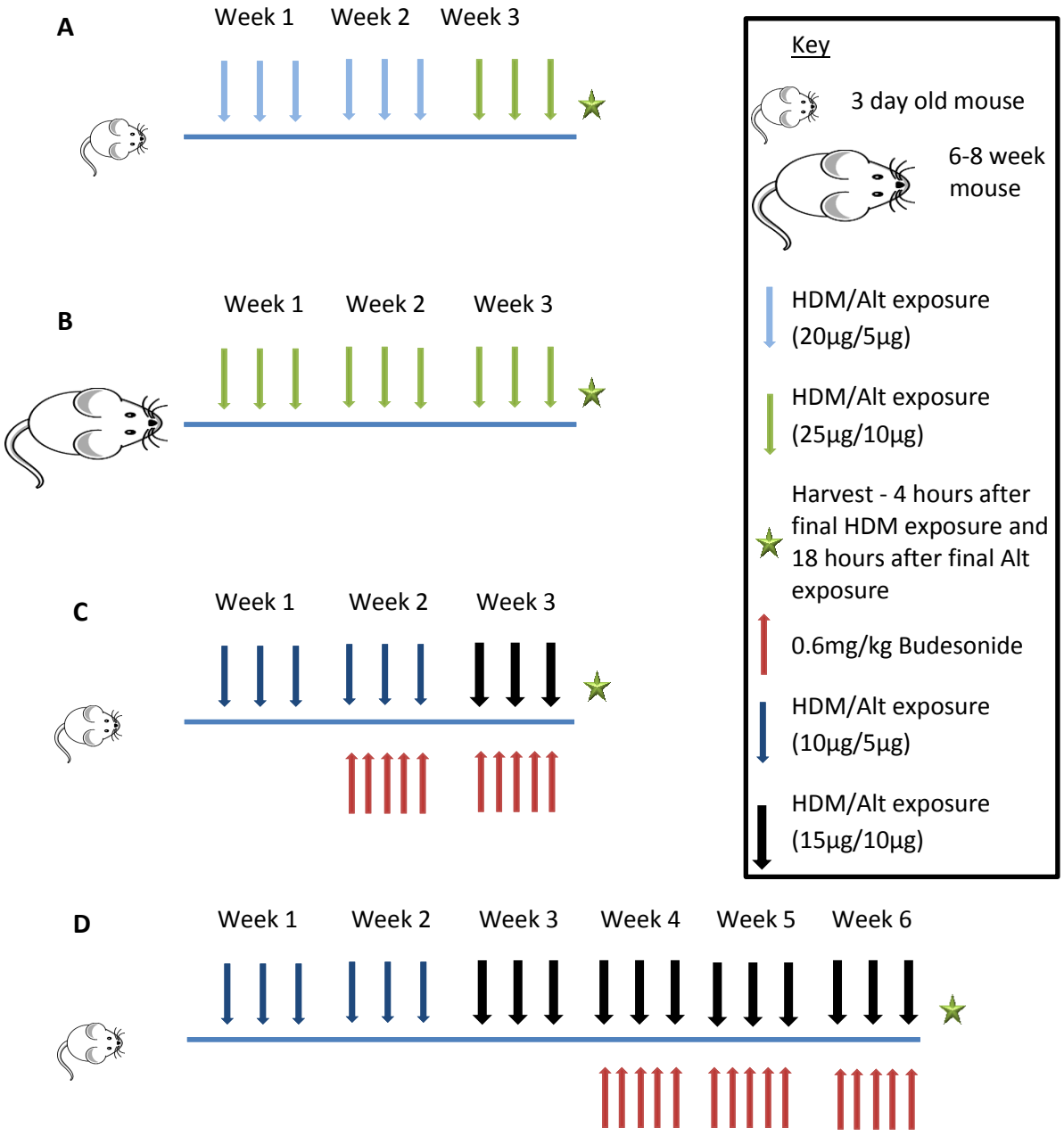

Figure 4.1 Methods

Three week neonatal HDM or Alt exposure protocol; from day three neonatal mice were exposed to HDM or Alt three times per week for three weeks **(A)**. Three week Adult HDM or Alt exposure protocol; adult mice were exposed to HDM or Alt three times per week for three weeks **(B).** Neonatal preventative budesonide protocol; from day 3 of life mice were exposed to HDM or Alt for one week. For the following two weeks mice were exposed to allergen as well as i.n. budesonide 5 times per week **(C)**. Neonatal therapeutic budesonide protocol; neonatal mice were exposed to HDM from day 3 of life for three weeks. For the next three weeks, mice were exposed to both HDM and budesonide **(D)**.

4.3 Results

4.3.1 Alt induces enhanced airway resistance compared to HDM in adult, but not neonatal mice

Neonatal mice exposed to either HDM or Alt (Fig 4.1 A) developed a similar airway resistance response to increasing doses of methacholine (Figure 4.2 A). However, airway resistance to 100mg/ml MCh was elevated in response to HDM compared to Alt (Figure 4.1 C). In contrast, after three weeks of allergen exposure, adult mice had higher airway resistance in response to Alt exposure when compared to HDM exposure (Figure 4.2 B, D).

HDM and Alt induced significant BAL and lung inflammation in both adults and neonates above the PBS control following both allergens, but Alt exposure resulted in significantly more lung inflammation in comparison to HDM in both neonates and adults (Fig 4.3 A-D).

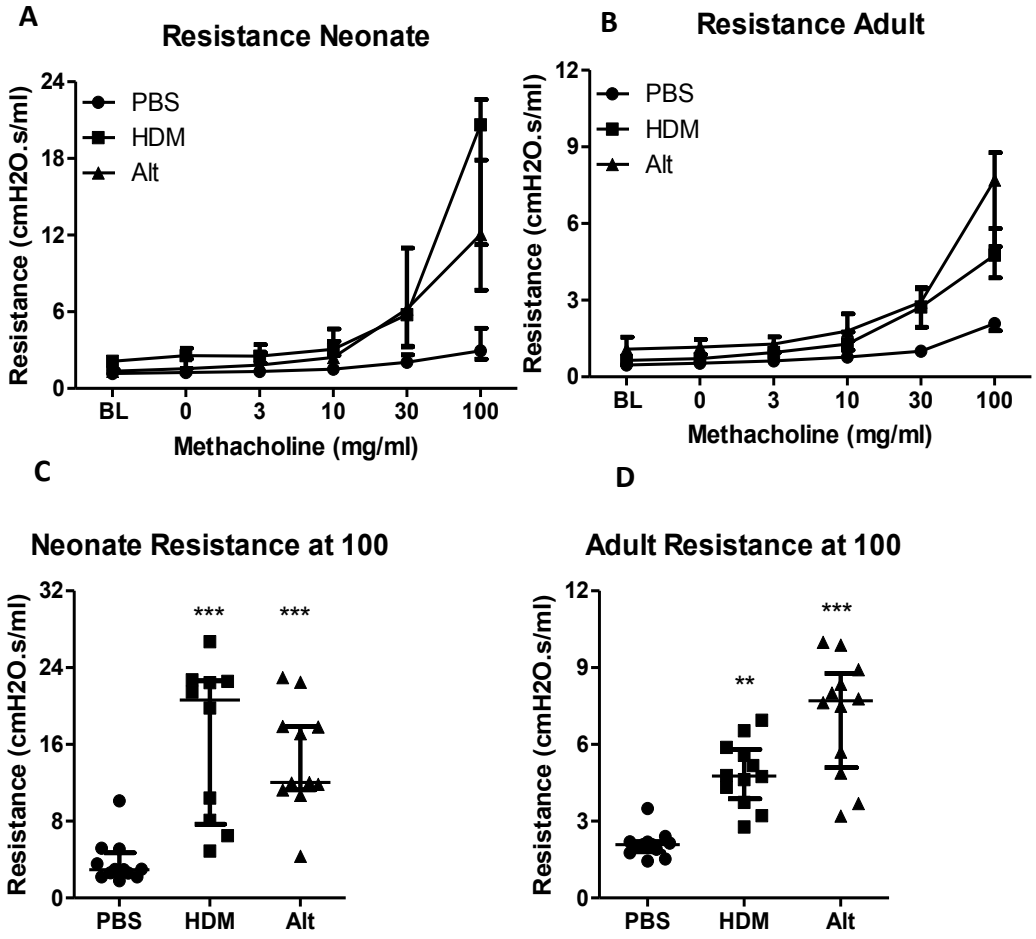

Figure 4.2 Adult and neonatal airway resistance in response to HDM or Alt.

AHR measured by flexivent. Airway resistance over increasing concentrations of methacholine in mice dosed for three weeks with HDM in neonates **(A)** and adults **(B)**. Resistance at 100mg/ml Methacholine in neonates **(C)** and adults **(D)**. Circles represent PBS, squares HDM and triangles Alt. n= 8-12 mice per group, results from two separate experiments. ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

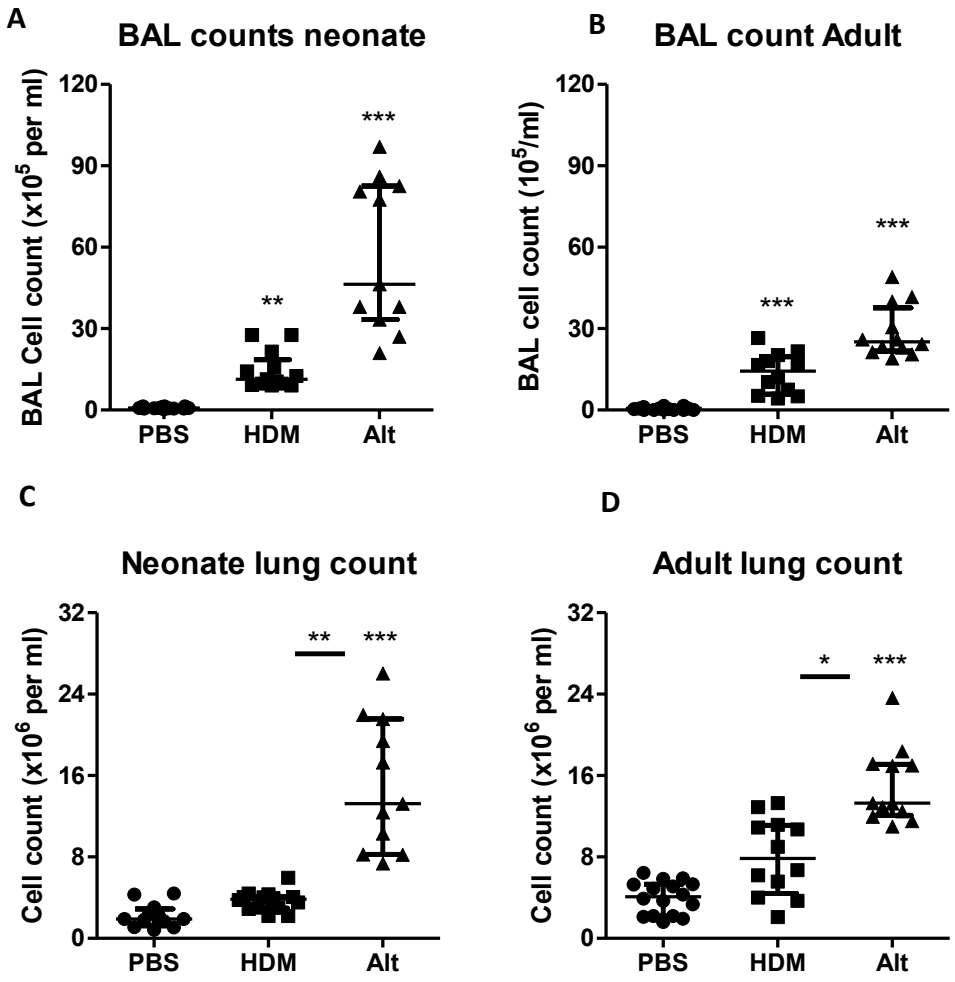

Figure 4.3 Adult and neonatal airway inflammation

Lungs and BAL were collected after three weeks of HDM or Alt exposure and processed for cell counts. Cell count per ml of BAL fluid in neonates **(A)** and adults **(B)**. Lung cell count per ml lung digest in neonates **(C)** and adults **(D)**. Circles represent PBS, squares HDM and triangles Alt. n= 8-12 mice per group, results from two separate experiments. *p<0.05, ** p<0.01 and *** p<0.001 from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

4.3.2 Inflammatory cytokines and sensitisation

After three weeks of allergen exposure in neonates and adults; lung, BAL supernatant and serum were analysed by ELISA for Th2 inflammatory cytokines and IgE. Lung IL-5 and IL-4 were elevated in response to HDM and Alt in both neonates and adults with significantly more IL-5 induced in response to Alt compared to HDM exposure in adult lungs only (Fig 4.4 A-D). Alt exposure resulted in the induction of significantly more IgE than HDM exposure in both neonates and adults (Fig 4.4 E, F). Alt also resulted in a greater increase in IL-33 when compared to HDM in neonatal lungs while exposure to HDM or Alt resulted in similar IL-33 levels in adult mice (Fig 4.5 A, B). IL-13 was elevated in the lung and BAL in response to HDM and Alt exposure in both neonates and adults. With the exception of lung levels in neonatal mice, more IL-13 was induced following Alt than HDM exposure (Fig 4.5 C-F).

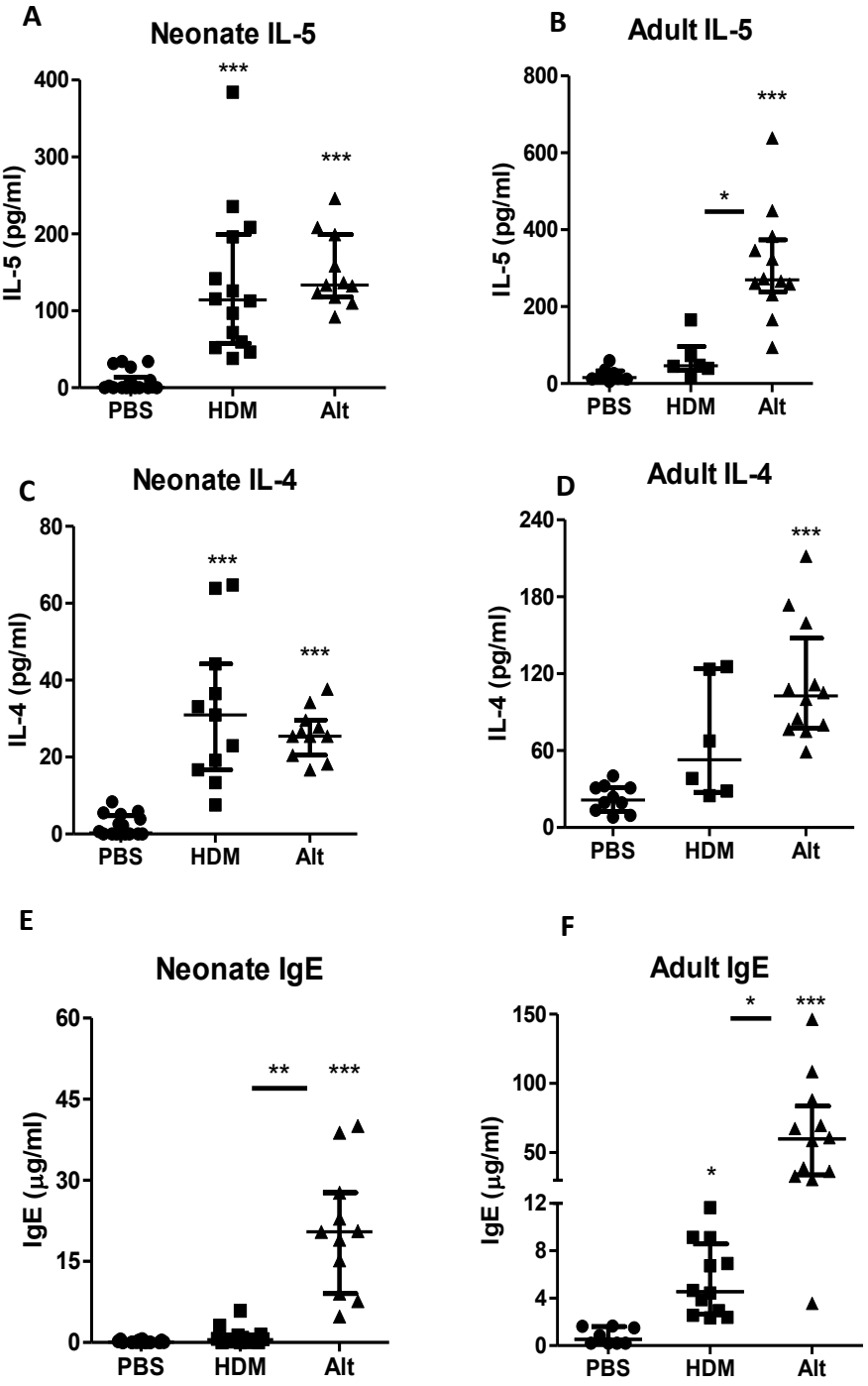

Figure 4.4 HDM and Alt sensitisation in neonates and adults.

Serum and lung was collected after three weeks of intranasal HDM or Alt and lung was homogenised. IL-5 in neonatal lung **(A)** and adult lung **(B)**. IL-4 in neonatal lung **(C)** and adult lung **(D)** as concentration per ml lung homogenate (50mg/ml). Total serum IgE in neonates **(E)** and adults **(F)**. Circles represent PBS, squares HDM and triangles Alt. n= 8-12 mice per group, results from two separate experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

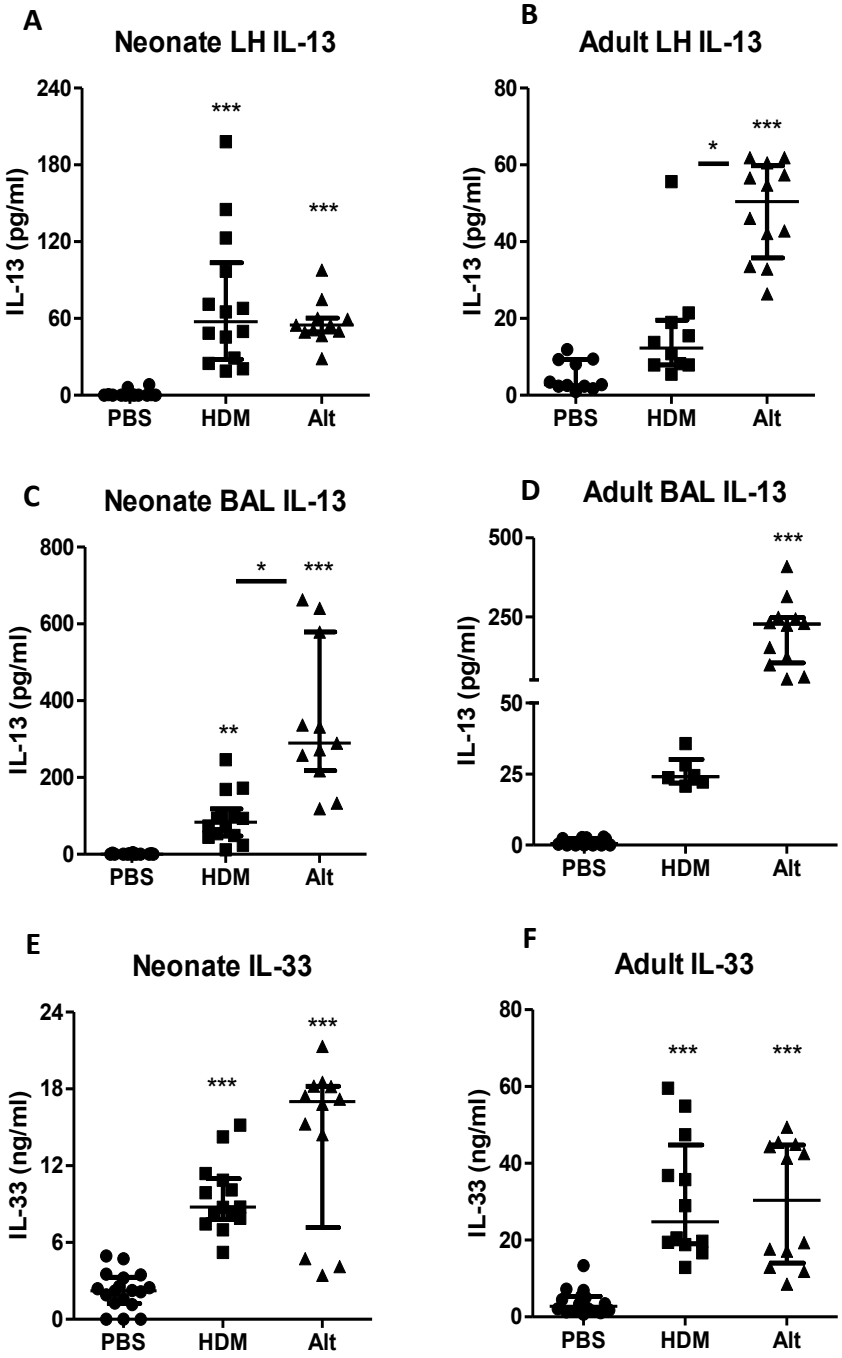

Figure 4.5 Inflammatory cytokines in HDM and Alt exposed adults and neonates

Lung and BAL was collected after three weeks of intranasal HDM or Alt exposure and lung was homogenised. IL-33 in neonatal lung **(A)** and adult lung **(B).** IL-13 in neonatal lung **(C)** and adult lung **(D)** as concentration per ml lung homogenate (50mg/ml). BAL IL-13 in neonates **(E)** and adults **(F)**. Circles represent PBS, squares HDM and triangles Alt. n= 8-12 mice per group, results from two separate experiments. n= 8-12 mice per group, results from two separate experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

4.3.3 ILC and T cell populations in response to ALT and HDM exposure in neonates and adults

Flow cytometry was used to determine numbers of T cells and ILCs as cellular sources of IL-13 contributing to the higher levels of IL-13 observed in the BAL of both adult and neonatal mice and the lung tissue of adult mice after Alt exposure. After three weeks of allergen exposure, both adults and neonates had increased total ILCs in the lung compared to PBS control mice. In neonatal mice, Alt induced a significantly larger ILC population than HDM (Fig4.6 A) while in adult mice, exposure to both HDM and Alt resulted in a similar population (Fig4.6 B). Both allergens caused a significant induction of IL-13$^+$ ILCs in both neonates and adults with Alt exposure generating a significantly larger population than HDM in neonates (Fig 4.6 C, D).

CD3$^+$CD4$^+$ T cells were significantly higher in the lungs of neonatal mice exposed to Alt while the overall population of T cells in adult mice was not increased by allergen exposure (Fig 4.7 A, B). Despite the limited increase in the overall T cell population, IL-13$^+$ T cells were increased above the PBS control in response to Alt and HDM in both neonates and adult, but Alt exposure induced a significantly larger population than HDM (Fig 4.7 C, D). As the number of IL-13$^+$ T cells after both HDM and Alt exposure were lower in neonates when compared to adults (Fig 4.7 C, D) the proportion of T cells expressing IL-13 was also investigated to determine if the reduced populations were due to an overall reduction in numbers or less activation of the T cells. Both allergens caused an increase in the percentage of T cells expressing IL-13 compared to the PBS control with Alt leading to a higher percentage of IL-13$^+$ T cells in both adults and neonates. As with total number, the percentage of IL-13$^+$ T cells was also substantially increased in adults compared to neonates (Fig 4.7 E, F).

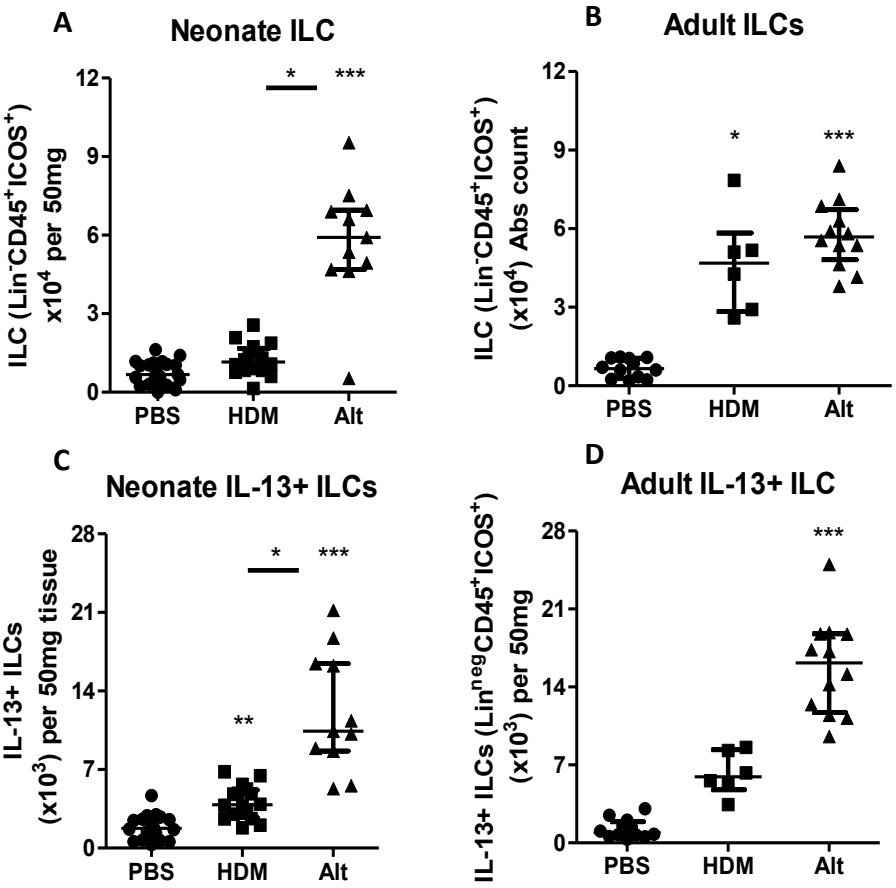

Figure 4.6 ILCs induced by HDM and Alt sensitisation

Lungs were collected and cells isolated for flow cytometry analysis after three weeks of HDM or Alt exposure. Total ILCs ($Lin^-CD45^+ICOS^+$) in neonates **(A)** and adults **(B)**. IL-13^+ ILCs in neonates **(C)** and adults **(D)** determined by flow cytometry. Circles represent PBS, squares HDM and triangles Alt. n= 6-12 mice per group, results from two separate experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

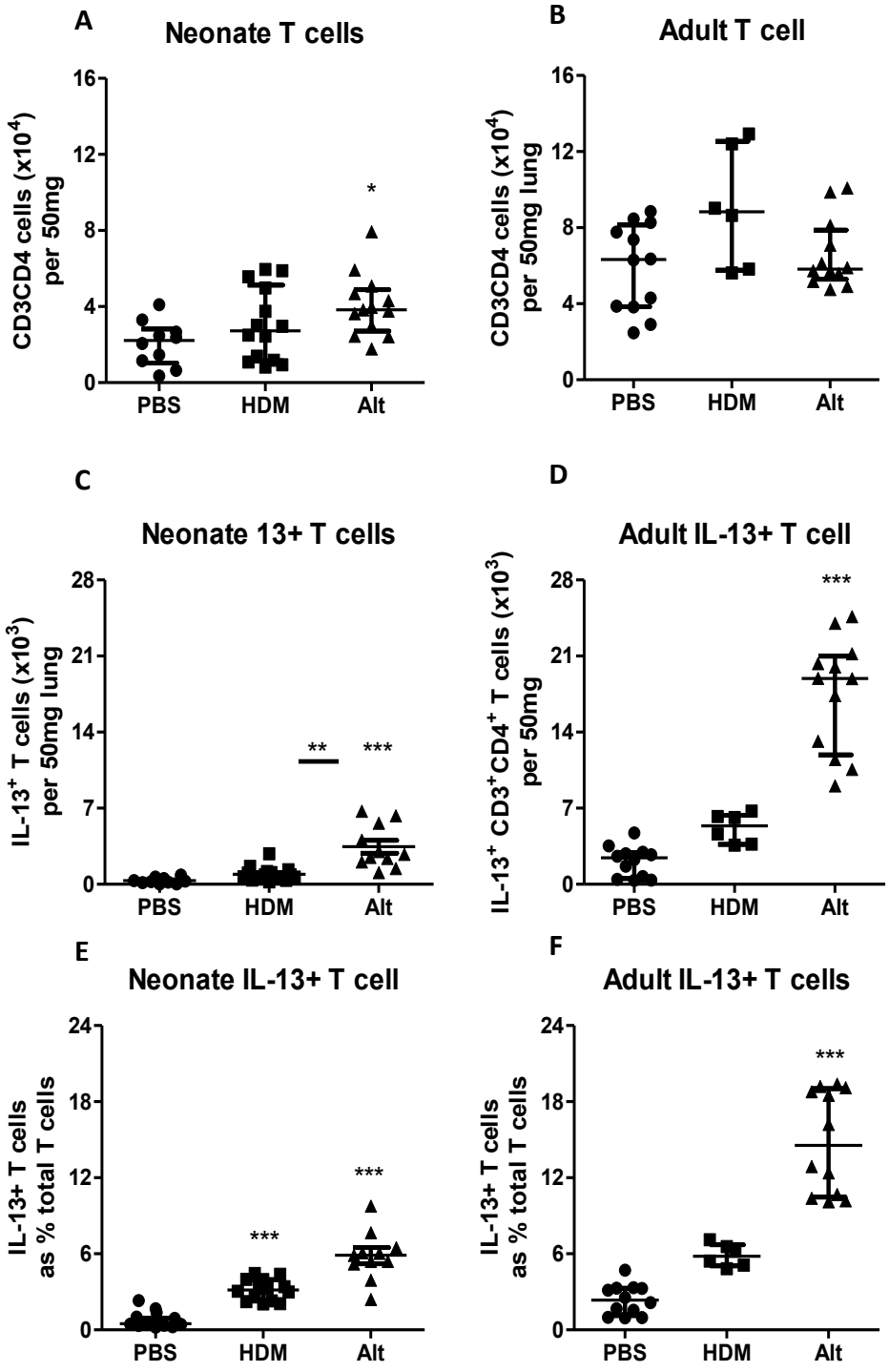

Figure 4.7 T cells induced by HDM and Alt sensitisation

Lungs were collected and cells isolated for flow cytometry analysis after three weeks of HDM or Alt exposure. Total T cells ($CD3^+CD4^+$) in neonates **(A)** and adults **(B)**. Total IL-13^+ T cells in neonates **(C)** and adults **(D)** and percentage of T cells expressing IL-13 in neonates **(E)** and adults **(F)** determined by flow cytometry. Circles represent PBS, squares HDM and triangles Alt. n= 6-12 mice per group, results from two separate experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

4.3.4 Steroid treatment using a preventive regimen inhibits HDM, but not Alt induced AAD

Patients with severe asthma are often treated with high doses of steroids, however for a subset of these patients, steroids have no affect and the disease is extremely difficult to control. Sensitisation to one or more fungal allergens is known to be associated with severe asthma (Blatter et al., 2014; O'Driscoll et al., 2009; Zureik et al., 2002). Our lab previously published that steroid resistance in neonatal mice was due to elevated IL-33 in response allergen sensitisation (Saglani et al., 2013). To investigate whether fungal sensitisation is associated with more steroid resistant disease, neonatal mice were exposed to either HDM or Alt for one week before exposure to both allergen and daily i.n bud for a further two weeks as a preventative steroid protocol (Fig 4.8 A).

The addition of daily budesonide to a neonatal model of HDM exposure prevented the development of AHR; this was significantly reduced compared to mice exposed to HDM without budesonide (Fig 4.8 B, C). In contrast, mice dosed with daily budesonide throughout Alt exposure still had AHR and budesonide did not reduce Alt induced AHR (Fig 4.8 D, E).

Dosing mice with budesonide in a prevention regimen with HDM exposure did not resulted in increased BAL inflammation (Fig 4.9 A). In contrast, BAL inflammation in mice exposed to both Alt and budesonide was elevated above the control, although not significantly (Fig 4.9 B). Lung inflammation in response to both HDM and Alt was significantly reduced by Bud in comparison to allergen exposure alone (Fig 4.9 C, D). To elucidate the composition of BAL inflammatory infiltrates, eosinophils were determined by flow cytometry. In HDM exposed mice, the inflammatory infiltrate in BAL was composed of 60% eosinophils. However, when mice were exposed to daily budesonide and HDM, no eosinophils were recruited above the PBS control (Fig 9 E). In contrast, budesonide did not prevent the recruitment of eosinophils to the BAL during Alt and budesonide (Fig 4.9 F).

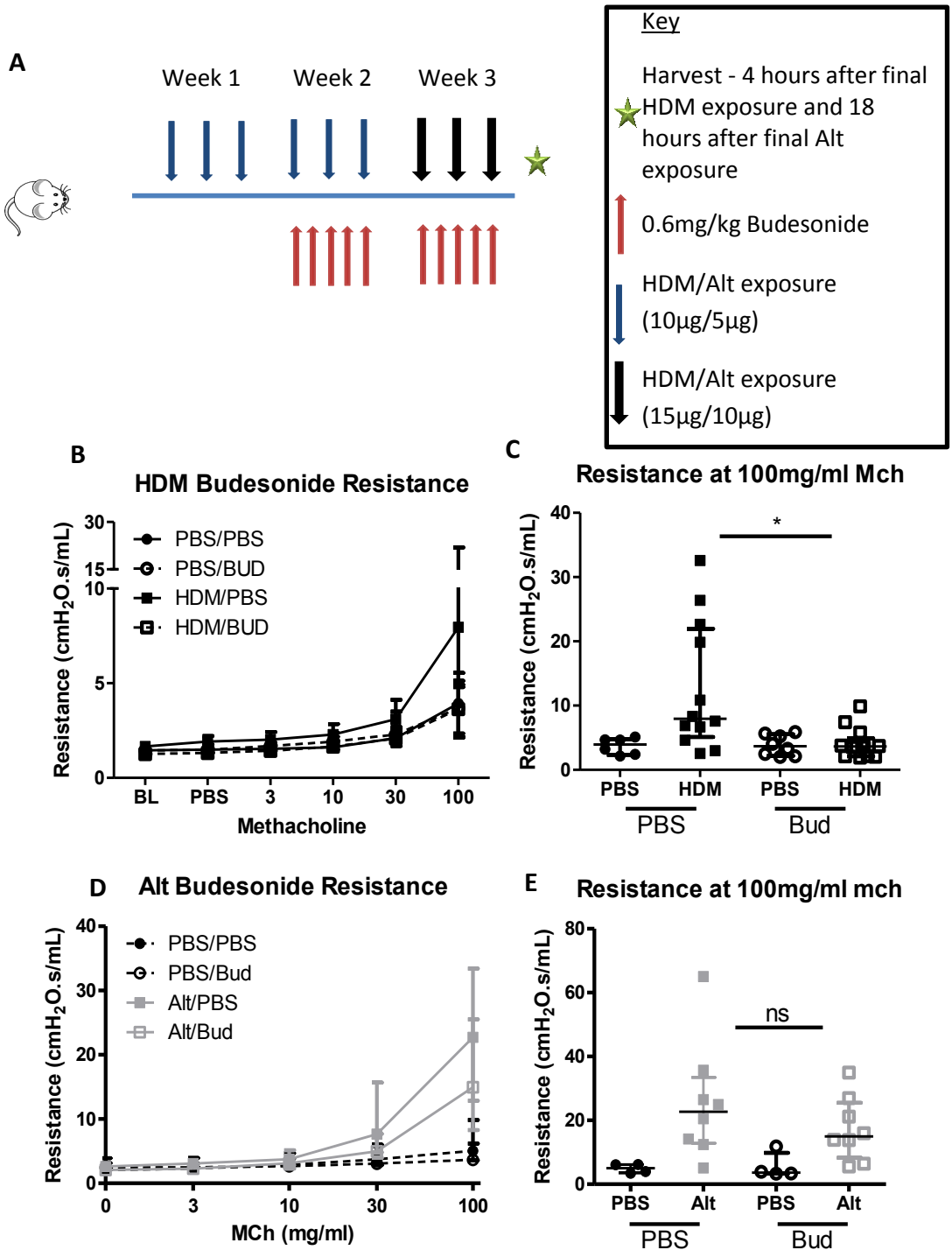

Figure 4.8 Airway resistance in response to allergen and budesonide exposure

Neonatal mice were dosed daily with budesonide throughout HDM or Alt exposure **(A)**. Airway resistance over increasing concentrations of methacholine in HDM exposed mice **(B)** and resistance at 100mg/ml Methacholine **(C)**. Airway resistance over increasing concentrations of methacholine in mice dosed with budesonide and exposed to Alt **(D)** and resistance at 100mg/ml Methacholine **(E)**. n= 4-12 mice per group, HDM results from two separate experiments, Alt results from one. * p<0.01 ns=not significant by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

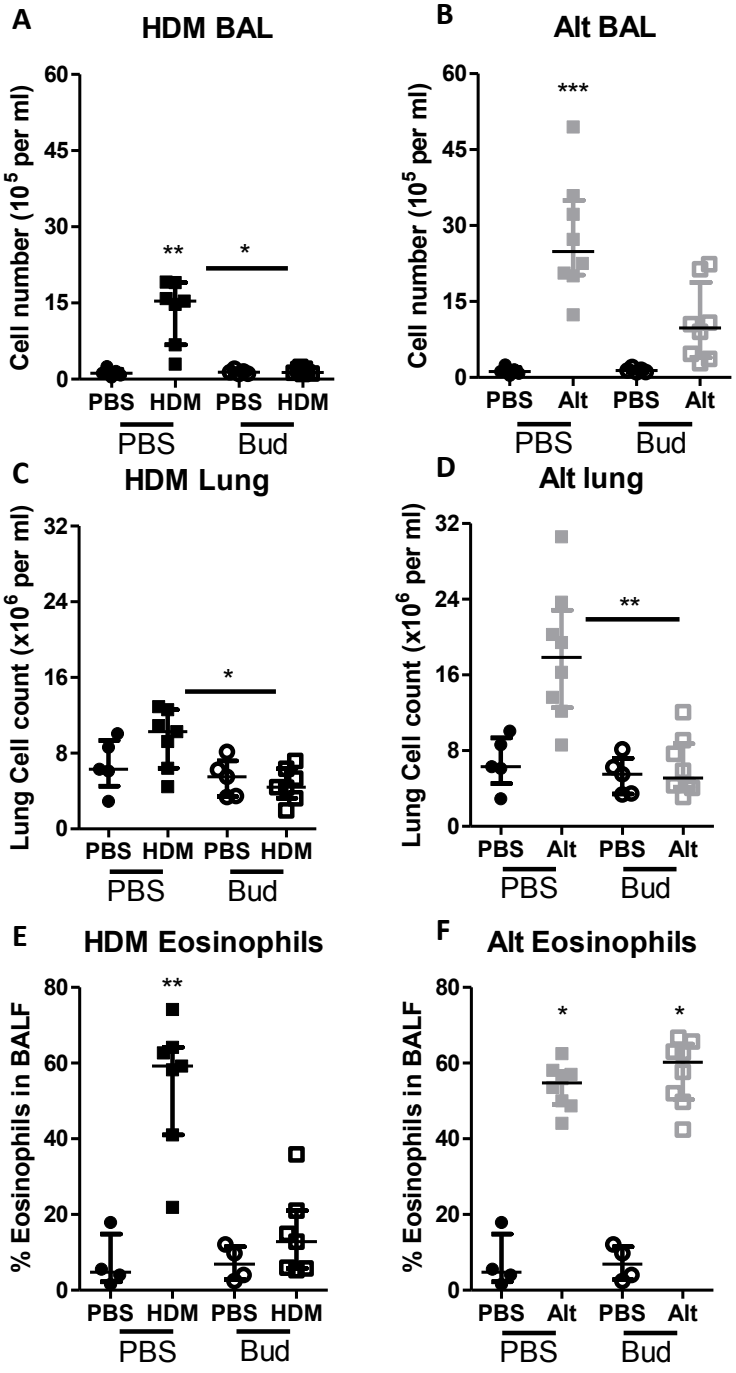

Figure 4.9 Lung and BAL inflammation in response to allergen and budesonide exposure.

Neonatal mice were dosed daily with budesonide throughout HDM or Alt exposure before collection of BAL and lungs. BAL counts in mice dosed with budesonide and exposed to HDM **(A)** and Alt **(B)**. Lung counts in mice dosed with budesonide and exposed to HDM **(C)** and Alt **(D)**. BAL eosinophils as percentage of live BAL cells in mice dosed with budesonide and exposed to HDM **(E)** and Alt **(F)**. n= 4-8 mice per group. *p<0.05, ** p<0.01 and *** p<0.001 from relevant PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

4.3.5 Budesonide caused a significant reduction in IL-13, but not IL-33, during allergen exposure

To determine which cytokines were affected by the administration of steroids during allergen exposure lungs and BAL were collected and analysed by ELISA. Serum was also collected to determine the effect of steroid dosing on IgE.

Levels of IL-4 were unaffected by treating neonatal mice with budesonide during exposure to HDM, however budesonide treatment with Alt exposure resulted in a reduction in IL-4 levels compared to Alt alone (Fig 4.10 A, B). IL-5 induction was unaffected by daily budesonide dosing with HDM and Alt exposure (Fig 4.10 C, D). Serum total IgE was similar regardless of budesonide treatment during HDM exposure (Fig 4.10 E). In contrast, Alt induced IgE was only significantly elevated in Alt alone exposed mice, although levels in budesonide treated mice were still substantially higher than IgE generated in response to HDM (Fig 4.10 F).

IL-13 was detected in the BAL and lung of mice exposed to either HDM or Alt. The addition of daily budesonide treatment caused the complete suppression of BAL IL-13 in response to both HDM and Alt exposure (Fig 4.11 A, B). In addition to BAL IL-13 secretion, budesonide also prevented the generation of lung IL-13 in response to HDM exposure (Fig 4.11 C). In contrast, lung IL-13 remained elevated in mice treated with budesonide during Alt exposure (Fig 4.11 D). IL-33 levels were unaffected by budesonide dosing in both HDM and Alt exposed neonates (Fig 4.11 E, F).

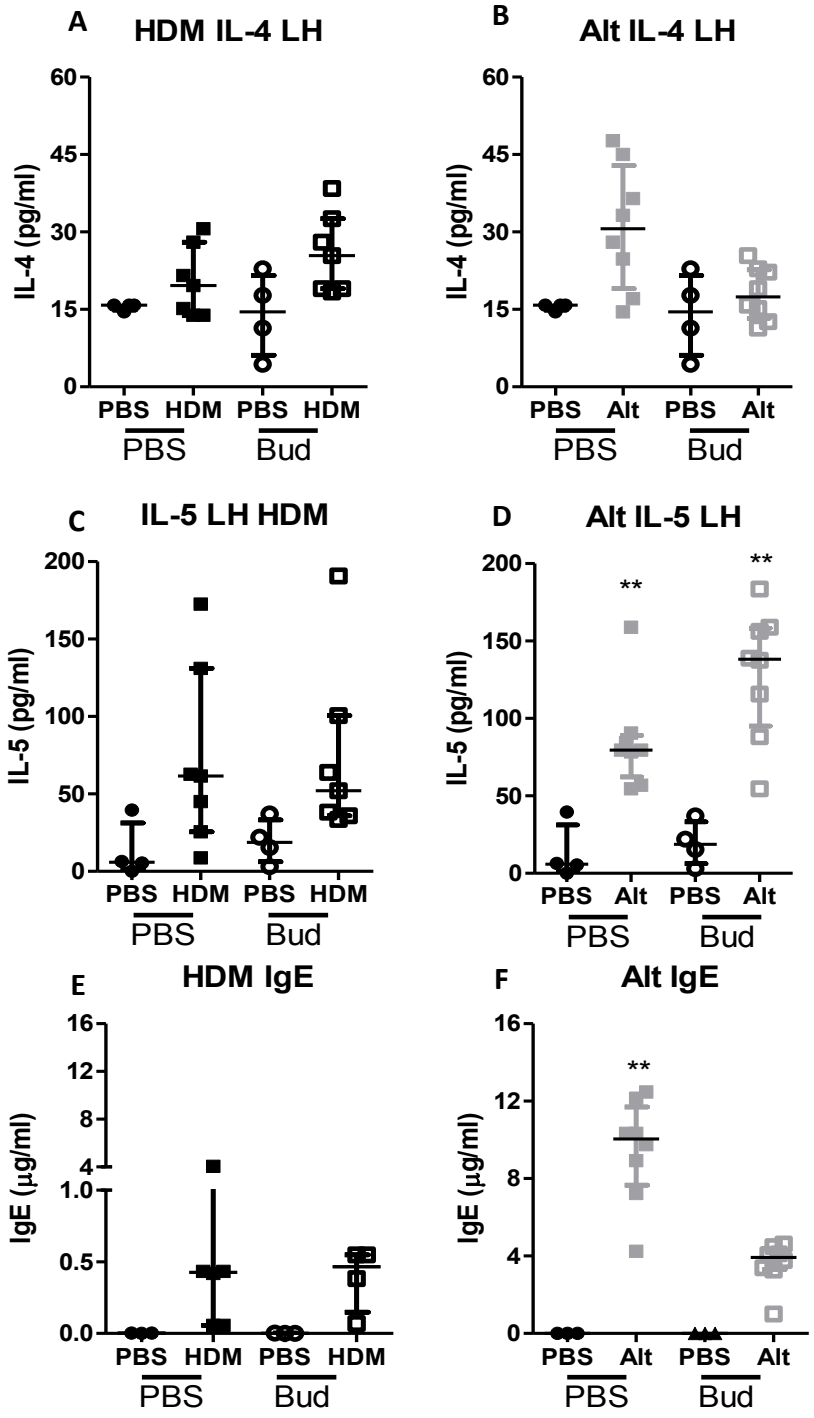

Figure 4.10 Inflammatory cytokines and IgE

Neonatal mice were dosed daily with budesonide throughout HDM or Alt exposure before collection of blood and lungs. IL-4 levels after HDM exposure **(A)** and Alt exposure **(B)** as concentration per ml lung homogenate (50mg/ml). IL-5 levels after HDM exposure **(C)** and Alt exposure **(D)** as concentration per ml lung homogenate (50mg/ml). Serum IgE after HDM **(E)** and Alt **(F)** exposure as µg/ml serum. n= 4-8 mice per group. ** $p<0.01$ from relevant PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

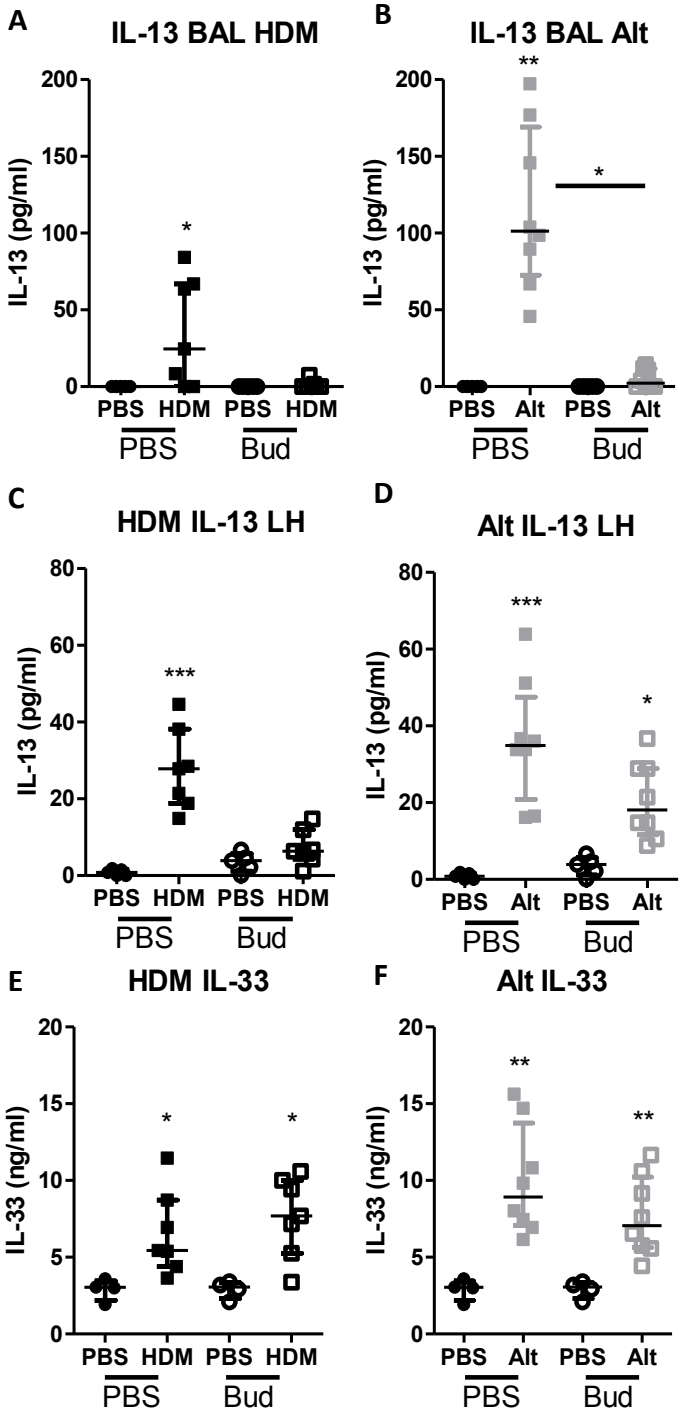

Figure 4.11 Lung and BAL cytokines in response to allergen and budesonide

Neonatal mice were dosed daily with budesonide throughout HDM or Alt exposure before collection of BAL and lungs. BAL IL-13 after HDM exposure **(A)** and Alt exposure **(B)** as pg/ml BAL. Lung IL-13 levels after HDM exposure **(C)** and Alt exposure **(D)** as concentration per ml lung homogenate (50mg/ml). Lung IL-33 levels after HDM exposure **(E)** and Alt exposure **(F)** as concentration per ml lung homogenate (50mg/ml). n= 4-8 mice per group. * $p<0.05$, $p<0.01$ and *** $p<0.001$ from relevant PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

4.3.6 Budesonide completely suppressed pulmonary cellular inflammation following HDM but not Alt exposure

To determine the cellular populations affected by steroid treatment during allergen exposure lungs were collected and cells isolated for analysis by flow cytometry.

Total ILC populations were completely suppressed by adding budesonide with both HDM and Alt exposure (Fig 4.12 A, B). IL-13^{+} ILCs were also completely abrogated by budesonide treatment during HDM exposure (Fig 4.12 C). In contrast, mice exposed to Alt and budesonide still had elevated IL-13^{+} ILCs above the PBS control, although they were reduced by budesonide with Alt exposure (Fig 4.12 D). IL-13^{+} T cell populations followed the same pattern as IL-13^{+} ILCs; with complete suppression by Bud in HDM exposed mice compared to only partial suppression by Bud treatment in Alt exposed neonates (Fig 4.12 E, F).

As steroids have been shown to increase regulatory T cells (Tregs) in paediatric patients with asthma (Hartl et al., 2007), Treg populations were examined by flow cytometry to ascertain whether these cells played a role in the suppression of inflammation observed in response to Bud treatment. The populations of Helios^{+} and Helios^{-} cells were also assessed as the emergence of a Helios^{-} subset if Tregs at day 15 of life is vital for the period of non-responsiveness to allergen described in the previous chapter (Gollwitzer et al., 2014). In contrast to the hypothesised result, the population of total Tregs was reduced in response to budesonide treatment with both HDM and Alt exposure compared to allergen exposure alone (4.13 A, B). The reduction in Tregs in response to budesonide during allergen exposure also existed in both the Helios^{+} and Helios^{-} subsets in both HDM and Alt exposed mice (Fig 4.13 C-F).

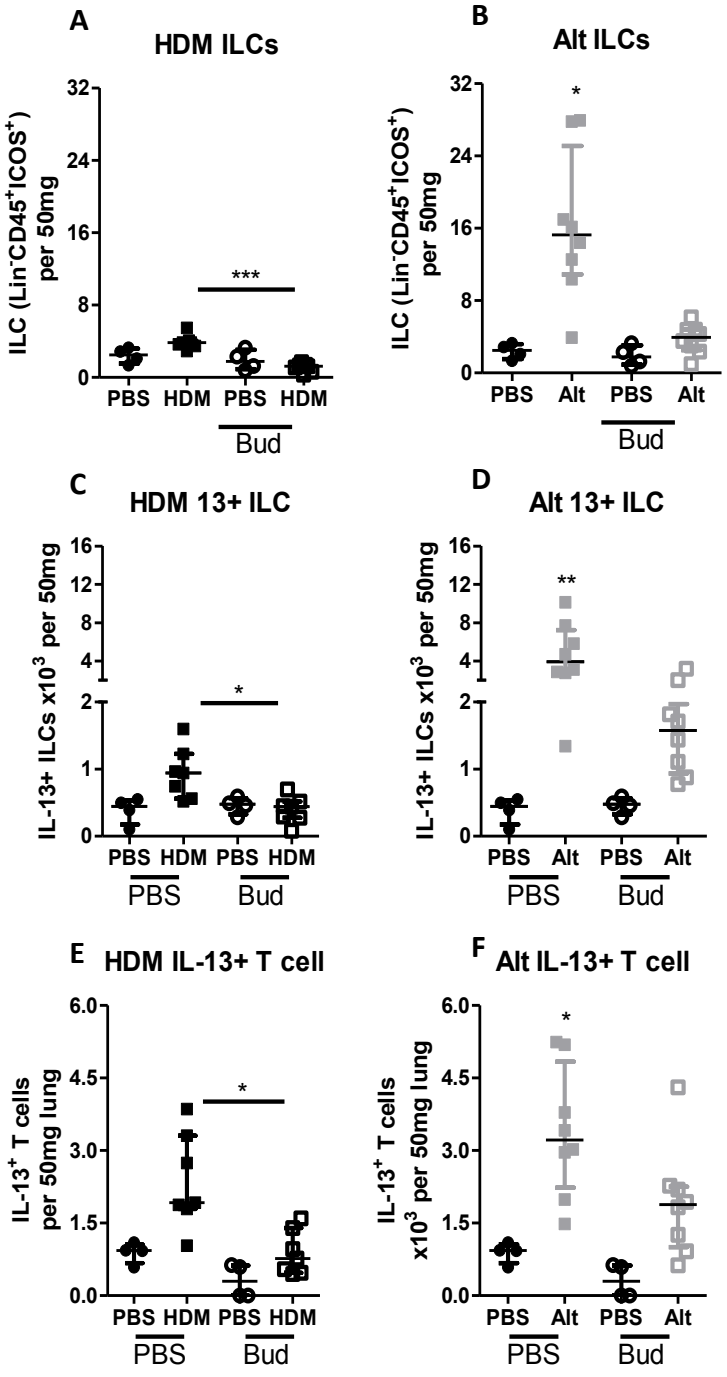

Figure 4.12 Inflammatory cells in response to allergen and budesonide

Neonatal mice were dosed daily with budesonide throughout HDM or Alt exposure before collection and preparation of lungs for facs analysis. ILCs ($lin^-CD45^+ICOS^+$) after HDM exposure **(A)** and Alt exposure **(B)**. IL-13^+ ILCs after HDM exposure **(C)** and Alt exposure **(D)**. IL-13^+ T cells ($CD3^+CD4^+$) after HDM exposure **(E)** and Alt exposure **(F)**. n= 4-8 mice per group. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from relevant PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

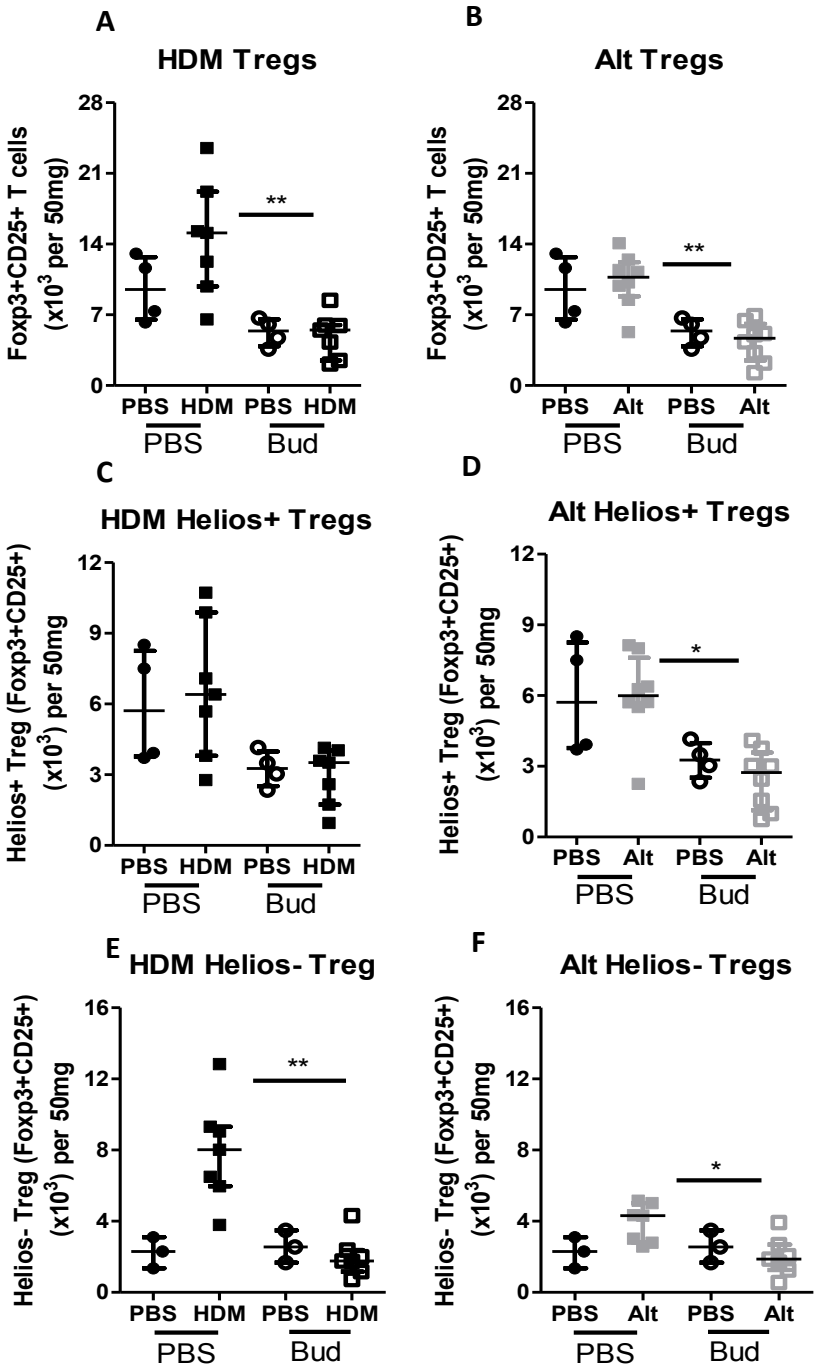

Figure 4.13 Regulatory T cells in response to allergen and budesonide

Neonatal mice were dosed daily with budesonide throughout HDM or Alt exposure before collection and preparation of lungs for facs analysis. Tregs ($Foxp3^+CD25^+$) after HDM exposure **(A)** and Alt exposure **(B)**. $Helios^+$ Tregs after HDM exposure **(C)** and Alt exposure **(D)**. $PD\text{-}1^+$ Tregs after HDM exposure **(E)** and Alt exposure **(F)**. n= 4-8 mice per group. . * $p<0.05$ and ** $p<0.01$ as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

4.3.7 Budesonide treatment during established disease did not significantly reduce airway resistance following HDM exposure

Although preventative budesonide treatment resulted in complete suppression of both AHR and IL-13 in the lung, steroid treatment in a prevention regimen, prior to allergen sensitisation is not a realistic option for treatment of patients. In reality, patients will only be treated with steroids once asthma is established. A therapeutic budesonide protocol was therefore developed to begin budesonide treatment after three weeks of HDM exposure when AAD is established (Fig 4.14 A).

Budesonide administered in a therapeutic regimen with HDM did not cause a significant reduction in airway resistance (Fig 4.14 B, C). However, therapeutic budesonide did cause a down regulation of both BAL and lung inflammation (Fig 4.14 D, E). The percentage of eosinophils in the BAL was partially suppressed from approximately 55% to 45% through Bud treatment in comparison to HDM exposure alone (Fig4.15 A). IL-13 in the lung homogenate was partially down regulated, while IL-33 was not altered by budesonide treatment (Fig 4.15 B, C), as observed after a preventative budesonide regimen.

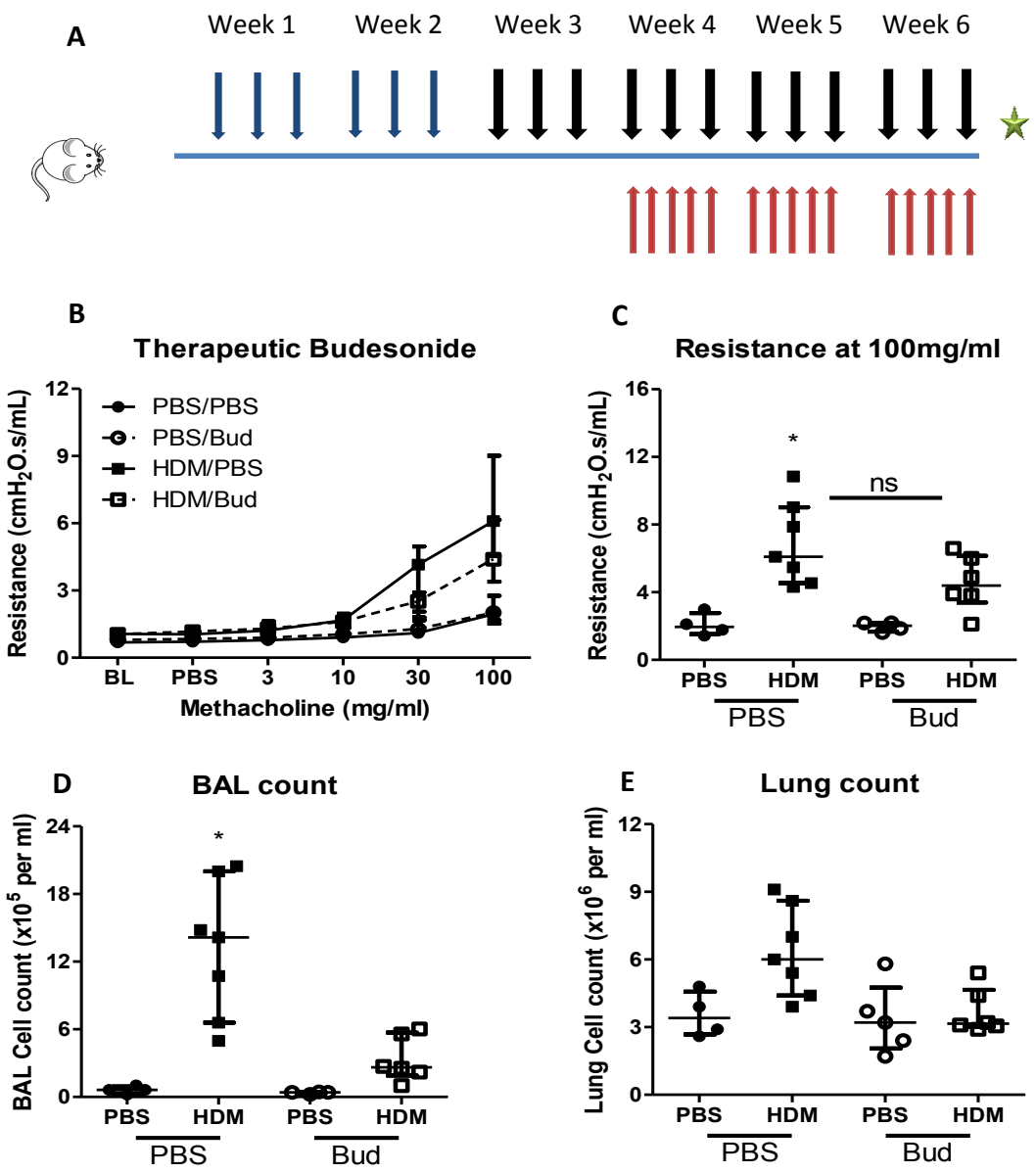

Figure 4.14 Airway resistance in response to therapeutic budesonide

Neonatal mice were exposed to three weeks of HDM before daily budesonide treatment and measure of airway resistance by flexivent and collection of BAL and lungs **(A)**. Airway resistance over increasing concentrations of methacholine **(B)** and resistance at 100mg/ml Methacholine **(C)**. BAL **(D)** and lung **(E)** inflammatory counts. n= 4-7 mice per group. * $p<0.05$, ns= non-significant from PBS control or as indicated by bars by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

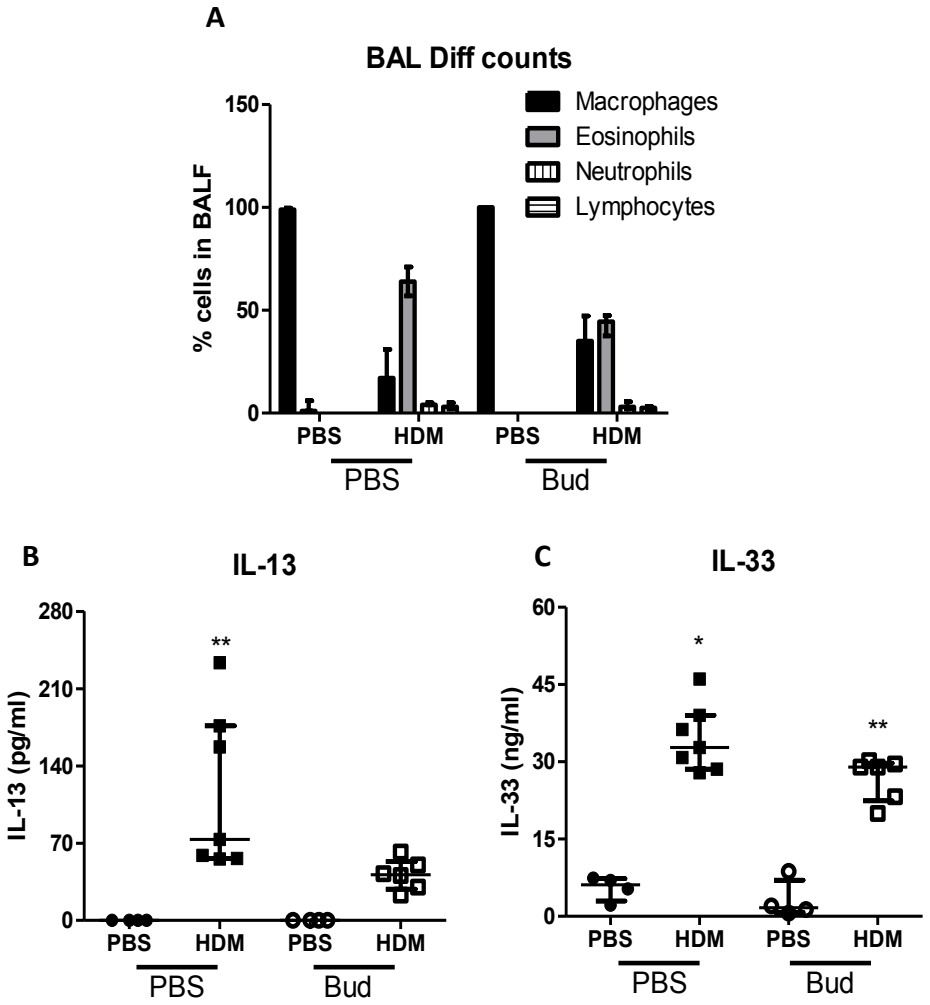

Figure 4.15 BAL inflammation and lung inflammatory cytokines after therapeutic Bud

Neonatal mice were exposed to three weeks of HDM before daily budesonide dosing before collection of BAL and lungs. Differential counts of BAL cells as percentage of live BAL cells **(A)**. Lung IL-13 levels **(B)** and Lung IL-33 levels after HDM exposure **(C)** as concentration per ml lung homogenate (50mg/ml). n= 4-8 mice per group. * $p<0.05$ and ** $p<0.01$ from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

4.4 Discussion

The aim of this chapter was to ascertain the differences in responses of adult and neonatal mice to allergen exposure and investigate the development and severity of disease in both. We also aimed to determine differences in response to the common household allergen HDM compared to Alt, a fungal allergen associated with severe, steroid resistant asthma.

This study demonstrated that neonatal mice responded to allergen exposure with markedly higher AHR and increased IL-13 levels in comparison to adult mice. This occurred even though neonatal mice had lower levels of IgE, IL-33 and IL-13^{+} T cells. The fungal allergen Alt did not cause increased AHR compared to HDM exposure in neonates; however it did result in increased eosinophilic lung inflammation and a number of pro-inflammatory Th2 mediators were further increased from levels induced by HDM. In contrast, Alt did cause increased AHR, compared to HDM exposure, in adult mice and resulted in increased lung inflammation. Preventative steroid treatment administered throughout allergen exposure in neonates prevented airway resistance in response to HDM exposure while Alt induced AHR developed despite steroid treatment. However, once HDM induced disease had been established, steroid treatment did not reduce airway resistance, although lung inflammation and IL-13 levels were reduced. The level of IL-33 was not affected by either steroid regimen or allergen.

The neonatal immune system is primed to limit inflammatory responses to the constant exposure of new antigens at mucosal surfaces by the predisposition to both Th2-type and regulatory responses (Adkins et al., 2003; Al-Garawi et al., 2011; Krishnamoorthy et al., 2012). This tendency towards the development of Th2 responses is one hypothesis to explain why 95% of asthmatics develop asthma before age 6 (Masoli et al., 2004). Interestingly, in the current study, a number of Th2 associated parameters were increased in neonatal compared to adult mice although some classical Th2 cytokines – such as IL-4 – were higher in adults in response to allergen exposure. This provides some insight into the drivers of paediatric asthma. One outstanding difference between adults and

neonates was AHR generated in response to both HDM and Alt exposure. Airway resistance was approximately 2 fold higher in neonates compared to adults and allergen type did not affect this. The physical size of the mouse, and therefore smaller airways, may be a factor in the increased measured resistance although body weight is factored into FlexiVent analysis. Studies investigating the methacholine response in children and adults have shown that age has a significant effect on responsiveness, whereby children demonstrate greater bronchial reactivity (Hopp et al., 1985). This suggests that paediatric smooth muscle may be more sensitive and/or responsive to stimulation. Increased smooth muscle mass in both adult and paediatric asthmatic patients is well documented (Berair et al., 2013; Regamey et al., 2008), however, the effect of age on the development or responsiveness of smooth muscle has not been documented to date. Human smooth muscle cells in culture release inflammatory cytokines upon stimulation including IL-6, IL-8 and GM-CSF (Johnson and Knox, 1997a), investigating the potential contribution of smooth muscle function to the onset of childhood asthma would therefore increase the understanding of the marked differences in AHR generated in adult and neonatal mice.

In contrast to neonatal AHR, adult AHR was affected by allergen type with Alt causing an increase in comparison to HDM. Alt is a fungal allergen that has been associated with a severe asthma phenotype in both paediatric and adult asthmatics, the allergen has also been associated with extreme and often fatal asthma exacerbations asthma (Blatter et al., 2014; Denning et al., 2014; Halonen et al., 1997; O'Driscoll et al., 2009; Zureik et al., 2002). However, mechanisms underlying severe asthma with fungal sensitisation (SAFS) remain largely unexplored and results of clinical trials using anti-fungal agents have been disappointing (Agbetile et al., 2014). The molecular mechanisms underlying Alt induced asthma exacerbations were recently examined by Snelgrove *et. al*. (2014). In an adult murine model of Alt exposure, intrinsic serine protease activity of the fungal allergen was responsible for immediate release of IL-33 from exposed pulmonary epithelium. This rapid release of IL-33 led to increased inflammation and enhanced AHR in a model of asthma exacerbation (Snelgrove et al., 2014). Indeed, in the current study, three weeks of Alt exposure caused

substantially increased BAL and lung inflammation in comparison to HDM exposure in both neonatal and adult mice. Results published by Willart *et. al* (2012) show the generation of IL-33 from the murine pulmonary epithelium in response to 5 days of HDM exposure (10μg/dose) that was shown to be dependent on IL-1α signalling, although this group was unable to generate consistent proof of IL-33 release from air-liquid interface (ALI) cultures (Willart et al., 2012). Unfortunately, the contribution of released IL-33 following Alt exposure could not be investigated in the current study as our time-point of investigation was 18 hours after the last Alt dose and IL-33 is known to be optimally detected in BAL at 1 hour (Snelgrove et al., 2014). Loss of IL-33 by 18 hours may be due to either rapid uptake of the cytokine or degradation. IL-33 was detectable in the lung tissue homogenate and shown to be significantly elevated in response to Alt over HDM in neonates only. In adult mice IL-33 levels following allergen exposure were higher than in neonatal mice and were unaffected by allergen type. The relationship between lung levels and BAL secreted IL-33 would be of interest as lower lung IL-33 in neonatal mice may be due to increased release of the cytokine. Interestingly, the Th2 cytokines known to be induced by IL-33 via ILCs and T cells, IL-4, IL-5 and IL-13, were not increased in the lung tissue of neonates in response to Alt compared to HDM, while all three cytokines were increased in response to Alt in adult lungs. This may therefore represent a maximal Th2 cytokine response of neonates to both HDM and Alt as neonatal immune cells are primed to respond to antigen challenge with a Th2 response (Adkins et al., 2003; Al-Garawi et al., 2011; Krishnamoorthy et al., 2012). An 'exaggerated' response to less potent allergens, such as HDM, may provide a mechanistic reason for the development of allergic asthma during early life in the vast majority of patients.

In contrast to similar levels of cytokines induced by both HDM and Alt in the lung of neonates, Alt caused an increase in the level of IL-13 secreted into the BAL; this was also observed in adult mice albeit at lower levels. IL-13 is known to be a critical driver of AHR and mucus production (Wills-Karp et al., 1998b) and the increased levels of IL-13 in the airway could therefore contribute towards the elevated AHR observed in neonates compared to adults and could provide an attractive target for

therapy of asthma in early life. The contribution of ILCs and T cells to the generation of IL-13 was examined by flow cytometry. Despite the higher levels of pulmonary IL-33, which is known to be an inducer of IL-13$^+$ ILC proliferation, in adult mice, IL-13$^+$ ILCs were similar between adult and neonatal allergen exposed mice. Neonatal ILCs may therefore be more sensitive to lower levels of cytokine and investigation of differences in ST2 receptor expression between adult and neonatal cells will therefore be of interest. At both ages, a larger population of IL-13$^+$ ILCs was present in the lungs of mice exposed to Alt compared to HDM. The number of IL-13$^+$ T cells was also higher in response to Alt, compared to HDM, in both neonates and adults.

Unlike total ILCs, T cell numbers were substantially decreased in neonatal mice compared to adult mice – both in PBS and allergen exposed mice. Both ILCs and T cells are known to differ phenotypically in neonates and adults. T cells isolated from neonates have a reduced ability to activate B cells through co-stimulation (Splawski et al., 1991), differentiate to a regulatory phenotype under stimulation (Wang et al., 2010b) and also display reduced proliferation and IL-2 production when stimulated (Hassan and Reen, 1997) – a characteristic that could explain the reduced T cell numbers in this study. A recent publication has also identified the existence of an IL-8 producing T cell population in new-born infants, these have been hypothesised as having important roles in the differentiation between pathogens and a newly colonising microbiome (Gibbons et al., 2014). The behaviour of neonatal ILCs is less clearly defined, although a larger population of RORγt$^+$ ILCs are known to exist in new-borns, these cells play a role in the protection against intestinal parasites (Sawa et al., 2011). To date, no information is available on phenotypic differences between adult and neonatal ILC2s. This study shows no difference in the overall number of ILC2s generated in response to allergen between adult and neonatal mice, however, the dramatic differences in T cell numbers between ages may be due to phenotypic differences in the ILCs as these have been shown to be vital for the generation of antigen specific T cells (Halim et al., 2014). A number of *in vitro* and *in vivo* experiments could be used to determine phenotypic differences between adult and neonatal ILCs and T cells. This including *in vitro* culture of individual cell types with allergen to determine the

cytokine profile in response to stimulation and adoptive transfer of adult cells to neonatal knockout recipients and vice versa.

The total number of IL-13^{+} ILCs and T cells did not correlate with the higher levels of secreted IL-13 in the BAL of neonates. Future work is required to determine the contribution of other cell types – such as mast cells, NKT cells and smooth muscle cells - to the generation and secretion of IL-13 in both adults and neonates. Mast cells will be of particular interest as IL-13^{+} mast cells have been identified as infiltrating smooth muscle in asthmatic patients and are therefore ideally located for released IL-13 to cause rapid smooth muscle contraction (Brightling et al., 2003). As the number of cells expressing intracellular IL-13 does not necessarily correlate to the level of cytokine secreted, *in vitro* assays of IL-13 secretion from individual cell types in response to allergen would determine whether neonatal cells secreted more cytokine than adult cells.

Despite the comparable levels of IL-4 in response to HDM and Alt in neonatal mice, total serum IgE was significantly elevated in response to Alt compared to HDM. The maturation of B cells in neonatal mice means IgE is not produced until week 2-3 of life (Haba and Nisonoff 1988). Neonatal mice in this study were analysed at week 3, on the cusp of them having the ability produce IgE in response to allergen and therefore immune responses could be the result of an innate response to allergen stimulation rather than allergic sensitisation. However, elevated IgE was detectable in the serum of both HDM and Alt exposed neonates and therefore the neonatal exposure model represented allergic sensitisation of the mice over a three-week allergen exposure model. Due to the higher level of IgE detectable in Alt exposed neonates it is likely to cause a more rapid and strong induction of IgE compared to HDM.

In addition to the association of Alt with increased asthma exacerbations and a more severe disease phenotype, sensitisation to Alt has also been linked to therapy resistant disease (Castanhinha et al., 2012). To investigate the development of steroid resistant disease with fungal sensitisation, in the current study, budesonide treatment was started after a single week of allergen exposure before

allergen induced AAD had developed. Allergen was given for a week to mimic normal exposure in early life as, in reality, no child would begin inhaled steroids naïve to allergen exposure. Daily budesonide completely abrogated HDM induced AHR while no significant reduction was caused by budesonide treatment with Alt exposure. Interestingly, despite no effect on airway resistance in response to Alt exposure, Bud treatment did have an effect on a number of inflammatory parameters including cell counts and cytokine generation. Lung inflammation was reduced to PBS control levels while BAL inflammation was substantially reduced with budesonide and Alt exposure. However, the percentage of eosinophils in the BAL infiltrate remained elevated in Alt exposed mice treated with Bud, constituting roughly 60% of cells. Steroid treatment also resulted in the reduction of both IL-4 and IgE in response to Alt exposure and a dramatic reduction in secreted IL-13 detected in the BAL. A similar disconnect between the effects of steroids on airway function and inflammatory parameters has been documented in paediatric patients with severe therapy resistant asthma. Aggressive steroid treatment resulted in very low detectable BAL IL-13 (Bossley et al., 2012), while eosinophilia persisted. However, these children were not categorised according to fungal sensitisation so definitive similarities to the murine model cannot be drawn. It may therefore be that the term 'steroid resistance' only applies to features of asthma such as airway resistance and eosinophilia while other components are steroid sensitive. However, as quality of life is heavily determined by airway function in severe asthmatic patients any treatment which does not improve this parameter may not be worthwhile. Importantly, mechanisms underlying the persistent steroid resistant eosinophilia in children with severe asthma remain unknown and investigation into eosinophil chemokines would therefore be beneficial.

Despite significantly reduced BAL IL-13 in response to steroid treatment, the level of lung homogenate IL-13 remained elevated from control in Alt exposed mice, while in HDM exposed mice IL-13 was not high in the lung either. Steroid treatment in HDM exposed mice caused complete ablation of both IL-13^{+} T cell and ILC populations while during Alt exposure these cells were reduced but remained elevated from the PBS control. Future work to define the steroid resistant nature of

these cell subsets in response to Alt sensitisation and to determine further cellular sources of IL-13, and the role of secreted vs. non-secreted IL-13, will therefore be of great importance as it could reveal a steroid resistant cell population and therefore provide possible therapeutic targets for disease intervention. Interestingly, IL-33 was not reduced in either HDM or Alt exposed mice treated with steroids, implicating this cytokine as another possible therapeutic target for severe, steroid resistant disease.

Regulatory T cells have been identified as a possible mediator of disease control by steroids as they are elevated in steroid treated paediatric asthmatics (Hartl et al., 2007). However, in the current study the population of $CD25^{+}FoxP3^{+}$ T cells was reduced in response to steroid intervention of both HDM and Alt induced disease. The contribution of both $Helios^{+}$ and $Helios^{-}$ Tregs was also examined as the emergence of these cells, in response to the developing lung microbiota, at day 15 of life has been implicated in the period of non-responsiveness described in the previous chapter. Both subsets of Tregs were also reduced in response to steroid treatment. Investigation of the human microbiota in chronic obstructive pulmonary disease (COPD) patients have described the lung microbiome-altering effect of inhaled steroids (Pragman et al., 2012). Investigation of the relationship between early life steroid use and the development of these important Treg populations will therefore be of importance, along with alterations of Treg populations in later life. One interesting observation from the current study including Treg cell subsets was the two fold higher level of $Helios^{-}$ Tregs in response to HDM exposure compared to Alt exposure alone. The type of allergen may therefore interfere with the natural expansion of these cells in response to exposure throughout early life.

In contrast to the complete abrogation of HDM induced AHR in response to a preventative steroid regimen, steroids administered after the onset of AAD resulted in a partial, but non-significant, reduction of airway resistance. Lung IL-13 was significantly reduced while IL-33 remained elevated, further highlighting IL-33 as a steroid resistant cytokine and a possible target for intervention in severe asthma.

Future studies of steroid resistance in allergic disease would focus on other cell subsets affected by steroid dosing, including eosinophils, and the effect on lung remodelling will be of importance.

Throughout the previous two chapters the importance of age and allergen type have been highlighted, however, a number of potential limitations in the experimental protocols exist which have the potential to affect the relevance of results in the setting of human disease. Although neonatal exposure to allergen was shown to result in a more severe disease phenotype compared to adult exposure, approximately one third of adults with severe asthma develop disease in adulthood with no prior respiratory disease in childhood (Miranda et al., 2004). The neonatal models used in the present study are therefore highly relevant to childhood onset asthma, which accounts for two thirds of patients with severe asthma, however do not model late onset severe adult asthma.

The doses of HDM used in these chapters were chosen to allow comparisons to previously published data describing the effects of age (Al-Garawi et al., 2011) and steroid resistance (Saglani et al., 2013) on sensitisation to HDM. The number and amount of mites per home vary greatly globally due to differences in climate, allowing exposure levels to be directly related to risk of developing sensitisation to HDM. Two studies have identified a risk of sensitisation in homes where HDM allergen levels are detectable at or above 2µg/g of household dust (Platts-mills, 1989; Wickman and Korsgaard, 1996). People are exposed to a wide variety of dust concentrations known to range from 15-1000µg/m^3 of air dependent on the environment (Mølhave et al., 2004). As adult humans have an average intake of 20m^3 of air per day (Calabrese and Kenyon, 1991) there is therefore a potential exposure rate of 0.6-40µg HDM per day or 4.2-280µg per week in an average male adult. The high-dose protocol of 25µg HDM three times per week (75µg per week) in adult mouse studies therefore falls in the range of potential human exposure, however, direct comparison is difficult due to the difference in size between mice and men and administration of HDM in saline for murine models. The steady state exposure over a long period in humans also differs from the three exposures per week in the murine model so may represent a different sensitisation pathway. Ideally, a similar

protein weight of allergen extract would have been used for exposure to Alt for comparison with HDM induced responses, however, mice responded adversely to a dose of 25µg Alt extract over time so a smaller dose of 10µg was selected for multiple dose experiments. This dose was found to cause comparable airway resistance measurements to HDM after three weeks of exposure in both a neonatal and adult mouse model. As with HDM exposure, the physiological exposure of humans to Alt has also been investigated. The mean concentration of Alt in household dust of homes in the USA has been identified as 4.88µg/g, meaning a male adult has a potential exposure of 1.46-97.6µg per day or 10-683µg per week in indoor environments (Salo et al., 2006). As with HDM, the dose selected for this study of 30µg per week falls within this range, not accounting for the difference in size and lung capacity of humans and mice and the different durations of exposure.

The final possible limitation for these chapters was the fact that lung volume was not taken into consideration when deciding an allergen dose at different ages; essentially meaning younger mice with a smaller lung volume would receive more allergen per area of lung surface. This has the potential to cause greater allergic responses in smaller animals. Between ages 3 and 8 days the lung volume of a C57BL/6J mouse has been shown to increase more than two-fold from 0.1ml to 0.25 ml (Soutiere and Mitzner, 2006). However, the current study did not identify any substantial differences in airway resistance or inflammation when allergen exposure began at day 3 or 7 (Figure 3.2 and 3.4), although consideration of lung volume to allergen dose may be of importance to future work comparing the response of neonatal and adult mice to allergen exposure. A possible future experiment to determine if neonatal lungs are more responsive could focus on comparing ALI cultures of epithelial cells isolated from neonates and adults. As these cultures would have equal surface areas, the direct cytokine response to allergen exposure could be quantified without the need to adjust allergen exposure to the volume of the lung.

4.4.1 Conclusion

This chapter described the differences between the development of allergic airway disease during childhood and adulthood in response to the common household allergen HDM and an allergen associated with severe, therapy resistant disease – Alt. It was hypothesised that neonatal mice would have an exaggerated response to allergen exposure, which was shown to be accurate through increased airway resistance, and secreted IL-13 in neonatal mice despite reduced IgE and IL-13^{+} T cells compared to adults. A further hypothesis for this chapter was that exposure to the fungal allergen Alt would induce a more severe AAD phenotype, which was less sensitive to steroid intervention. In adult mice, Alt caused elevated AHR in comparison to HDM while the opposite occurred in neonatal mice, indicating that neonatal mice may be hyper-responsive to less allergenic stimuli. This was further indicated through equal levels of Th2 associated cytokines in response to HDM and Alt in neonatal mice while levels were increased in response to Alt in adult mice. An age–dependent effect of T cell responses was also identified with fewer T cells responding to allergen exposure in neonates and a lower proportion of those cells expressing IL-13. In contrast to this, ILCs were not affected by age and therefore may act as a potent driver of allergic disease in early life. The association of Alt sensitisation with steroid resistant disease was also investigated and was confirmed in a neonatal model of preventative steroid treatment as AHR was completely abrogated to HDM exposure but not exposure to Alt. IL-33 and the downstream cytokine IL-13 were identified as key components and possible drivers of steroid resistant disease.

Due to the observed importance of both IL-33 and the downstream cytokine IL-13 in the exaggerated neonatal response and the steroid resistant element of allergic disease, the next chapter will focus on the IL-33/ST2 signalling pathway in response to both HDM and Alt exposure.

Chapter 5 - Contrasting roles of the IL-33/ST2 signalling pathway in HDM and *Alternaria* induced allergic airways disease

5.1 Introduction

The previous chapter demonstrated enhanced immune responses to the fungal allergen Alt compared to HDM in both adult and neonatal mouse models. One of the main findings was the development of increased AHR in adult mice exposed to Alt, which was associated with increased levels of IL-13. This chapter focuses on an important innate signalling pathway involved in the induction of IL-13; IL-33 and its receptor ST2.

This pathway is of particular interest in asthma, since both IL-33 and ST2 have been identified as candidate genes for allergic asthma by genome wide association studies (GWAS) of asthmatic populations (Moffatt et al., 2010; Ramasamy et al., 2012; Torgerson et al., 2011). Therefore, investigation of the functional relevance of these molecules in disease pathogenesis seems essential. ST2 was an orphan receptor until the ligand IL-33 was discovered and described using Bioinformatics in 2005 (Schmitz et al., 2005). IL-33 signalling is known to play a vital role in host defence against pathogens acting as both an alarmin, released through cellular damage (Cayrol and Girard, 2009), and as a secreted cytokine from innate immune cells such as epithelial cells (Snelgrove et al., 2014; Willart et al., 2012), macrophages (Chang et al., 2011), dendritic cells (Yanagawa et al., 2011), mast cells (Hsu et al., 2010) and neutrophils (Lefrançais et al., 2012). A number of infections are known to be actively controlled by the action of IL-33 signalling through ST2. Mice were highly susceptible to *Toxoplasma Gondii* in $ST2^{-/-}$ models compared to WT (Jones et al., 2010) and also had impaired granuloma formation in response to initial infection with *Schistosoma mansoni* egg infection (Townsend et al., 2000) showing the IL-33 signalling pathway is vital for rapid induction of innate immune defences against pathogens. However, dysregulation of this signalling pathway has been implicated in a number of disease models including rheumatoid arthritis (Palmer et al., 2009), inflammatory bowel disease (Pastorelli et al., 2013), allergic asthma (Kim et al., 2012a; Saglani et al., 2013) and cardiac disease.

The recent discovery of type 2 innate lymphoid cells (ILC2s) has highlighted the role of IL-33 signalling in the pathogenesis of allergic asthma. ILC2s were discovered in an IL13-eGFP mouse model of parasite infection as a vital component in the innate immune system for helminth expulsion (Fallon et al., 2006; Neill et al., 2010). Neill *et. al*. demonstrated that the epithelial derived cytokine IL-33 was essential for the expansion of ILC2 cells. Following inhaled allergen exposure, ILCs migrate from the lymph nodes to IL-33 released from the pulmonary epithelium and produce Th2 cytokines, including IL-13, known to be essential to the progression of allergic asthma (Wills-Karp et al., 1998a). In addition to ILCs, a number of other cell types in the lung secrete IL-13 in response to IL-33 signalling through the ST2 receptor. These include basophils (Smithgall et al., 2008), Th2 cells (Schmitz et al., 2005) and mast cells both in the presence and absence of IgE cross-linking (Ho et al., 2007). IL-33 has also recently been identified as a vital contributor to the interaction between ILCs and Th2 cells for the generation of allergen specific T cell responses (Halim et al., 2014). The contribution of epithelial IL-33 release to the pathogenesis of allergic asthma is an important area of research as one of the clinical features of severe asthma is increased IL-33 expression in the bronchial epithelium (Préfontaine et al., 2009) and subepithelial cells (Saglani et al., 2013). IL-33 release from the pulmonary epithelium in response to allergen exposure has been investigated both *in vivo* and *in vitro* using air-liquid interface (ALI) cultures. Exposure to high dose HDM caused the production of IL-33 in a TLR4 dependent manner involving IL-1α signalling in murine models, however, the group could not determine definitively that IL-33 was released from human ALI cultures in response to HDM (Willart et al., 2012). In contrast, exposure to low dose HDM in murine models caused IL-33 production in a GM-CSF dependent manner (Llop-Guevara et al., 2014a). The release of IL-33 from the pulmonary epithelium in response to Alt exposure was shown to be dependent on serine protease activity and did not require other cytokines (Snelgrove et al., 2014).

IL-33 produced by innate immune cells is either released to mediate effects via ST2 signalling or remains in the nucleus and acts as a transcriptional regulator of NF-κβ (Choi et al., 2012). Choi *et. al*. demonstrated that nuclear IL-33 in endothelial cells acts via NF- κβ to increase expression of ICAM-1

and VCAM-1, which function to enhance recruitment of inflammatory cells. Due to the dual role of IL-33 as both a secreted and nuclear inflammatory cytokine it is crucial that studies investigating the activity of IL-33 are carried out in an appropriate model of IL-33 knockdown or knockout. However, due to the difficulty of producing an effective αIL-33 antibody the majority of research to date has been conducted by blocking ST2 signalling. One study conducted into the role of IL-33 in OVA induced AAD utilised IL-33$^{-/-}$ mice to show a significant reduction in eosinophil infiltration (Oboki et al., 2010). However, OVA sensitisation may not be the most appropriate model for investigating the role of IL-33 as sensitisation is not via a mucosal route and epithelial release of IL-33 has been shown to be vital for the initiation of innate immune responses (Neill et al., 2010). Although IL-33 is widely accepted as the ligand for ST2, one study using murine models of arthritis highlighted a disparity between the cytokine and receptor knockout models as ST2$^{-/-}$ mice were protected from disease while IL-33$^{-/-}$ mice were not (Martin et al., 2013). This group suggested the possibility of an additional ligand for ST2 although did not pursue the theory.

It is therefore vital to determine the individual roles of IL-33 and ST2 in our models of AAD in order to understand the contribution of each to disease pathogenesis and more accurately uncover their role as potential therapeutic targets. In order to evaluate specific roles of IL-33 and ST2 in the development of AAD WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were exposed to intermittent HDM or Alt for three weeks before assessment of AAD. As IL-33 is known to be rapidly released from the epithelium in response to Alt stimulation (Snelgrove et al., 2014) we also investigated how the absence of IL-33 or ST2 affected the immediate response to allergen after a single exposure or three exposures over one week. Finally, we aimed to generate an adeno-associated virus (AAV) containing murine IL-33 to emulate the overexpression of IL-33 observed in the lungs of patients with severe asthma (Préfontaine et al., 2009; Saglani et al., 2013).

5.1.1 Hypothesis

Both IL-33$^{-/-}$ and ST2$^{-/-}$ murine models will respond to allergen exposure with reduced attributes of allergic airway disease. However, responses may differ between the two due to the role of IL-33 as a nuclear factor, which will still be active in ST2$^{-/-}$ mice.

5.1.2 Aims

1. To determine the individual roles of IL-33 and ST2 in a three week exposure protocol with HDM or Alt.
2. To investigate the roles of IL-33 and ST2 in the inception of AAD in response to Alt through the utilisation of a one or three exposure allergen protocol.
3. To generate an IL-33 expressing AAV that allows the over-expression of IL-33 in murine pulmonary bronchial epithelial cells.

5.2 Methods

In protocol A, 6-8 week old female IL-33$^{-/-}$ with Balb/CJ wild type controls and ST2$^{-/-}$ with Balb/c wild type controls were exposed intranasally to either 25µg HDM or PBS three times per week for three weeks using recovery anaesthesia of inhaled isoflurane. HDM exposed mice were assessed for AHR 4 hours after the last exposure.

In protocol B, 6-8 week old female WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were exposed intranasally to either 10µg Alt or PBS three times per week for three weeks using recovery anaesthesia of inhaled isoflurane. Alt exposed mice were assessed for AHR 18 hours after the last exposure.

In protocol C, 6-8 week old female WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were exposed to either one or three intranasal administrations of Alt using recovery anaesthesia of inhaled isoflurane. Mice were assessed for AHR or inflammation 18 hours post last dose.

In protocol D, mice were exposed to intermittent Alt for three weeks as in protocol B. Blood was collected via tail bleed at 4 day intervals during allergen exposure.

Throughout this chapter, PBS values for all genotypes (WT, IL-33$^{-/-}$, ST2$^{-/-}$) were analysed for significant differences before being combined for graph clarity and analysis.

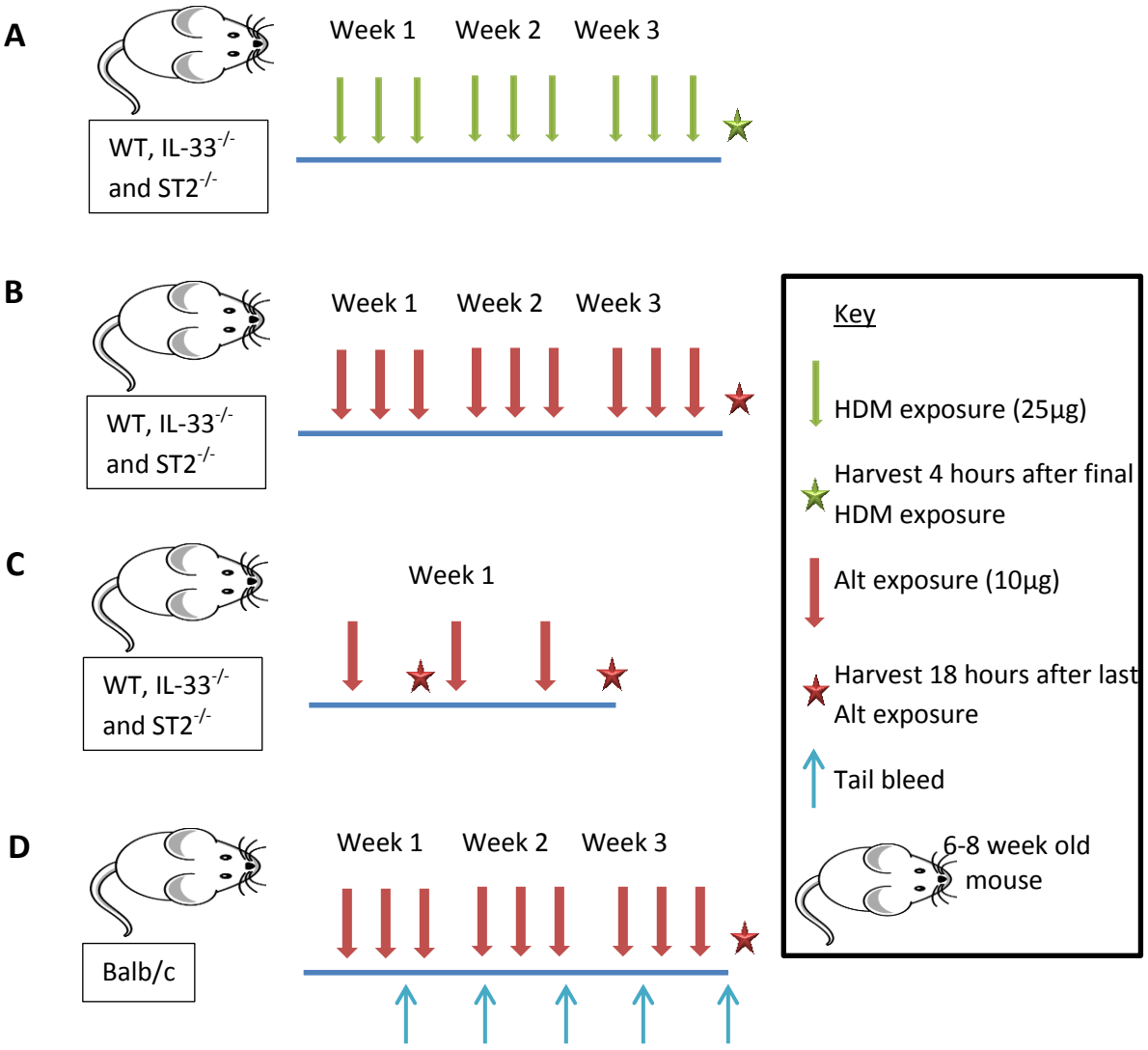

Figure 5.1 Chapter 5 Methods

WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice aged 6-8 weeks were exposed intranasally for three weeks to 25µg HDM **(A)**, 10µg Alt **(B)** or PBS three times per week before analysis or AHR by flexivent and collection of BAL, lung tissue and blood. Mice were exposed intranasally with 10µg Alt or PBS once or three times per week for one week **(C)**. A serial tail bleed on WT mice exposed to Alt over three weeks **(D)**.

5.3 Results

5.3.1 HDM induced allergic airways disease is ST2, but not IL-33 dependant

Both WT and IL-33$^{-/-}$ mice that had been exposed to HDM for three weeks (Fig 5.1 A) developed similar AHR (Fig 5.2 A, B). In contrast, HDM exposed ST2$^{-/-}$ mice did not develop AHR and their airway resistance at 100mg/ml MCh was not significantly different from PBS control mice. PBS groups from WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were analysed for any difference in baseline inflammation between genotypes and as none was present, PBS data was combined for graph clarity. BAL inflammation was significantly elevated following HDM exposure in all genotypes with no significant differences between groups (Fig 5.2 C). Differential counts of leukocytes recovered from the BAL revealed both WT and IL-33$^{-/-}$ mice had similar proportions of macrophages (approximately 30%), eosinophils (60%) and neutrophils and lymphocytes (5% each). In contrast, the airway inflammation in HDM exposed ST2$^{-/-}$ mice was predominantly neutrophillic (50% neutrophils) with smaller populations of macrophages and eosinophils (each accounting for approximately 25% of the total inflammatory cells) and few lymphocytes (Fig 5.2 D). The number of inflammatory cells recruited to the lung tissue in HDM exposed mice was a similar level in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice. However, haematoxylin and eosin stained lung sections revealed the inflammatory infiltrates were not concentrated around the airways and vessels in ST2$^{-/-}$ mice as they were in both WT and IL-33$^{-/-}$ mice but rather, dispersed throughout the parenchyma (Fig 5.3 A, B).

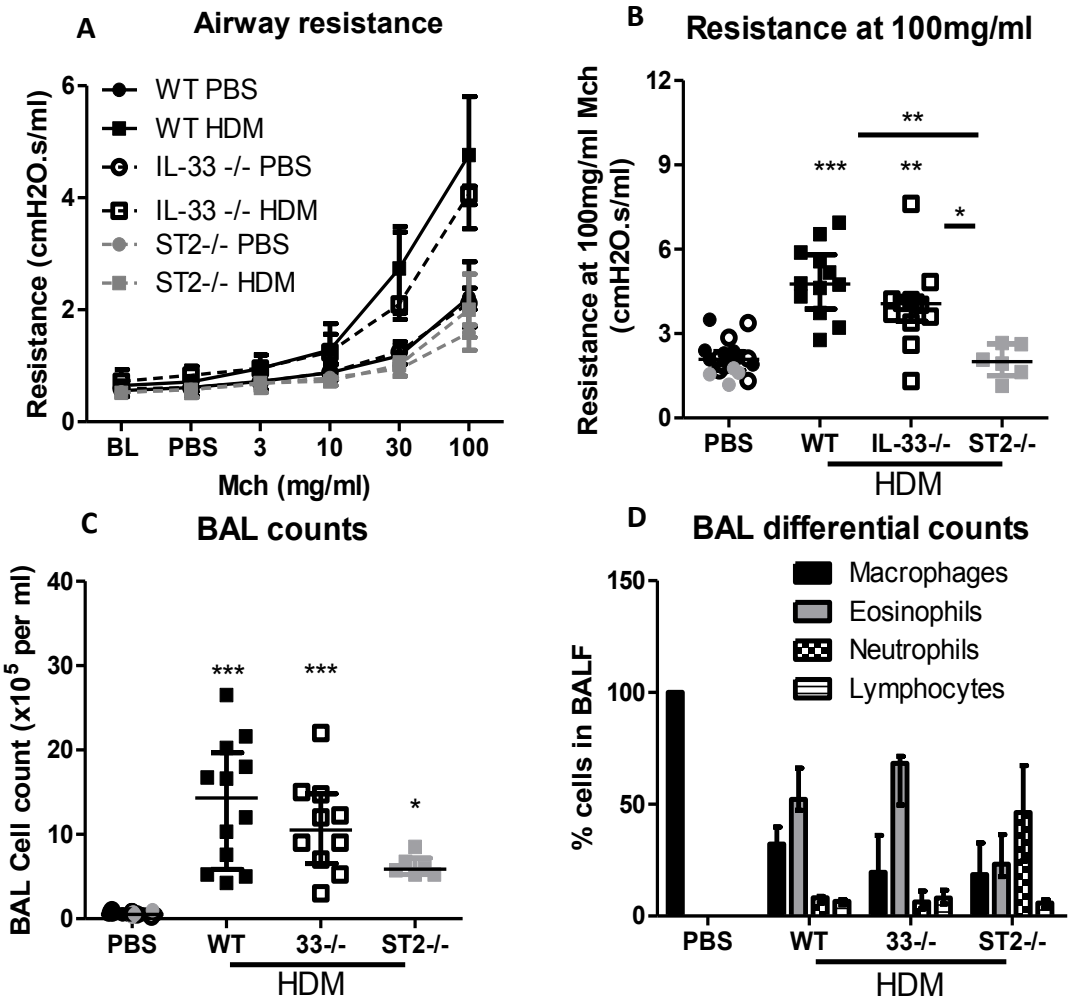

Figure 5.2 HDM induced airway resistance and BAL inflammation

AHR measured by flexivent. Airway resistance to increasing concentrations of methacholine in mice dosed for three weeks with HDM **(A)**. Resistance at 100mg/ml methacholine **(B)**. Cell count per ml of BAL fluid **(C)**. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. Differential counts of BAL cells **(D)**. n= 4-12 mice per group, results from two separate experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

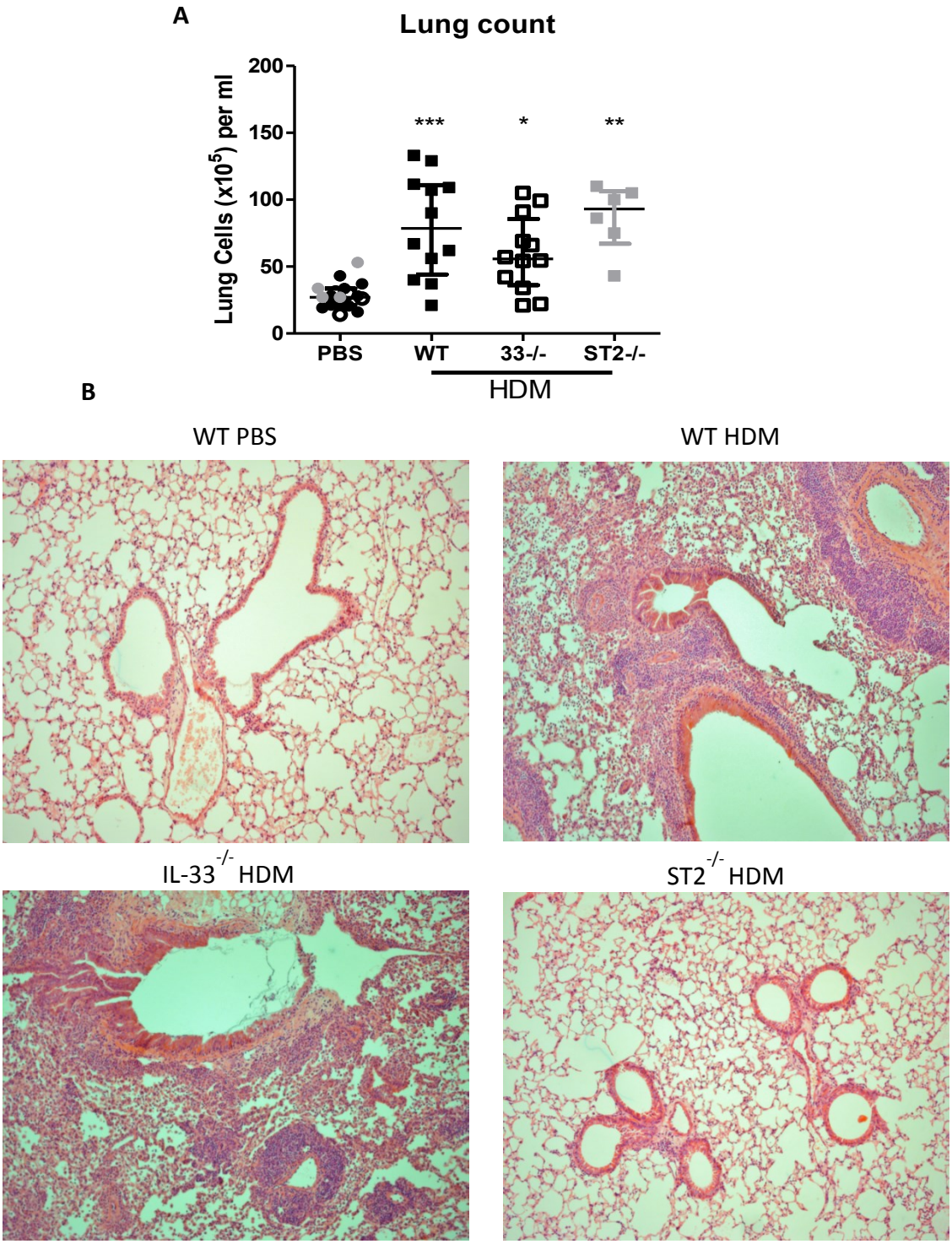

Figure 5.3 HDM induced lung inflammation

Lung cell count per ml in mice exposed to HDM for three weeks **(A)**. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. Representative H&E stained lung sections at 20x magnification **(B)**. n= 4-12 mice per group, results from two separate experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

5.3.2 Inflammatory mediators were reduced in ST2$^{-/-}$ mice, while IL-33 was elevated in response to HDM.

Pulmonary IL-5 was equally elevated in WT and IL-33$^{-/-}$ mice in response to HDM exposure while levels in ST2$^{-/-}$ mice were not significantly increased from PBS control (Figure 5.4 A). IL-4 was not increased above PBS control levels in ST2$^{-/-}$ mice while equally elevated levels were generated in the lungs of both WT and IL-33$^{-/-}$ HDM exposed mice (Figure 5.4 B). This pattern was reflected in the total serum IgE and HDM specific IgE, which were only increased in response to HDM in WT and IL-33$^{-/-}$ mice (Figure 5.4 C, D).

Lung IL-33 levels were elevated in WT and ST2$^{-/-}$ mice in response to HDM exposure with no significant difference between the two genotypes (Figure 5.5 A). As expected, IL-33 was not detectable in IL-33$^{-/-}$ mice. Interestingly, despite the complete absence of IL-33, two cytokines known to be closely associated with IL-33 – IL-1α and IL-13 – were similarly elevated in the lungs of HDM exposed WT and IL-33$^{-/-}$ mice while neither was increased in ST2$^{-/-}$ mice administered HDM (Figure 5.5 B, C). IL-13 secreted into the BAL was also elevated in both WT and IL-33$^{-/-}$ but remained at baseline levels in ST2$^{-/-}$ mice after HDM exposure (Figure 5.5 D). Mast cells are known to be activated by both IL-33 and IgE cross-linking (Ho et al., 2007). We therefore measured serum levels of mast cell protease, MCPT-1. Serum MCPT-1 levels were increased in both WT and IL-33$^{-/-}$ mice, however, there was no evidence of mast cell activation in ST2$^{-/-}$ mice exposed to HDM (Figure 5.5 E).

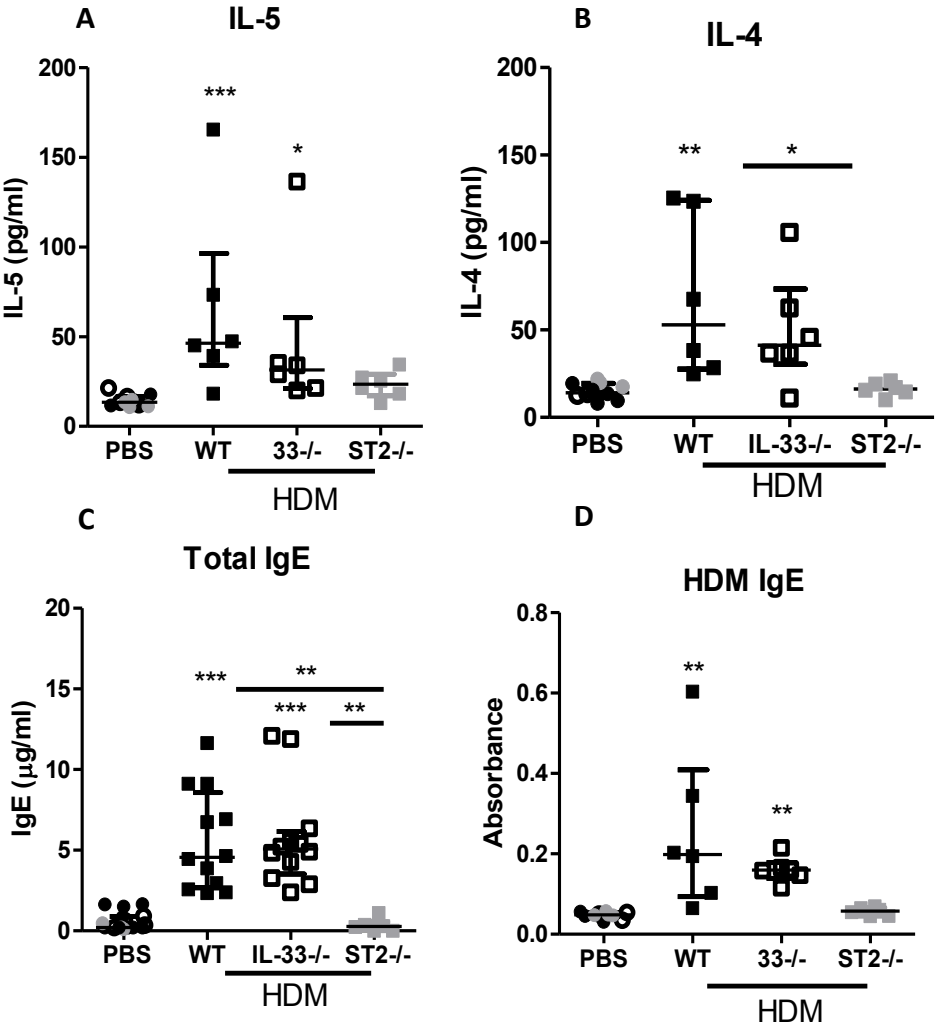

Figure 5.4. HDM sensitisation in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice.

Lung was collected and homogenised after three weeks of intranasal HDM and IL-5 **(A)**, IL-4 **(B)**, measured by ELISA and expressed as concentration per ml lung homogenate (50mg/ml). Total serum IgE **(C)** and HDM specific IgE **(D)** Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-12 mice per group * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

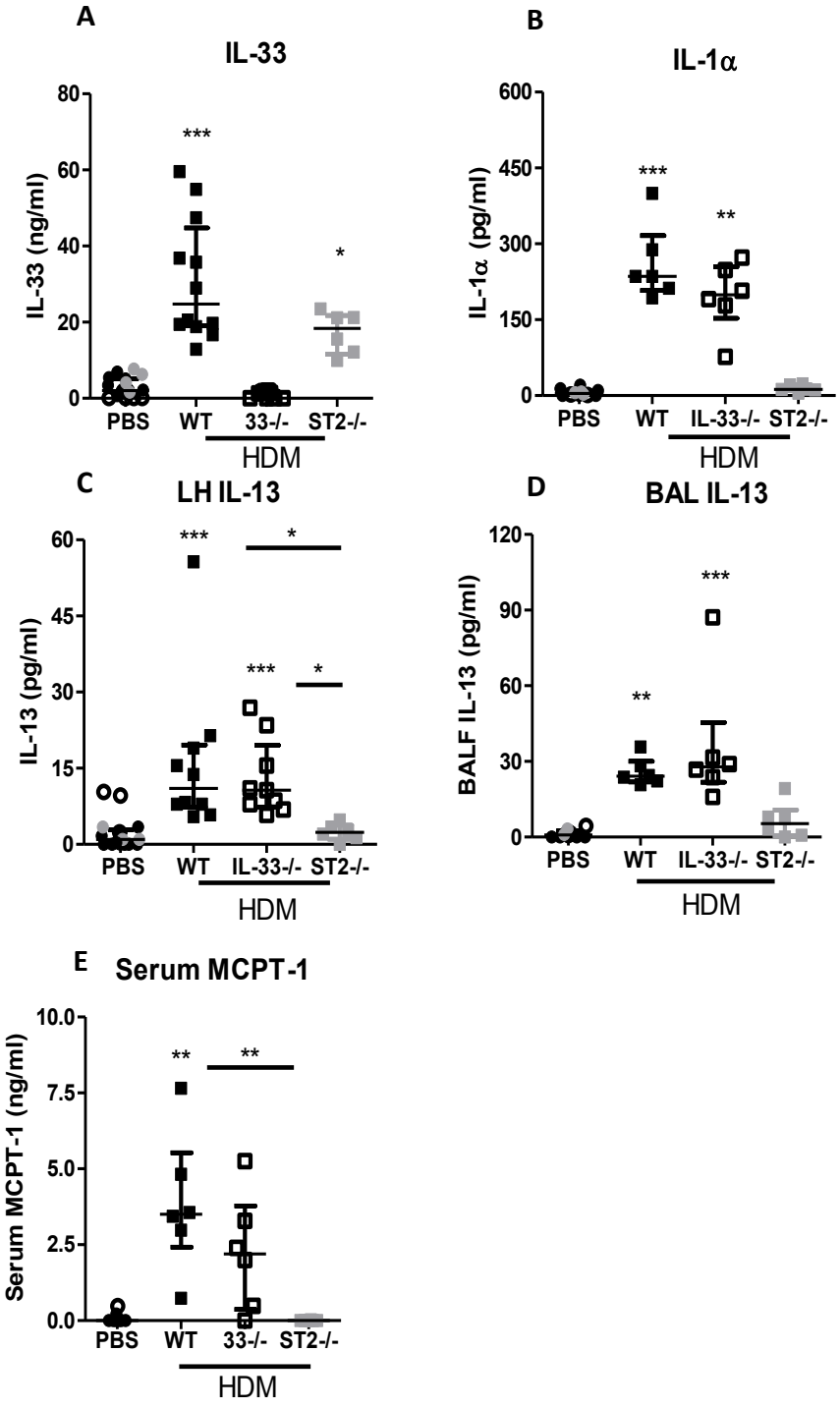

Figure 5.5 HDM induced Inflammatory cytokines in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice

Lungs, BAL and blood were collected after three weeks of intranasal HDM exposure. Lung tissue levels of IL-33 **(A)**, IL-1α **(B)** and IL-13 **(C)** as concentration per ml lung homogenate (50mg/ml). BAL IL-13 **(D)** as pg/ml BAL. Serum MCPT-1 **(E)** in ng/ml. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-12 mice per group ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

5.3.3 IL-13^{+} T cells and IL-13^{+} ILC recruitment to the lung following HDM exposure is partially dependent on ST2.

Total innate lymphoid cells (ILCs) (Lin^{-}ICOS^{+}CD45^{+}) were significantly elevated from baseline in all genotypes of mice exposed to HDM (Fig 5.6 A). Both IL-33$^{-/-}$ and ST2$^{-/-}$ mice that were exposed to HDM had a reduced population of IL-13^{+} ILCs in the lung when compared to WT mice, however all populations were increased from the PBS control (Fig 5.6 B). Total CD3^{+}CD4^{+} T cells were elevated in all genotypes with the largest population in the HDM exposed ST2$^{-/-}$ mice (Fig 5.6 C). Despite the larger population of total T cells, the IL-13^{+} subset was not significantly increased from the PBS control as it was in WT and IL-33$^{-/-}$ mice (Fig 5.6 D).

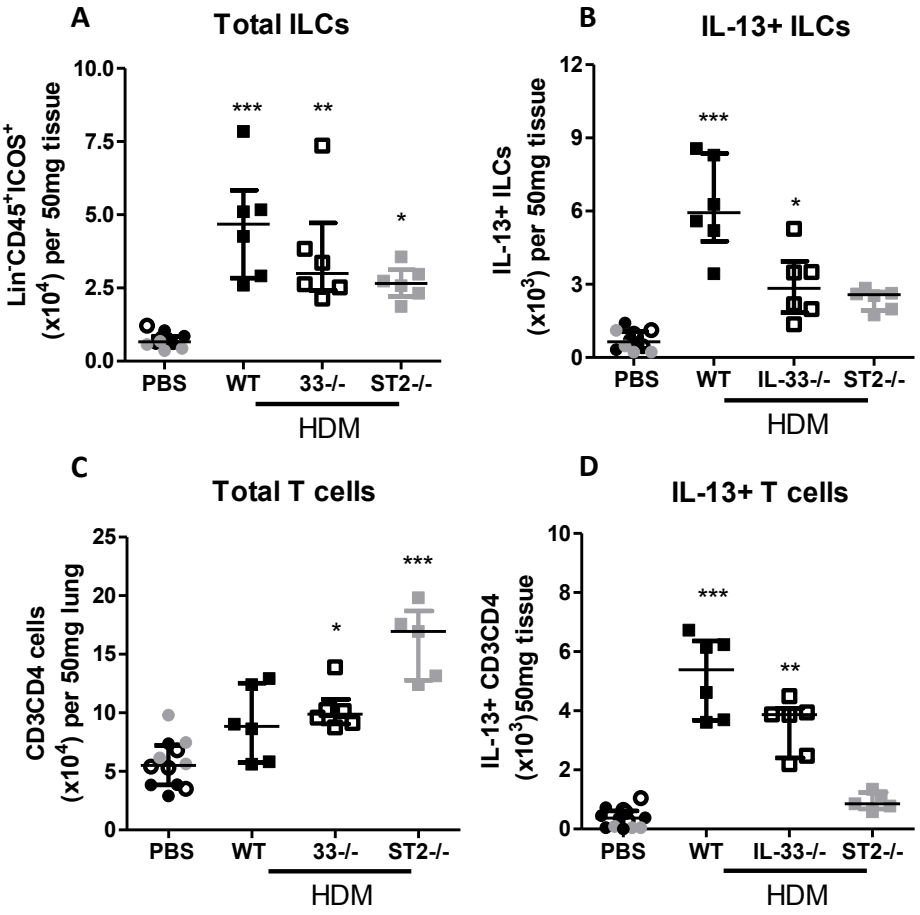

Figure 5.6 HDM induced ILC and T cell populations

Lungs were collected and cells isolated for flow cytometry analysis after three weeks of HDM. Total ILCs ($Lin^-CD45^+ICOS^+$) **(A)** ILC2s ($IL\text{-}13^+$) **(B)**, total T cells ($CD3^+CD4^+$) **(C)** and Th2 cell ($IL\text{-}13^+$) **(D)** determined by flow cytometry. Black symbols represent WT data points, hollow symbols $IL\text{-}33^{-/-}$ and grey symbols $ST2^{-/-}$ data points. n= 4-6 mice per group * $p<0.05$, **$p<0.01$ and *** $p<0.01$ from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

5.3.4 Alternaria induced AHR is not dependent on IL-33 or ST2

$ST2^{-/-}$ mice did not develop allergic airways disease in response to three weeks of HDM exposure. In order to determine whether this was an allergen specific effect, or if ST2 was required for sensitisation with all allergens, we exposed $ST2^{-/-}$, WT and $IL\text{-}33^{-/-}$ mice to three weeks of Alt (Fig 5.7 A) as this is a fungal allergen known to be associated with a more severe asthma phenotype (Agarwal, 2011; Neukirch et al., 1999).

All genotypes of mice developed similar AHR in response to Alt exposure with no significant difference in airways resistance at a dose of 100mg/ml MCh (Fig 5.7 B, C). $IL\text{-}33^{-/-}$ and $ST2^{-/-}$ mice that were exposed to Alt had lower numbers of inflammatory cells recovered from the BAL and lung compared to WT mice (Fig 5.7 D, E). The population of eosinophils present in the lung after Alt exposure represented 30% of live cells in both WT and $IL\text{-}33^{-/-}$ mice while this population was significantly lower in $ST2^{-/-}$ mice making up only 15% of live cells (Fig 5.8 A). A significant percentage of live cells in WT and $ST2^{-/-}$ mice were neutrophils, however, there was no neutrophilia in the lungs of $IL\text{-}33^{-/-}$ mice (Fig 5.8 B). Representative H & E stained lung sections showed WT, $ST2^{-/-}$ and $IL\text{-}33^{-/-}$ mice all had large inflammatory infiltrates around both airways and vessels in response to Alt sensitisation (Fig 5.8 C).

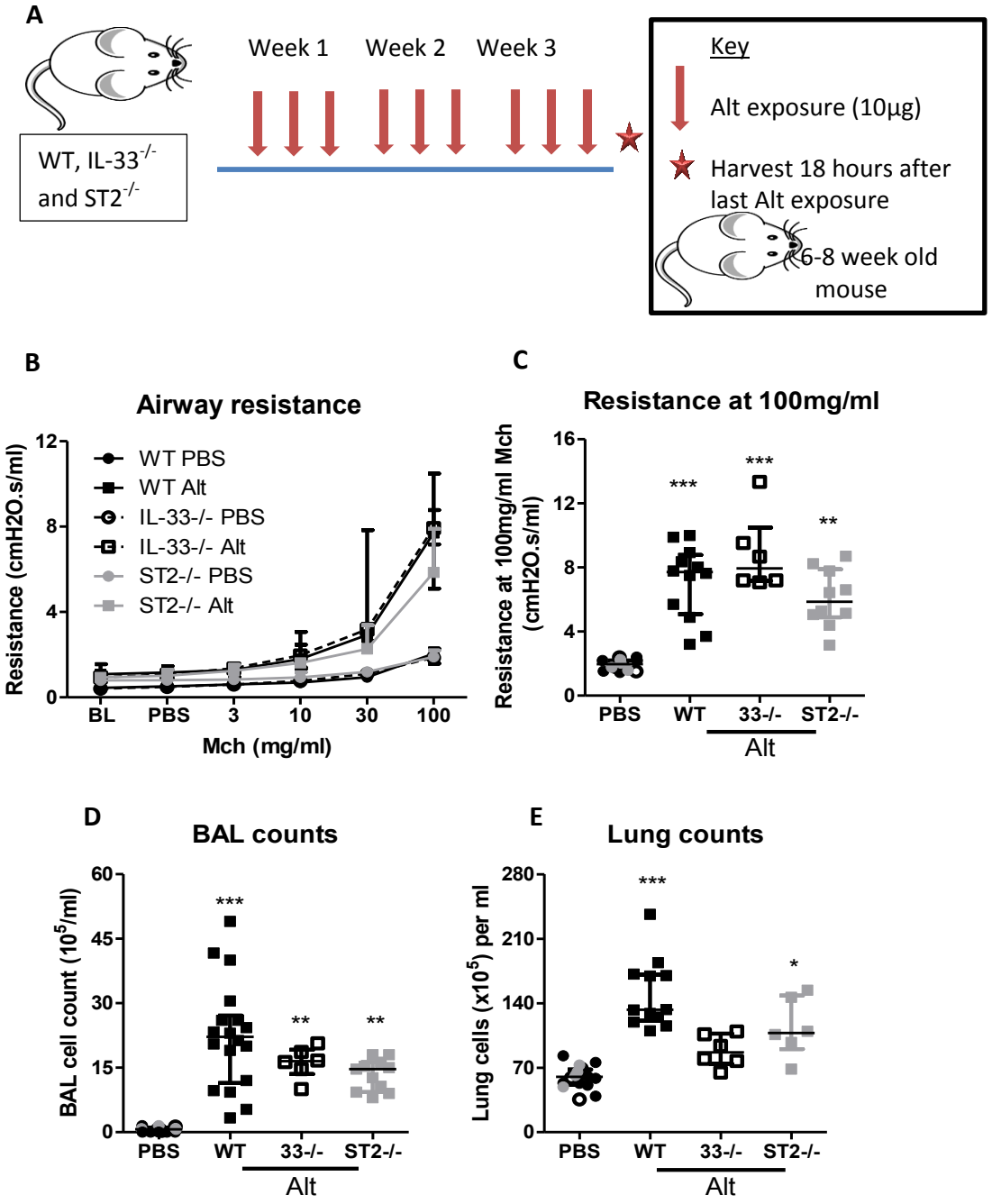

Figure 5.7 Alternaria induced AHR and inflammation

AHR measured by flexivent. Airway resistance over increasing concentrations of methacholine in mice dosed for three weeks with Alt **(A)**. Resistance at 100mg/ml Methacholine **(B).** Cell count per ml of BAL fluid **(C)**, lung cell count per ml **(D)** Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-12 mice per group, results from two separate experiments. * p<0.05 ** p<0.01 and *** p<0.001 from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

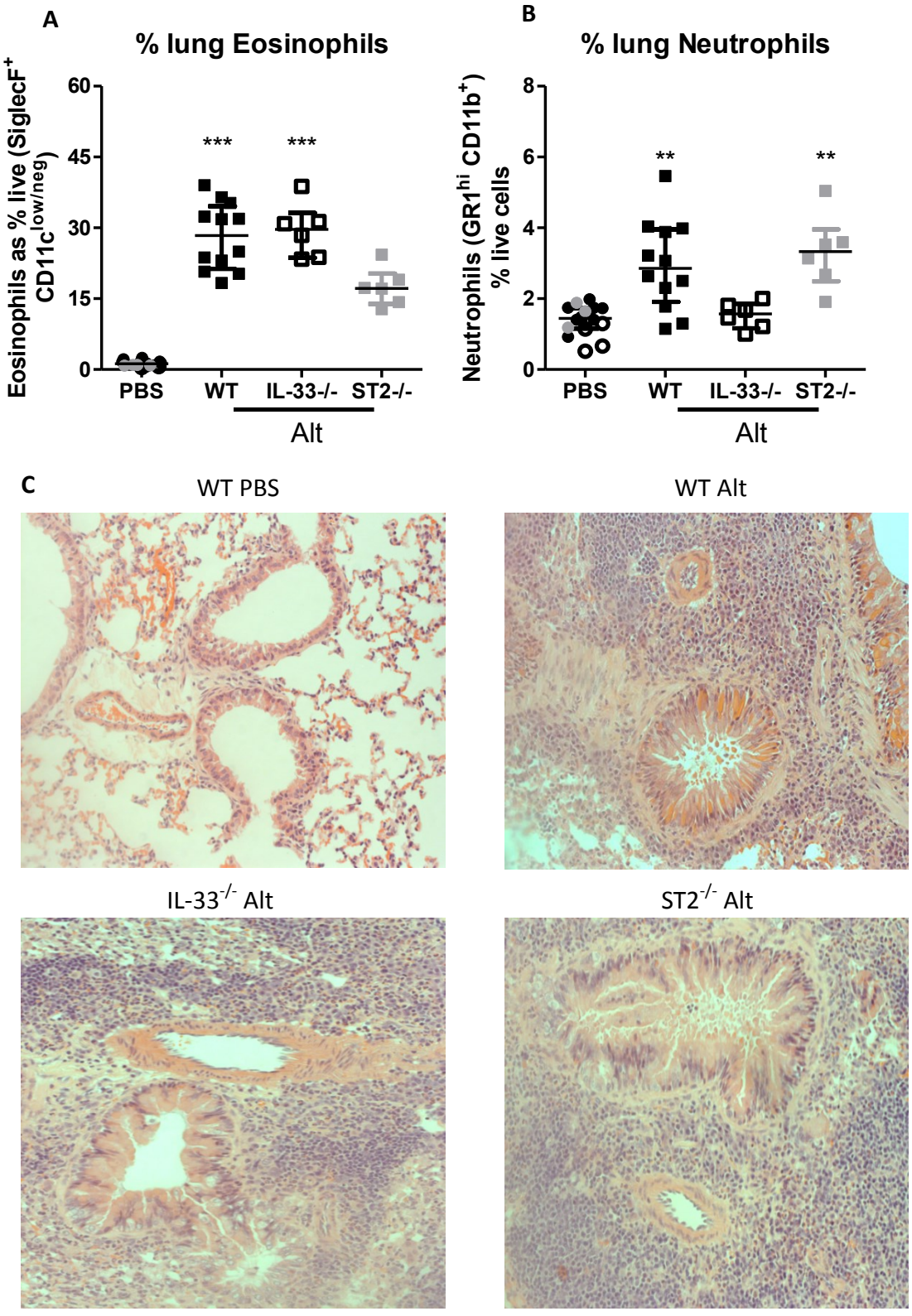

Figure 5.8 Alt induced lung inflammation

Lung inflammation after three weeks of exposure to Alt. Lung eosinophils **(A)** and neutrophils **(B)** as % live lung cells, determined by flow cytometry. Representative H&E stained lung sections of WT PBS, WT, IL-33$^{-/-}$ and ST2$^{-/-}$ Alt exposed mice at 20x magnification **(C)**. * $p<0.05$ and ** $p<0.01$ from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

5.3.5 Inflammatory cytokines induced by Alt in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice

IL-4 and IL-5 were significantly elevated above PBS control levels in WT and IL-33$^{-/-}$ mice exposed to Alt, however not in ST2$^{-/-}$ mice (Fig 5.9 A, B). In parallel with the pattern of IL-4 in ST2$^{-/-}$ mice, serum total IgE was lower in ST2$^{-/-}$ mice (25μg/ml) compared to WT and IL-33$^{-/-}$ mice (60-70μg/ml). Despite the lower overall total IgE, ST2$^{-/-}$ mice produced the same amount of Alternaria-specific IgE as WT mice, while IL-33$^{-/-}$ had more allergen specific IgE (Fig 5.9 C, D).

IL-33 was equally elevated in response to Alt exposure in the lungs of WT and ST2$^{-/-}$ mice (Fig 5.10 A). Lung IL-13 and IL-1α were induced in response to Alt similarly in all genotypes above the PBS control (Fig 5.10 B, C). In contrast to lung homogenate, lower levels of IL-13 were secreted into the BAL of IL-33$^{-/-}$ and ST2$^{-/-}$ mice in response to Alt when compared to WT mice (Fig 5.10 D). Serum MCPT-1 was elevated from PBS control in all genotypes of mice (Fig 5.10 E).

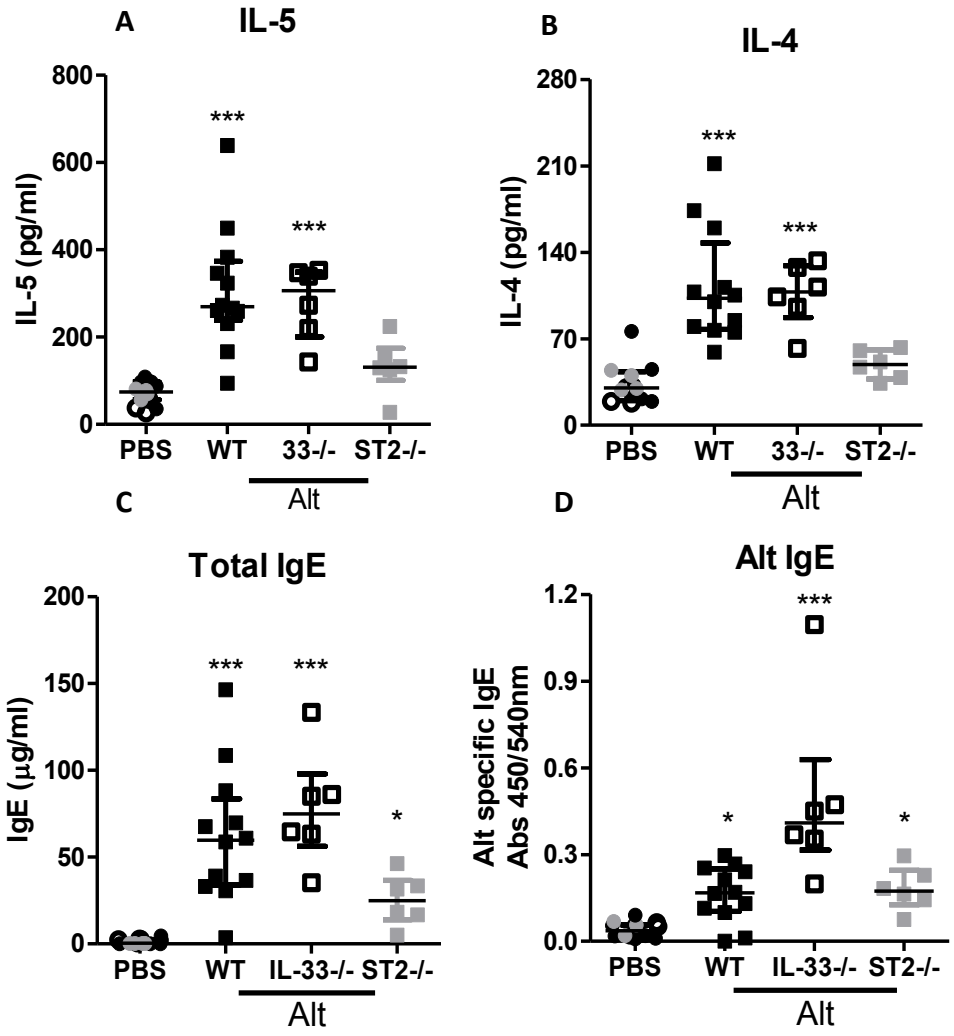

Figure 5.9 ST2 is partially responsible for Alt sensitisation

Lung and blood were collected and lung homogenised after three weeks of Alt exposure. IL-5 **(A)** and IL-4 **(B)**, as concentration per ml lung homogenate (50mg/ml). Total serum IgE **(C)** and Alt specific IgE **(D)** Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-12 mice per group * $p<0.05$ and *** $p<0.001$ from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

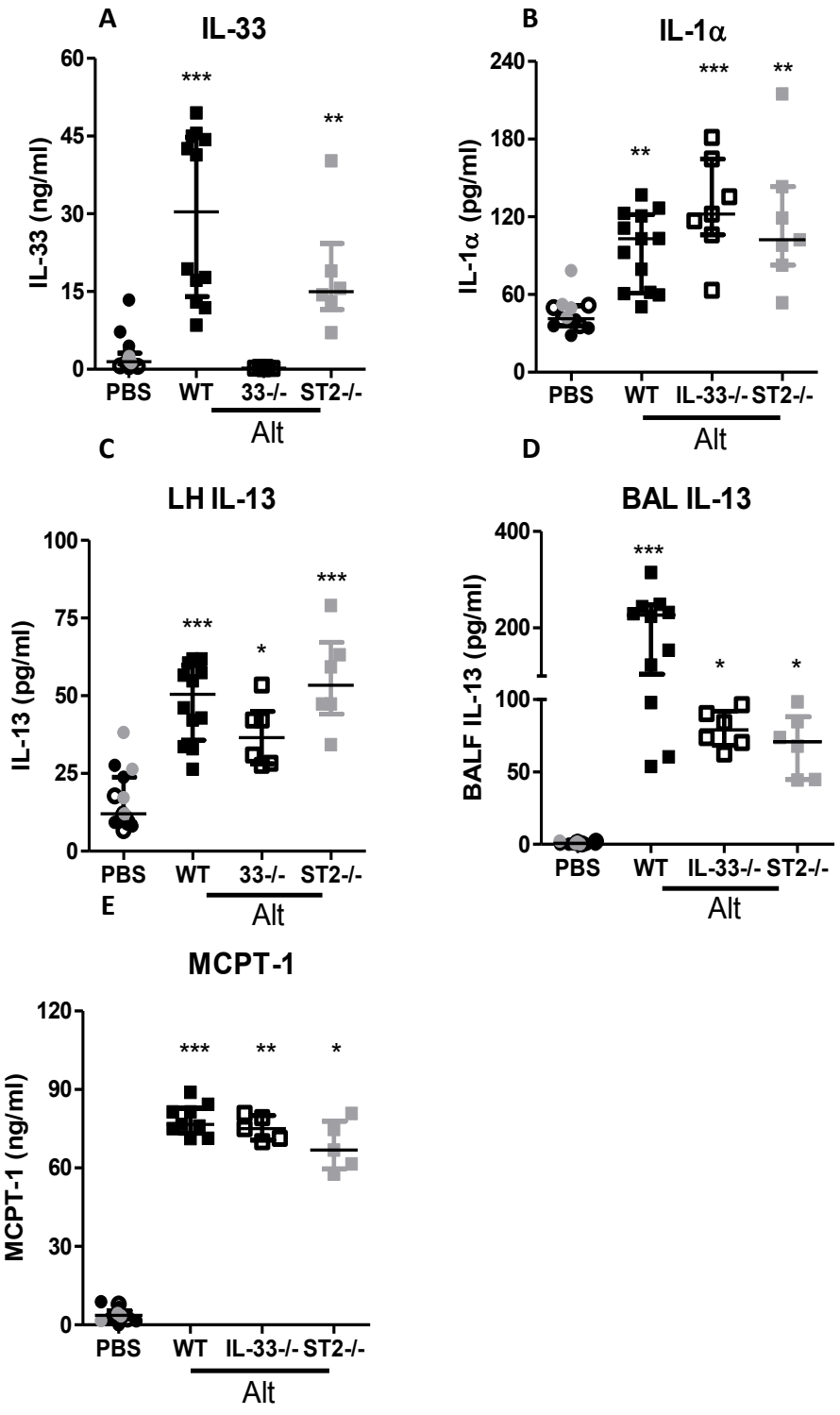

Figure 5.10 Alt induced Inflammatory cytokines in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice

Lung and BAL were collected and lung homogenised after three weeks of Alt exposure. IL-33 **(A)**, IL-1α **(B)** and IL-13 **(C)** as concentration per ml lung homogenate (50mg/ml). BAL IL-13 **(D)** as pg/ml BAL. Serum MCPT-1 **(E)** as ng/ml. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-12 mice per group. * p<0.05, **p<0.01 and *** p<0.001 from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

5.3.6 Alt induced T cells and ILCs

Total ILCs were elevated from the PBS control in all genotypes of mice in response to Alt exposure with approximately half the amount recruited to the lungs of $ST2^{-/-}$ and $IL\text{-}33^{-/-}$ mice in comparison to WT (Fig 5.11 A). The populations of $IL\text{-}13^{+}$ ILCs were also reduced from WT in both $ST2^{-/-}$ and $IL\text{-}33^{-/-}$ mice (Fig 5.11 B). Total $CD3^{+}CD4^{+}$ T cell populations were not increased by Alt exposure in any genotype (Fig 5.11 C). Despite this, $IL\text{-}13^{+}$ Th2 cells were elevated above the saline control in all genotypes in response to Alt exposure with a smaller number present in the lungs of $ST2^{-/-}$ mice (Fig 5.11 D).

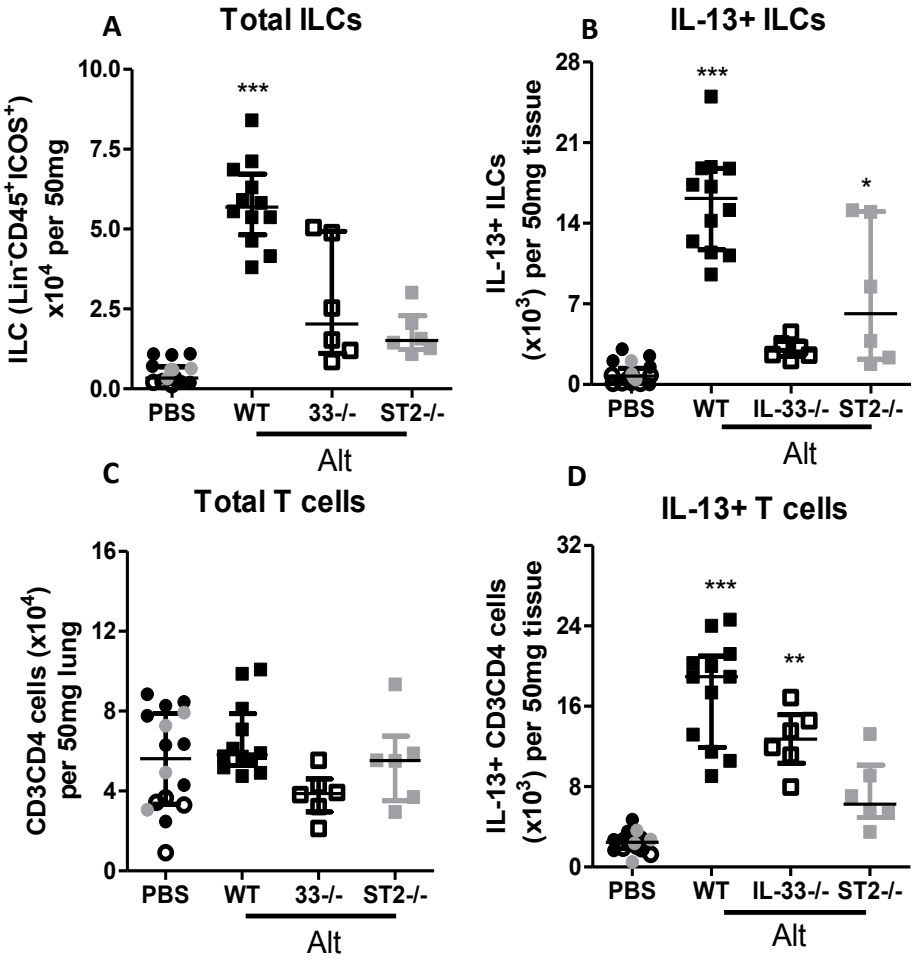

Figure 5.11 Alt induced ILC and T cell populations

Lungs were collected and cells isolated for flow cytometry analysis after three weeks of Alt exposure. Total ILCs ($Lin^-CD45^+ICOS^+$) **(A)** ILC2s ($IL\text{-}13^+$) **(B)**, total T cells ($CD3^+CD4^+$) **(C)** and Th2 cells ($IL\text{-}13^+$) **(D)** determined by flow cytometry. Black symbols represent WT data points, hollow symbols $IL\text{-}33^{-/-}$ and grey symbols $ST2^{-/-}$ data points. n= 4-12 mice per group * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

5.3.7 Pulmonary cells from ST2$^{-/-}$ mice secrete less IL-13 in response to Alt stimulation *in vitro*

Despite the reduced population of IL-13^{+} T cells in the lungs of ST2$^{-/-}$ mice exposed to Alt (Fig 5.11 D) the level of IL-13 in the lung homogenate was elevated to the same level as WT mice (Fig 5.10 C). In order to delineate the contribution of antigen specific T cells to pulmonary IL-13 levels, an *in vitro* Alt stimulation experiment was carried out. Pulmonary immune cells (unsorted mixed cell population) were isolated from WT, IL-33$^{-/-}$ and ST2$^{-/-}$ after three weeks of Alt or PBS exposure (Fig 5.1 B) and all cells were cultured *in vitro* with Alt. Supernatant was collected at 24 and 48 hours after stimulation and Th2 cells were identified by flow cytometry.

Despite elevated IL-33 in the lung homogenate of WT and ST2$^{-/-}$ mice after Alt exposure (Fig 5.10 A) no secreted IL-33 was detectable in the BAL 18 hours after the final Alt exposure (data not shown). Previous work in the lab shows IL-33 is rapidly secreted into the BAL of mice and can be detected one hour after Alt exposure (Snelgrove et al., 2014). Allergen challenge of human asthmatics caused IL-33 release into the BAL 10 minutes after challenge while the cytokine was no longer detectable at 18 hours post challenge (Fux et al., 2014) suggesting IL-33 is rapidly degraded or bound by sST2 and would therefore no longer be detectable at the 18 hour time point investigated in the current study. Secreted IL-33 measured in the cell supernatant was increased in cells isolated from Alt exposed mice over PBS controls and peaked at 24 hours of *ex vivo* culture with Alt (Fig 5.12 A). Lack of ST2 did not affect the capacity of cells to release IL-33. IL-13 release into the supernatant was equal from cells isolated from both WT and IL-33$^{-/-}$ mice and cytokine secretion was maintained over 48 hours (Fig 5.12 B). Lung cells cultured from ST2$^{-/-}$ mice that had been exposed to Alt secreted more IL-13 than cells isolated from PBS control mice, however the amount of IL-13 released was substantially less than cells isolated from WT and IL-33$^{-/-}$ Alt exposed mice (Figure 5.12 B). As Ki67 has been shown to act as an effective marker for antigen specific cell proliferation after *in vitro* culture (Soares et al., 2010) we used flow cytometry after 48 hours of culture to determine the percentage of T cells

expressing Ki67 and IL-13 to investigate if WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice have equal capacities to generate antigen specific T cells. The percentage of T cells as well as Ki67^{+} T cells with the ability to generate IL-13^{+} was equally increased from PBS in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ Alt exposed mice (Fig 5.12 C, D).

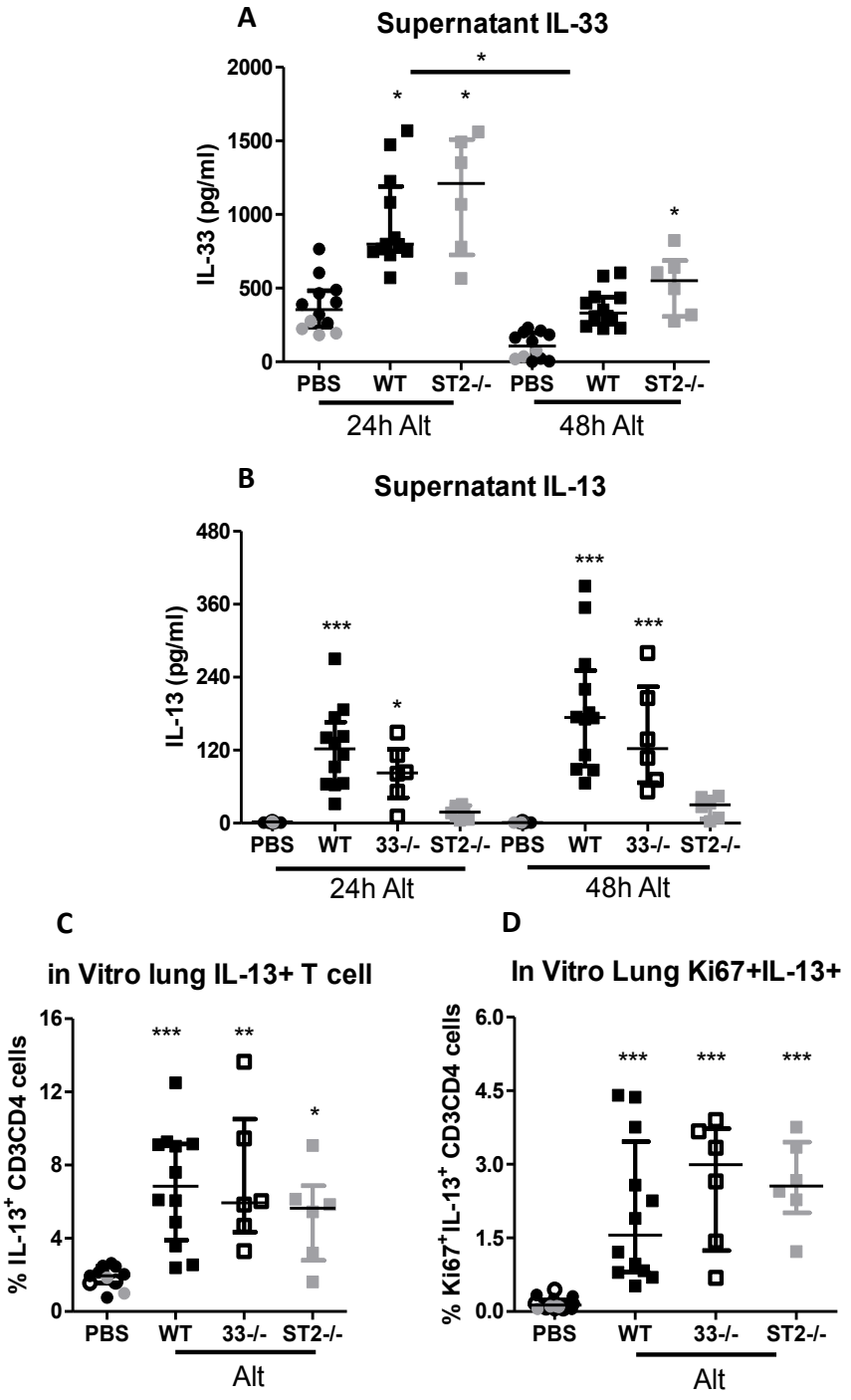

Figure 5.12 Cytokine release and proliferative T cell responses to culture with Alt *In vitro*

In vitro Alt stimulated lung cells isolated from mice exposed to PBS or Alt for three weeks. IL-33 **(A)** and IL-13 **(B)** in culture supernatant after 24 and 48 hours of Alt stimulation. **(C)** IL-13^{+} **(D)** and Ki67^{+}IL-13^{+} cells as % CD3CD4 cells after 48 hours *in vitro* Alt stimulation as determined by flow cytometry **(D)**. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-12 mice per group. * p<0.05 ** p<0.01 and *** p<0.001 from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

5.3.8 The impact of IL-33 or ST2 deletion on the inception of allergic airways disease

IL-33 is released from the pulmonary epithelium rapidly after Alt exposure (Snelgrove et al., 2014) and many lung resident cells have surface ST2 receptors. It was therefore essential to investigate the effect of loss of IL-33 or ST2 on the immediate response to allergen exposure in the lung.

To investigate the comparative role of IL-33 and ST2 in inducing early pulmonary inflammation a short-term protocol of allergen exposure using one and three exposures to Alt was carried out (Figure 5.13 A). Airway resistance was measured after three exposures to Alt and was increased in WT and IL-33$^{-/-}$ mice from the PBS control; however, no resistance was generated in ST2$^{-/-}$ mice (Fig 5.13 B). After a single intranasal administration of Alt, total BAL inflammation was increased in WT mice but was absent in ST2$^{-/-}$ and IL-33$^{-/-}$ mice. After three exposures to Alt, BAL inflammation was also increased in IL-33$^{-/-}$ mice and inflammation remained significantly reduced in ST2$^{-/-}$ mice compared to WT (Fig 5.13 C). BAL inflammation over the three weeks (9 exposures) peaked after three administrations of Alt in WT mice, but continued to increase in ST2$^{-/-}$ and IL-33$^{-/-}$ mice (Fig 5.13 D), indicating that BAL inflammation may reach similar levels in all genotypes if Alt exposure was continued for a longer period of time.

Lung inflammation was not induced after one Alt administration in any genotype and after three exposures was elevated in all groups of mice with lower numbers in IL-33$^{-/-}$ mice (Fig 5.14 A). In contrast to BAL inflammation over the course of Alt exposure, lung inflammatory counts rose concurrently in all genotypes over the timecourse of Alt exposure, however to a lesser extent in IL-33$^{-/-}$ and ST2$^{-/-}$ mice (Fig 5.14 B). Despite the lack of overall lung inflammation after one exposure to Alt, a significant population of eosinophils were attracted to the lungs of WT mice, this was absent in ST2$^{-/-}$ and IL-33$^{-/-}$ mice. After three exposures to Alt, eosinophils were recruited to the lungs of IL-33$^{-/-}$ mice, although populations were vastly lower than WT, and after 9 exposures eosinophil numbers were increased above PBS in all mice (Fig 5.14 C, D). In contrast to this, neutrophil numbers were not

elevated after a single Alt administration but numbers rose equally in WT and ST2$^{-/-}$ mice over 9 exposures, no increase in neutrophils occurred in IL-33$^{-/-}$ mice in response to Alt (Fig 5.14 E, F).

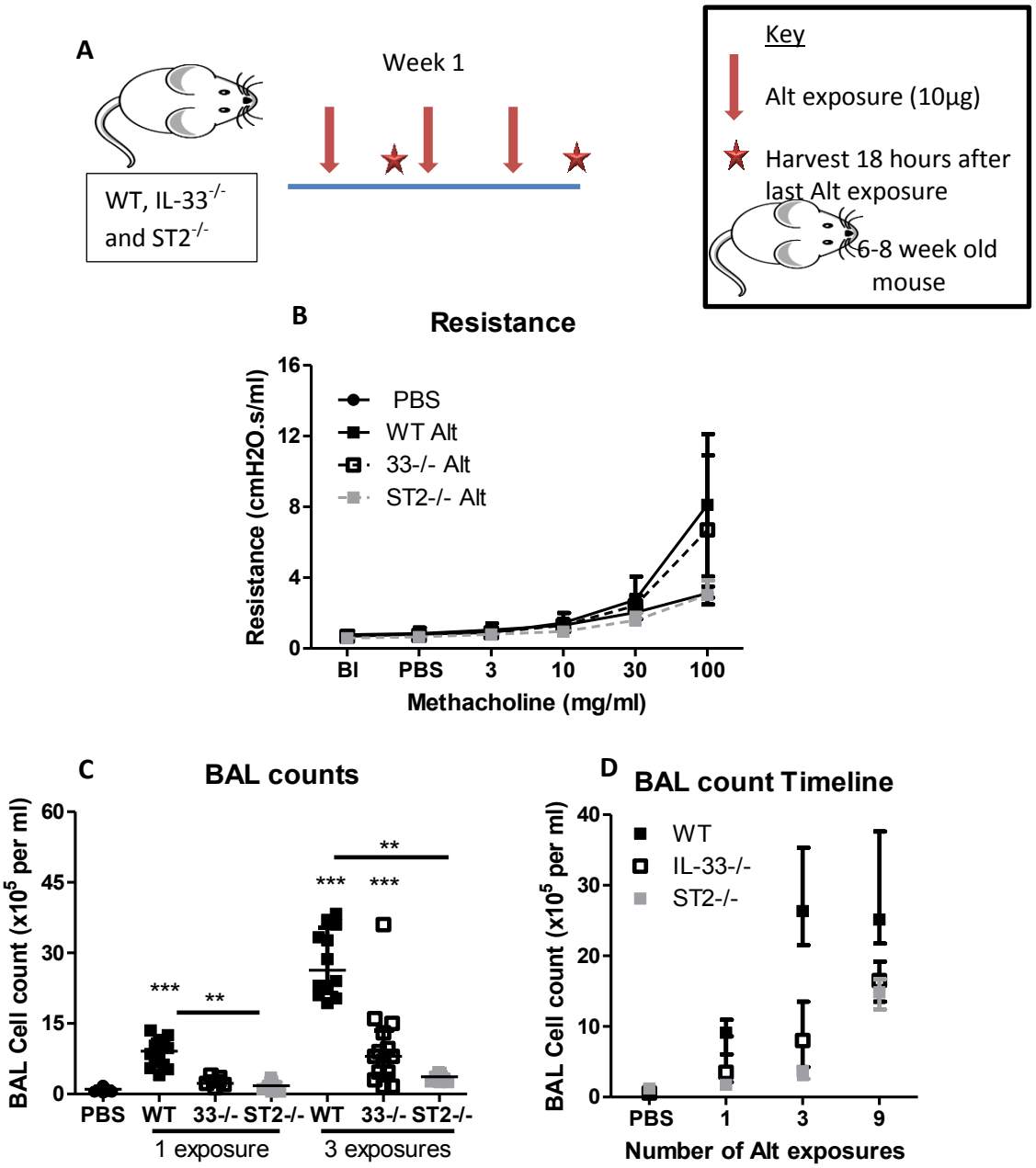

Figure 5.13 Resistance and inflammation in short term Alt exposure

Mice were exposed to one or three doses of Alt and analysed for AHR 18 hours after the final exposure **(A)**. Airway resistance in mice dosed with three doses of Alt **(B)** as determined by flexivent. BAL counts after one or three doses of i.n Alt **(C)** and collated data over the timecourse of Alt exposure **(D)** Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-12 mice per group . * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

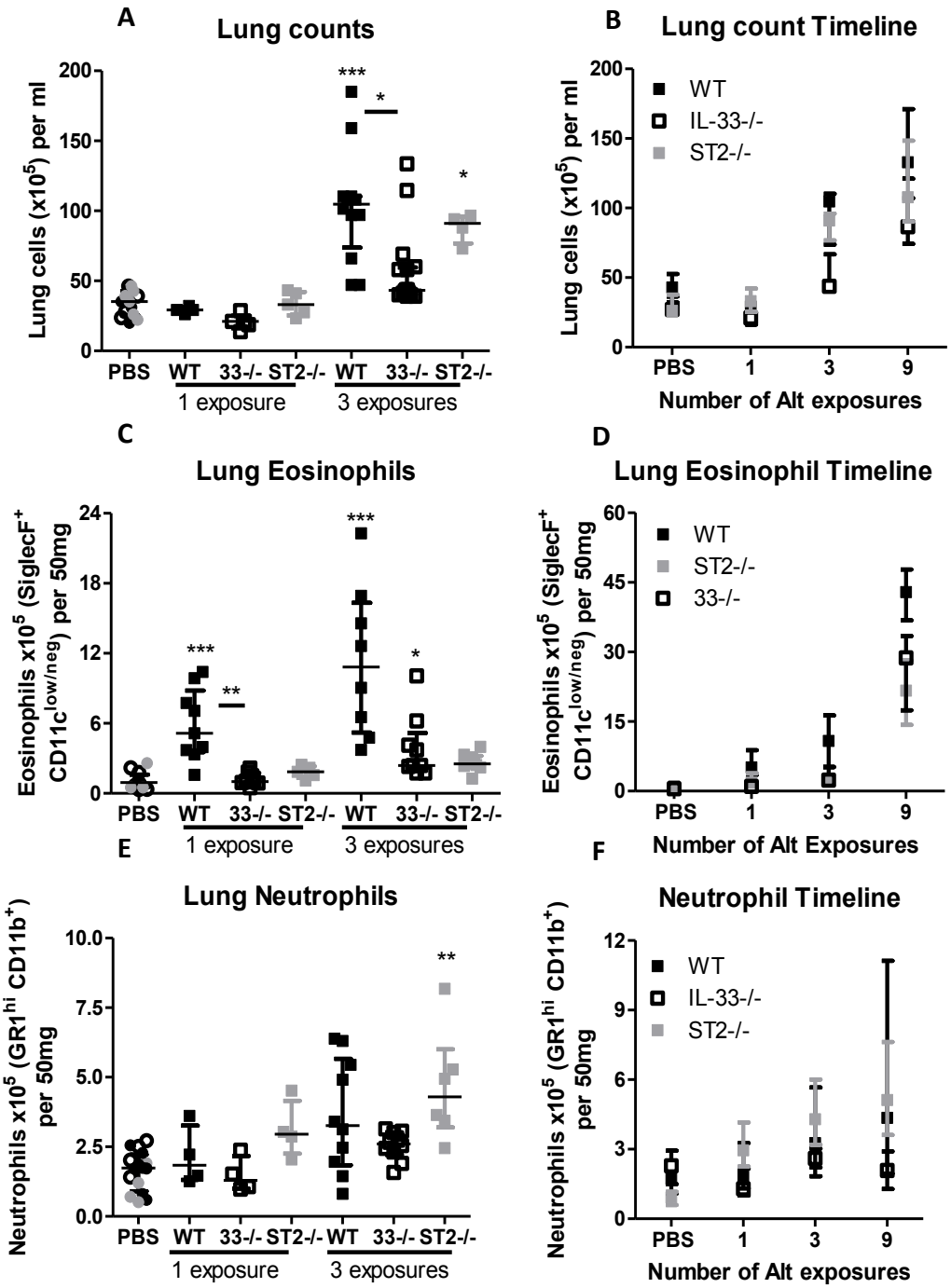

Figure 5.14 Resistance and inflammation in short term Alt exposure

Lungs were isolated 18 hours after one or three doses of i.n Alt. Lung cell counts after one and three exposures to Alt **(A)** and over the timecourse of exposure **(B)**. Lung eosinophils after one and three exposures **(C)** and over the timecourse of exposure **(D).** Neutrophils after one and three exposures **(E)** and over the timecourse of exposure **(F)** as determined by flow cytometry. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-8 mice per group * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

5.3.9 Early cytokine responses to Alt

Pulmonary IL-33 was not induced after one exposure to Alt in WT or ST2$^{-/-}$ mice and was elevated in both groups after three exposures, although levels were lower in ST2$^{-/-}$ mice (Fig 5.15 A). After three weeks of Alt challenge, levels of IL-33 were equal in WT and ST2$^{-/-}$ mice (Fig 5.15 B). No lung or BAL IL-13 was induced after a single exposure to Alt in any of the mice. IL-13 secreted into the BAL was induced by Alt in WT and IL-33$^{-/-}$ mice after three exposures, with reduced levels in IL-33$^{-/-}$ (70pg/ml) compared to WT mice (400pg/ml) (Fig 5.13 C). As with BAL inflammatory counts (Fig 5.13 C) maximal levels were detected after three exposures to Alt in WT mice while IL-13 was higher after nine exposures in both IL-33$^{-/-}$ and ST2$^{-/-}$ mice (Fig 5.15 D). As with BAL IL-13, lung IL-13 was induced in WT and IL-33$^{-/-}$ mice only, not ST2$^{-/-}$ mice after three exposures to Alt (Fig 5.15 E). Maximal levels of lung IL-13 were generated after a week of Alt exposure and declined over the three weeks in WT and IL-33$^{-/-}$ mice while maximal expression in ST2$^{-/-}$ mice was generated after three weeks of exposure (Fig 5.15 F).

IL-1α in the lung was induced after a single exposure to Alt to an equal level in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice. After three exposures IL-1α remained elevated to the same level in ST2$^{-/-}$ mice and decreased in WT and IL-33$^{-/-}$ mice. IL-1α levels then decreased over the three week timecourse of Alt exposure in all genotypes, indicating the role of IL-1α as an innate response to Alt exposure (Fig 5.16 A, B).

The surface expression of ST2 is prominent on Th2 cells, not Th1 cells, and is important for the induction of Th2 cytokines. One hypothesis was that the loss of ST2 caused a skewing towards a Th1 response following Alt exposure, causing the down regulation of IL-4, IL-13 and IgE observed in ST2$^{-/-}$ mice. To investigate the absence of ST2 on induction of a Th1 response, IFNγ was measured in the lung homogenate over the timecourse of Alt exposure. IFNγ was induced to a similar level in both WT and IL-33$^{-/-}$ mice following three exposures to Alt but was not induced in ST2$^{-/-}$ mice (Fig 5.16 C). Following 9 exposures, IFNγ was induced to the same level in all genotypes, equal to the level generated in response to three exposures of Alt in WT and IL-33$^{-/-}$ mice (Fig 5.16 D.

The ST2/IL-33 axis is important for the maturation and activation of mast cells (Allakhverdi et al., 2007). As active mast cells are known to play a role in the inception of AAD through the secretion of IL-13 (Ho et al., 2007), serum MCPT-1 was measured as a marker of mast cell activation. Serum MCPT-1 was elevated in all mice exposed to Alt three times although was substantially lower in $ST2^{-/-}$ mice compared to Alt exposed WT and $IL\text{-}33^{-/-}$ mice, levels of MCPT-1 continued to rise over the timecourse of Alt exposure with similar levels in all genotypes after three weeks (Fig 5.16 E, F).

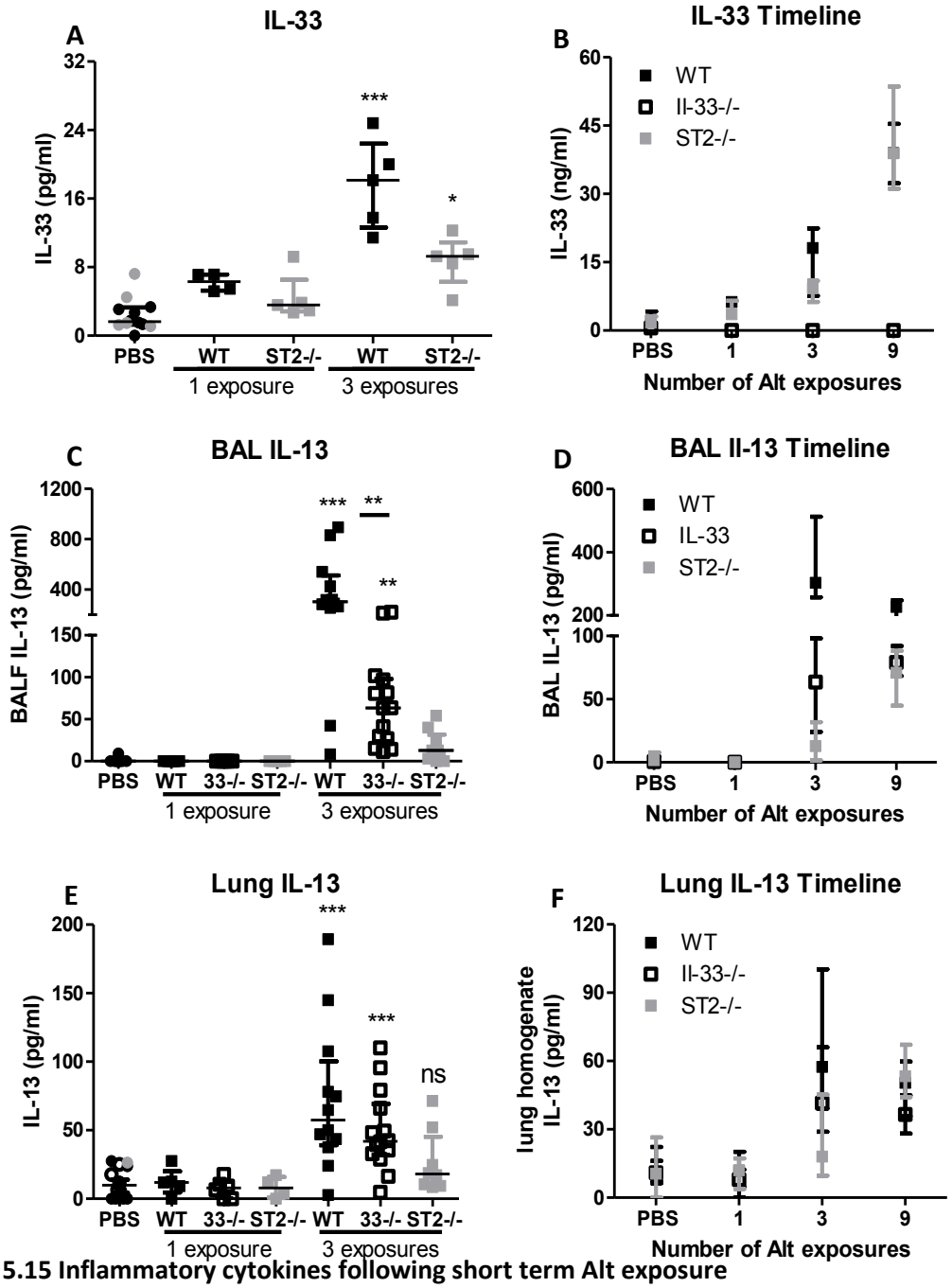

Figure 5.15 Inflammatory cytokines following short term Alt exposure

Lung and BAL were isolated after one or three exposures of Alt. lung IL-33 after one and three exposures to Alt **(A)** and over the timecourse of exposure **(B)** BAL IL-13 after one and three exposures **(C)** and over the timecourse of exposure **(D)** Lung IL-13 after one and three exposures to Alt **(E)** and over the timecourse of exposure **(F)** as determined by ELISA. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-12 mice per group * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.. ns = not significant.

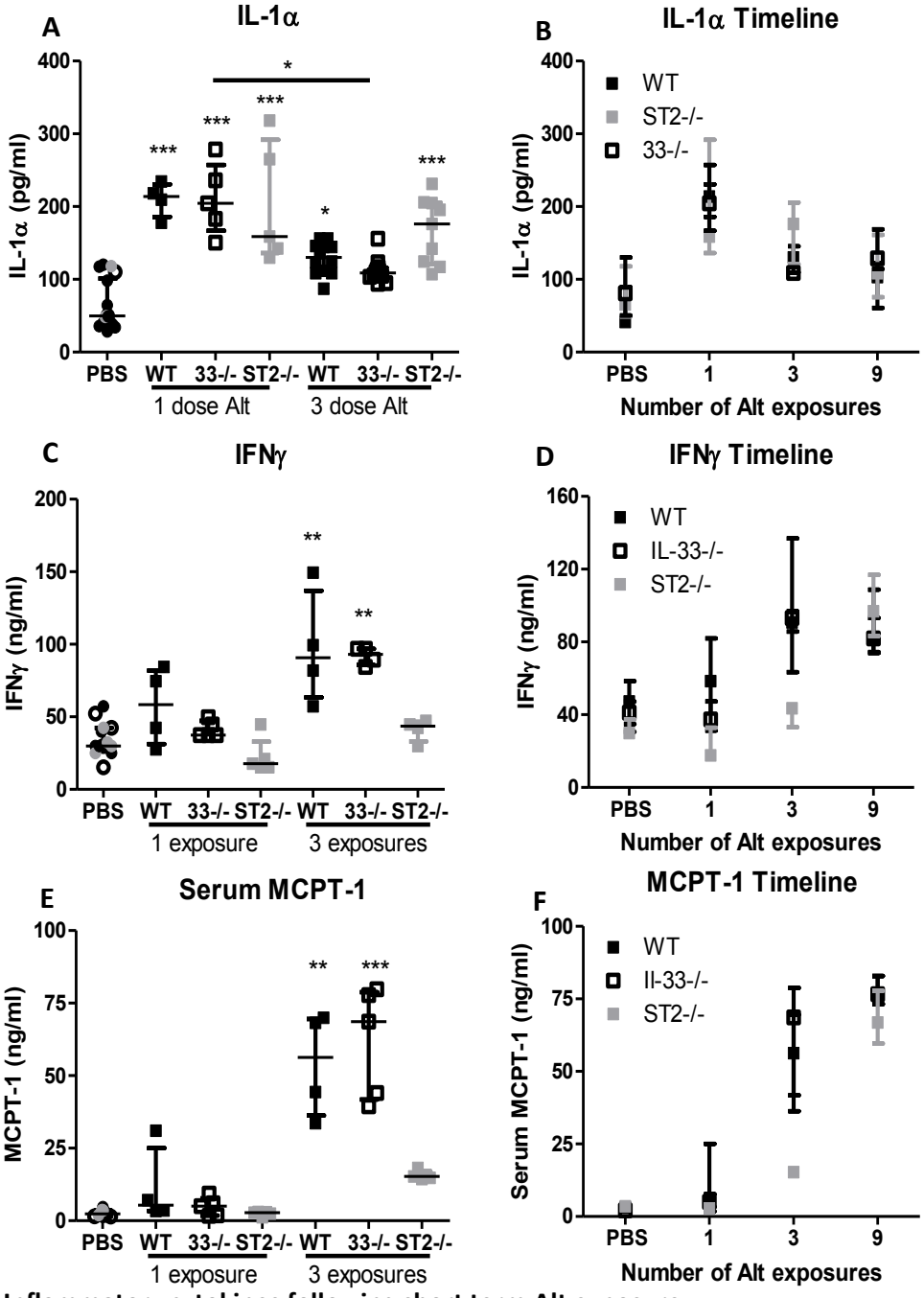

Figure 5.16 Inflammatory cytokines following short term Alt exposure

Lung and serum were isolated after one or three exposures of Alt. lung IL-1α after one and three exposures to Alt **(A)** and over the timecourse of exposure **(B)** Lung IFNγ after one and three Alt exposures **(C)** and over the timecourse of exposure **(D)** Serum MCPT-1 after one and three Alt exposures **(E)** and over the timecourse of exposure **(F)** as determined by ELISA. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-8 mice per group * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

5.3.10 Short term Alt exposure fails to induce Th2 cells in ST2$^{-/-}$ mice despite an increased percentage of Alt responsive T cells.

IL-13^{+} ILCs and T cells were not induced in the lungs of any mice after a single administration of Alt, with the exception of a small increase in IL-13^{+} ILCs in WT mice. After three exposures to Alt, all genotypes of mice had increased IL-13^{+} ILC populations although both IL-33$^{-/-}$ and ST2$^{-/-}$ mice had fewer ILC2s (Fig 5.17 A). Numbers declined vastly in WT mice following 9 exposures to Alt, confirming the innate nature of the cell type, while numbers remained similar in IL-33$^{-/-}$ and ST2$^{-/-}$ mice (Fig 5.17 B). IL-13^{+} T cells were induced in both WT and IL-33$^{-/-}$ mice while no increase occurred in the lungs of ST2$^{-/-}$ mice after three exposures to Alt (Fig 5.17 C). In contrast to ILCs, IL-13^{+} T cell numbers rose in all mice over the timecourse of Alt exposure, although to a lesser extent in both IL-33$^{-/-}$ and ST2$^{-/-}$ mice (Fig 5.17 D).

Mixed immune cells from lung digest were isolated from mice exposed to Alt three times and cultured *ex vivo* in the presence of Alt. After 24 and 48 hours of Alt stimulation WT and ST2$^{-/-}$ cells secreted an equal amount of IL-33 into the culture supernatant (Fig 5.18 A). The levels of IL-33 secreted from WT and ST2$^{-/-}$ cells did not increase after 9 Alt exposures after culture with Alt for 24 hours (Fig 5.18 B). In contrast, IL-13 secretion from cells isolated after three Alt administrations was not significantly elevated in either IL-33$^{-/-}$ or ST2$^{-/-}$ mice. However, after 9 exposures to Alt WT and IL-33$^{-/-}$ cells secreted equal levels of IL-13, elevated from 3 exposures, while IL-13 secretion from ST2$^{-/-}$ cells remained minimal. As a surrogate marker to detect the presence of antigen specific cells (Soares et al., 2010) flow cytometry was performed after 48 hours of culture with Alt to detect the percentage of T cells proliferating and producing IL-13 (IL-13^{+} Ki67^{+} T cells). The percentage of T cells capable of producing IL-13 was equal in cells isolated from all mice, despite the decreased level of IL-13 secretion from cells isolated from ST2$^{-/-}$ mice. However, the percentage of those T cells actively proliferating in response to Alt was significantly increased in ST2$^{-/-}$ mice compared to WT and IL-33$^{-/-}$

mice (Fig 5.18 F). This contrasts the equal percentage of T cells proliferating in all mice in response to Alt after three weeks of exposure (Fig 5.12 D).

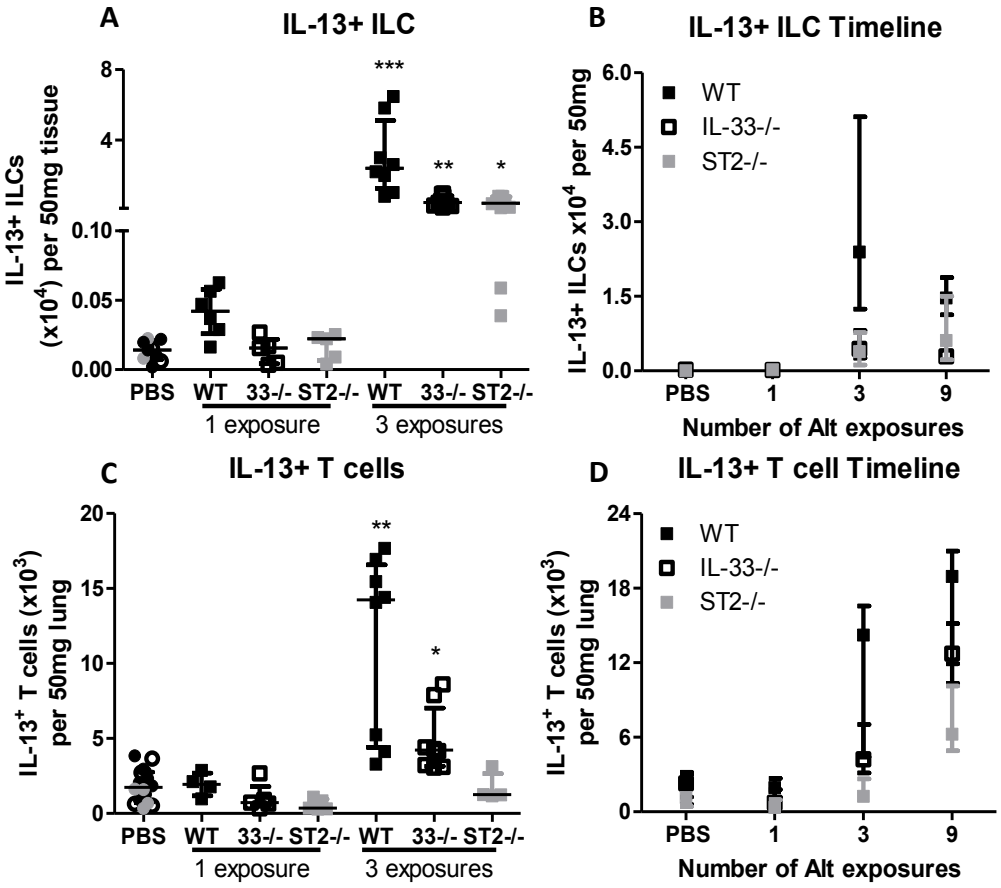

Figure 5.17 IL-13 ILCs and T cells after short term Alt exposure

Lungs were collected and cells isolated for flow cytometry analysis after one or three exposures of Alt exposure. IL-13$^+$ ILCs (IL-13$^+$Lin$^-$CD45$^+$ICOS$^+$) after one and three exposures **(A)** and over the timecourse of Alt exposure **(B)**. IL-13$^+$ T cells (IL-13$^+$CD3$^+$CD4$^+$) after one and three exposures to Alt **(C)** and over the three week Alt exposure timecourse **(D)** determined by flow cytometry. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-8 mice per group * p<0.05 ** p<0.01 and *** p<0.001 from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

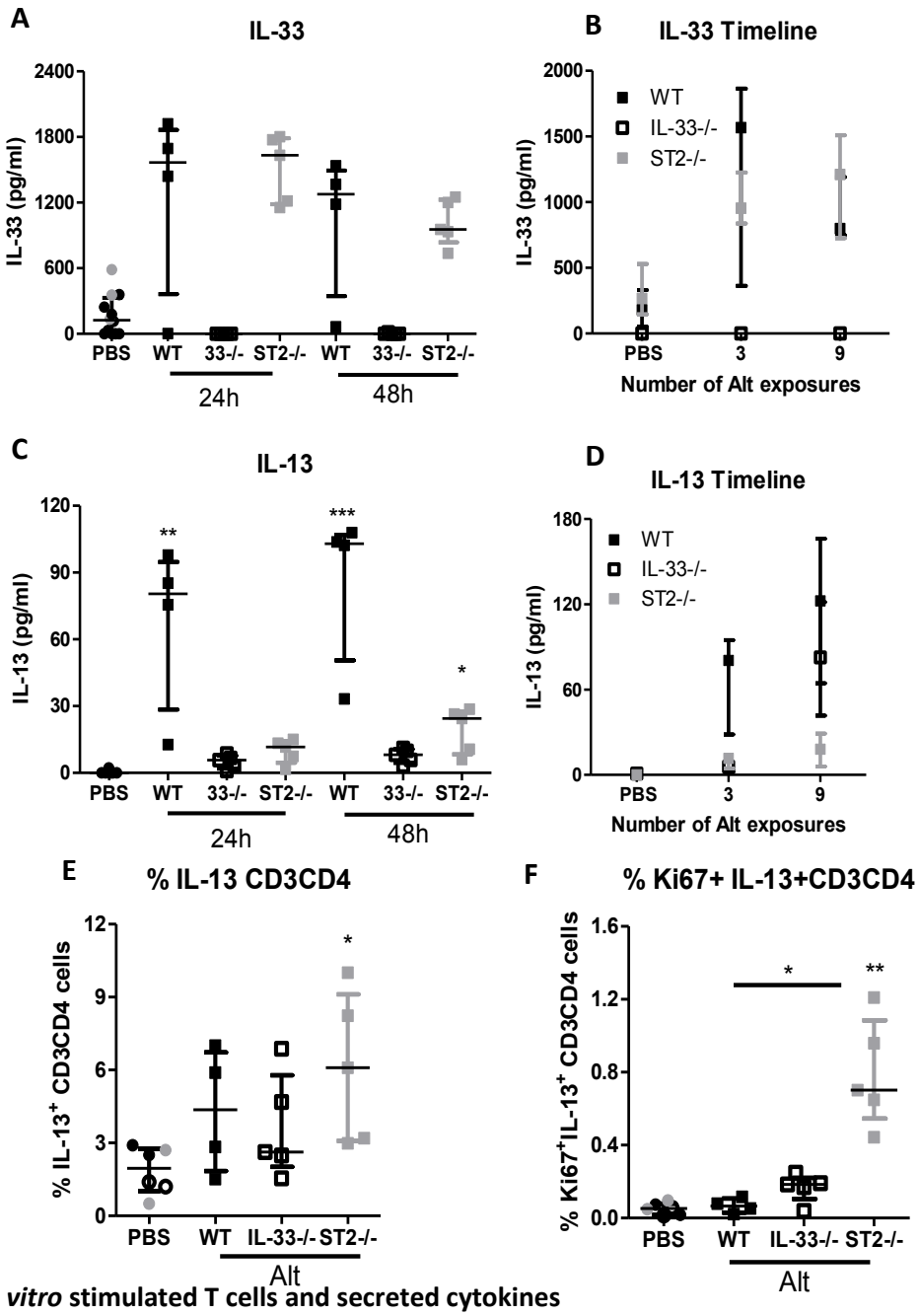

Figure 5.18 *In vitro* stimulated T cells and secreted cytokines

In vitro Alt stimulated lung cells isolated from mice exposed to Alt three times. IL-33 in culture supernatant after 24 and 48 hours of Alt stimulation **(A)** and IL-33 in culture supernatant after 24 hours from cells isolated after three or 9 Alt exposures **(B)**. IL-13 in culture supernatant after 24 and 48 hours of Alt stimulation **(C)** and IL-33 in culture supernatant after 24 hours from cells isolated after three or 9 Alt exposures **(D)**. IL-13^{+} **(E)** and KI67^{+}IL-13^{+} cells as % CD3CD4 cells after 48 hours *in vitro* Alt stimulation as determined by flow cytometry **(F)**. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4-5 mice per group * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range..

5.3.11 Generation of total and Alt specific immunoglobulin was not affected by the loss of ST2

$ST2^{-/-}$ mice had no increase in BAL Inflammation after one exposure to Alt (Fig 5.13 A) and no increase in lung or BAL IL-13 after three Alt exposures (Fig 5.15 D) in comparison to both WT and $IL-33^{-/-}$ mice. In order to determine whether absence of functional ST2 resulted in a delay in the generation of total and Alt specific Ig a serial tail bleed was carried out every 4 days over a three week Alt exposure protocol (Fig 5.19 A) and both total and Alt specific Ig were measured by ELISA. In all genotypes, both total and Alt specific IgE and IgG1 rose concurrently over the three weeks (Fig 5.19 B-E). Total IgG2a was elevated in WT mice only; however, Alt specific IgG2a was not generated in any of the mice exposed to Alt over three weeks (Fig 5.19 F, G).

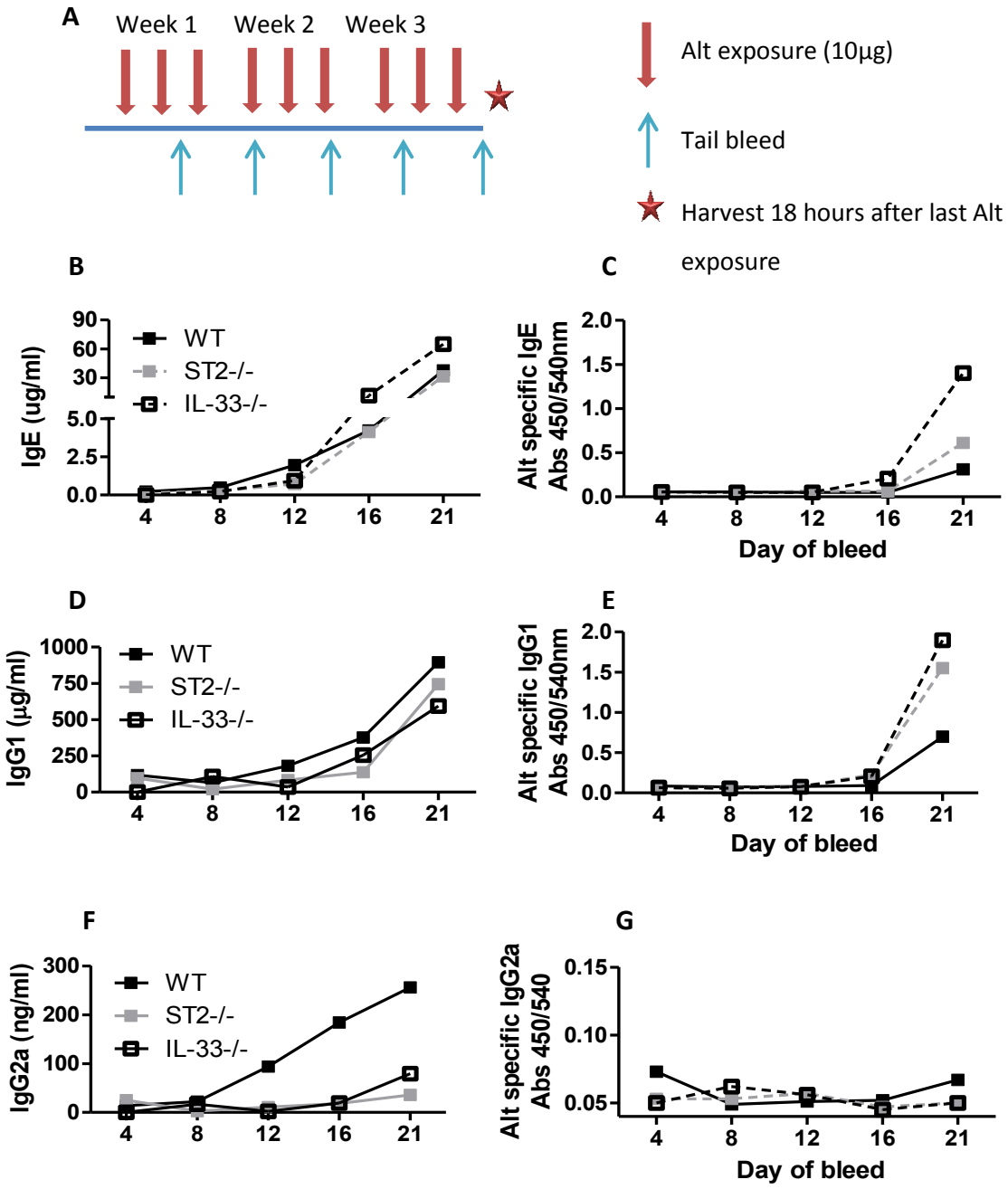

Figure 5.19 Total and Alt specific Immunoglobulin development during Alt dosing.

Serum was collected by tail bleed every 4 days over a three week dosing protocol with Alt **(A)**. Serum IgE **(B)**, Alt specific IgE **(C)**, IgG1 **(D)**, Alt specific IgG1 **(E)**, IgG2a **(F)** and Alt specific IgG2a **(G)**. Black symbols represent WT data points, hollow symbols IL-33$^{-/-}$ and grey symbols ST2$^{-/-}$ data points. n= 4 mice per group. Data represented as median.

5.3.12 Overexpression of IL-33 in macrophage cultures is unaffected by HDM, Alt or LPS stimulation.

As the global knockout of IL-33 did not affect sensitisation to allergens, we aimed to determine if tissue specific dysregulation of IL-33 influenced the development of AAD. A growing body of research has shown that in addition to inflammation, IL-33 also has roles in homeostasis and protection against diseases such as obesity and atherosclerosis (Brestoff et al., 2014; Miller et al., 2008). A global IL-33$^{-/-}$ would therefore interfere with any anti-inflammatory roles of IL-33 during the development of AAD which had not yet been identified. One of the hallmarks of patients with severe asthma is increased expression of IL-33 in both bronchial epithelial and subepithelial cells (Préfontaine et al., 2009; Saglani et al., 2013). IL-33 release from the epithelium has been shown to be a defining feature of the exposure to HDM and Alt and subsequent allergen sensitisation (Llop-Guevara et al., 2014b; Snelgrove et al., 2014; Willart et al., 2012). In order to emulate the increased epithelial IL-33 observed in severe asthmatic patients an adeno-associated virus (AAV) to express murine IL-33 was developed. The pulmonary expression of IL-33 differs in mice and humans with expression restricted to alveolar epithelial cells in mice (Hardman et al., 2013) and bronchial epithelial cells in humans (Préfontaine et al., 2010). Overexpression of IL-33 specifically in the bronchial epithelium of mice would allow the effect of dysregulated epithelial IL-33 observed in human asthmatics to be investigated.

An Origene plasmid containing IL-33 under a CMV promoter was purchased (Biolegend c.n. MC200369). This plasmid was used to assess whether the full length IL-33 sequence, once translated to protein, could be detected by ELISA. Macrophage cultures were prepared to provide a single cell culture that could be readily transfected with the plasmid using Lipofectamine. Bone marrow cells were isolated from femurs of adult Balb/c mice and differentiated to macrophages by culturing with M-CSF. Macrophages were then transfected with the IL-33 containing plasmid and after 18 hours

PBS, HDM, Alt or LPS were added to the media. Cell supernatant was collected after 1, 2, 4 and 24 hours and assessed for the presence of secreted IL-33 by ELISA. No IL-33 was secreted from the cells at any time point with any stimulant (Data not shown). Cell pellets were collected after 24 hours and assessed by ELISA. Untransfected macrophages had no detectable IL-33 in response to any stimulation while IL-33 transfected cells generated substantial levels of IL-33 protein, this was unaffected by exposure of these cells to HDM, LPS or Alt (Fig 5.20).

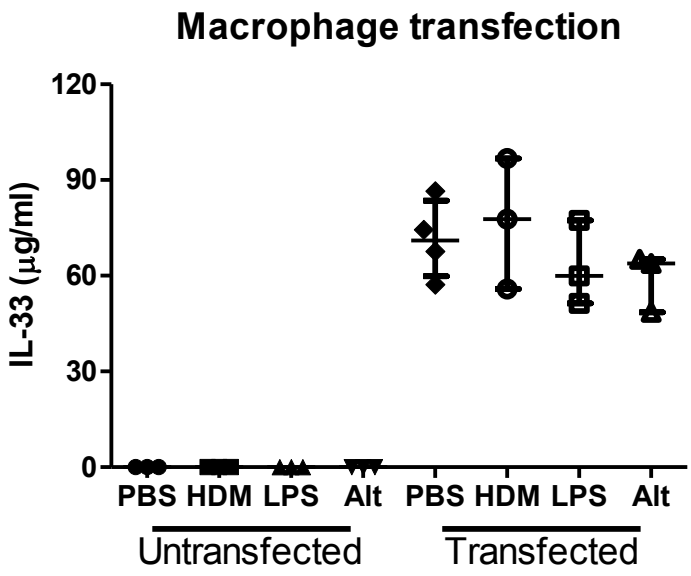

Figure 5.20 IL-33 overexpression in macrophage cultures

Bone marrow derived macrophages were transfected with a plasmid containing IL-33 under the control of a CMV promoter. Transfected and untransfected macrophages were cultured with PBS, HDM, LPS or Alt before assessment of cell pellets for IL-33 by ELISA. n=3-4 per group. Data represented as median and interquartile range.

5.3.13 AAV9 infects epithelial cells allowing epithelial expression of genes

In order to achieve IL-33 overexpression in bronchial epithelial cells an adeno-associated virus (AAV) serotype was selected. The virus is a human parvovirus which was originally discovered as a co-infector or patients with an adenoviral infection and did not cause any symptoms or pathology (Blacklow et al., 1968). AAVs have since been identified as a promising viral vector for gene therapy as they have a diverse host range and lead to long-term expression of genes *in vivo* with no apparent pathogenicity (Büning et al., 2003). A library of AAV serotypes containing GFP-encoding plasmids was screened to identify the most suitable serotype for the infection of bronchial epithelial cells *in vivo*. The viral constructs were administered both i.v and i.n to mice on the first day of life and after 6 weeks mice were culled and lung sections were stained for GFP. Intranasal dosing with AAV2 resulted in detectable GFP in the epithelium (Fig 5.21 A) but other cells resident in the lung the parenchyma were also transfected (Fig 5.21 B), so this serotype was excluded. Administration of the viral constructs through the superficial temporal vein frequently resulted in expression of GFP in the pulmonary perivascular smooth muscle, as illustrated in figure 5.21 C, depiction AAV2/6. Intranasal dosing with this serotype was also shown to be inappropriate for this study, as epithelial cells did not express the marker gene (Fig 5.21 D). Infection with AAV9 using the i.n route resulted in targeted expression of GFP in the epithelium only (Fig 5.21 E, F).

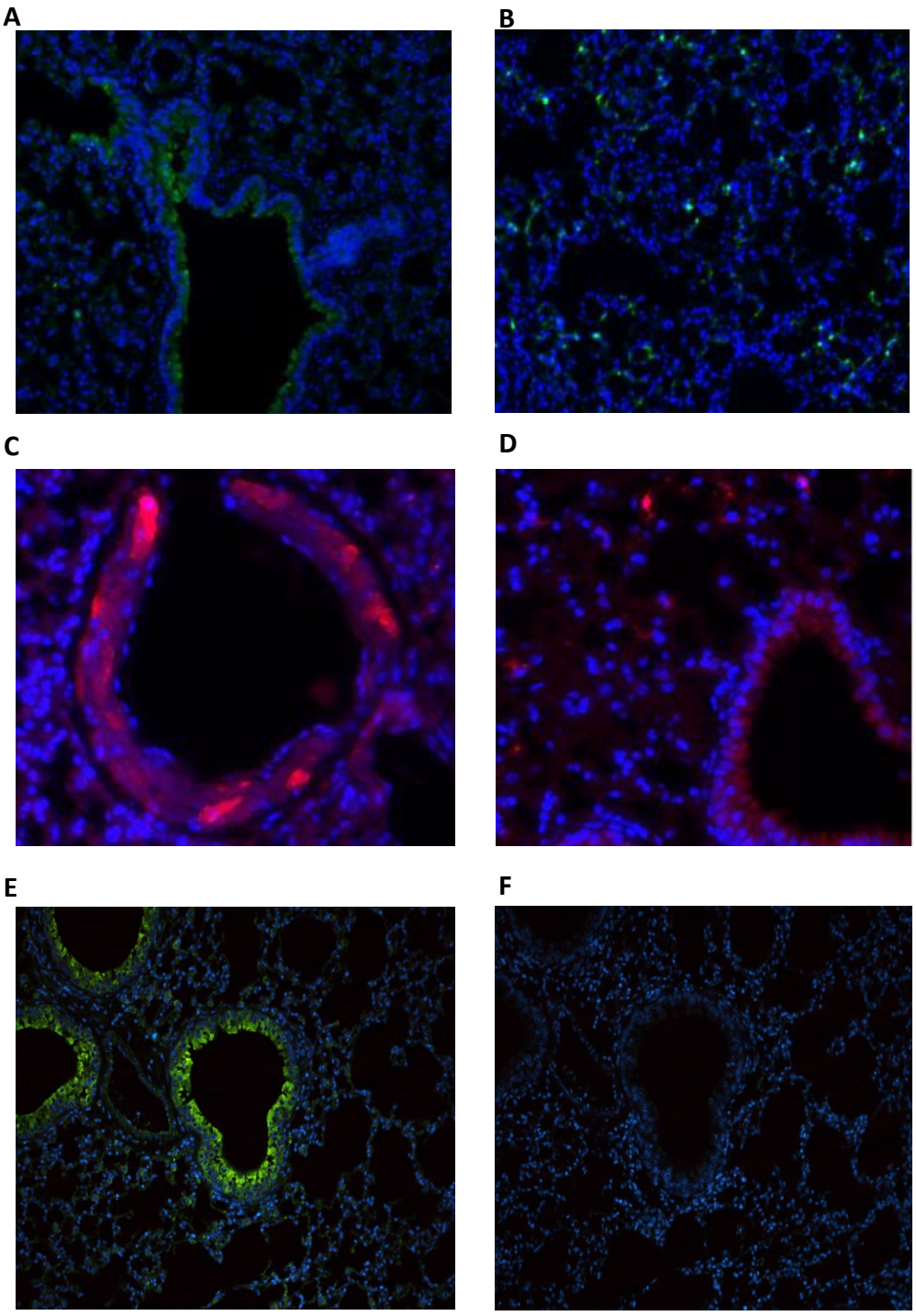

Figure 5.21 AAV selection for epithelial expression.

Lungs were collected 6 weeks after viral administration of AAV serotypes containing a plasmid encoding GFP. Sections were probed with anti-GFP and either Alexa 594 or Alexa 488 conjugated secondary antibodies. i.n AAV2 infection showing staining in the airways **(A)** and parenchyma **(B)** x20 magnification. I.v administration of AAV2/6 **(C)** and i.n administration **(D)** x40 magnification. I.n administration of AAV9 **(E)** and a no primary control **(F)** x20 magnification.

5.3.14 Insertion of IL-33 into the AAV backbone plasmid

Before expansion of the IL-33 containing plasmid a single nucleotide at position 595 (Genbank NM_133775.2) was corrected to be identical to the Genbank sequence with a forward primer spanning the area to be corrected containing the correct base pair. Forward and reverse primers were 5'-phosphorylated to allow linear DNA fragments to re-circularise and form the plasmid construct. Primers were also created to isolate the IL-33 sequence with the addition of restriction sites Xho1 (highlighted in blue) and Not1 (highlighted in green) (Fig 5.22 B) for integration into the AAV backbone plasmid pZac2.1. This PCR product was then cloned into the AAV plasmid using restriction digest enzymes Xho1 and Not 1 (Fig 5.22 C) and the plasmid was expanded using Sure2 super competent cells to avoid disruption of the inverted terminal repeats (ITR).

After amplification of the final plasmid, a number of restriction digests were performed to verify that all parts of the plasmid were in the correct orientation and there had been no rearrangements. Both ITRs were isolated and represented in green and red. The CMV promoter and IL-33 insert were identified as inserted in the plasmid and the correct size as shown in pink and yellow respectively (Fig 5.23 A, B). This construct was then shipped to the University of Pennsylvania for generation of an AAV9 vector with our IL-33 insert. The intention was to overexpress IL-33 in the bronchial epithelium to assess the effect of increased IL-33 on allergen exposure, however, due to time constraints this will be continued in the lab in the future.

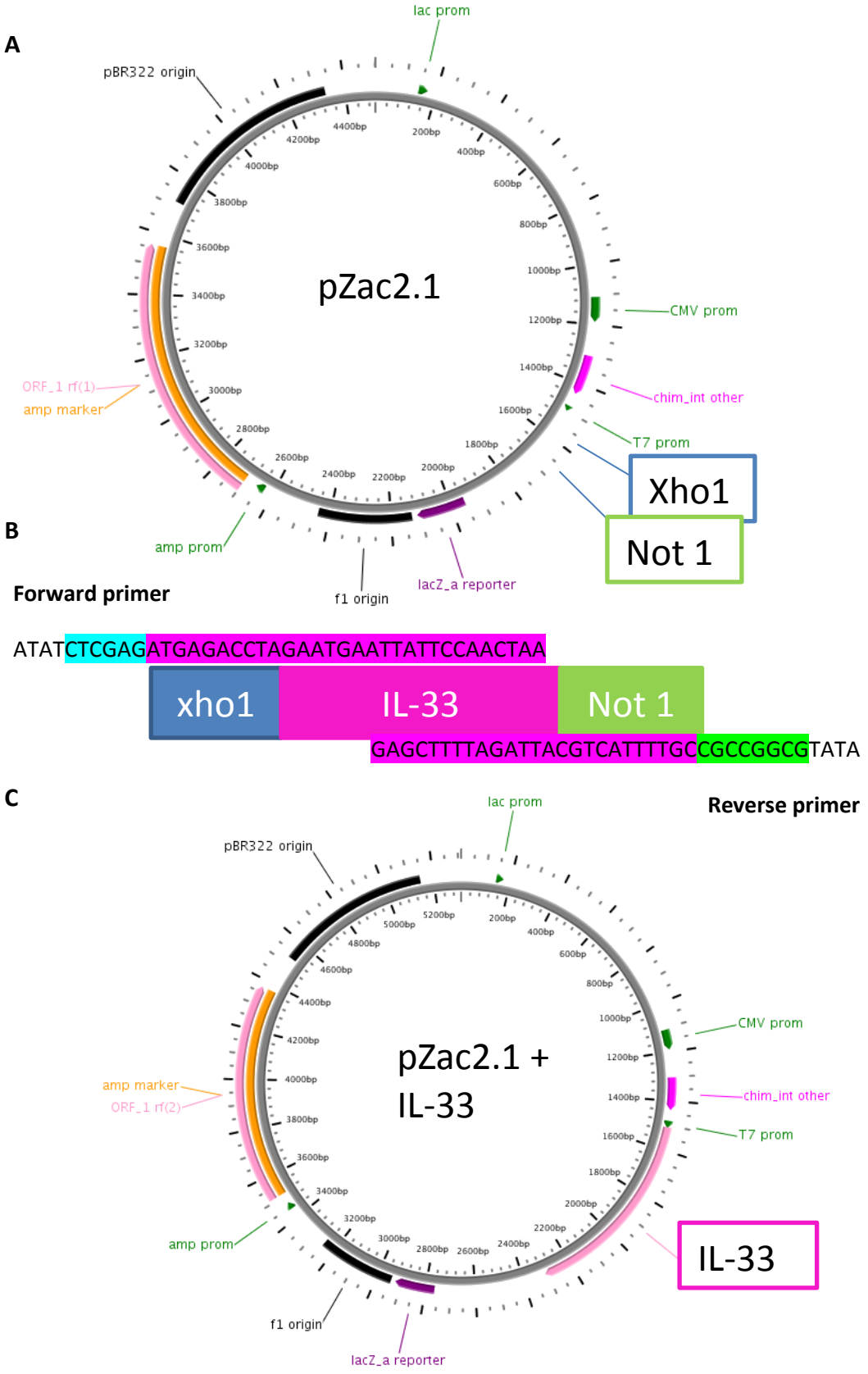

Figure 5.22 Insertion of IL-33 gene into pZac2.1

AAV backbone plasmid (pZac2.1) with xho1 and Not 1 restriction sites highlighted in blue and green respectively **(A)**. IL-33 was flanked with complementary restriction sites using primers **(B).** pZac2.1 with IL-33 insert **(C)**.

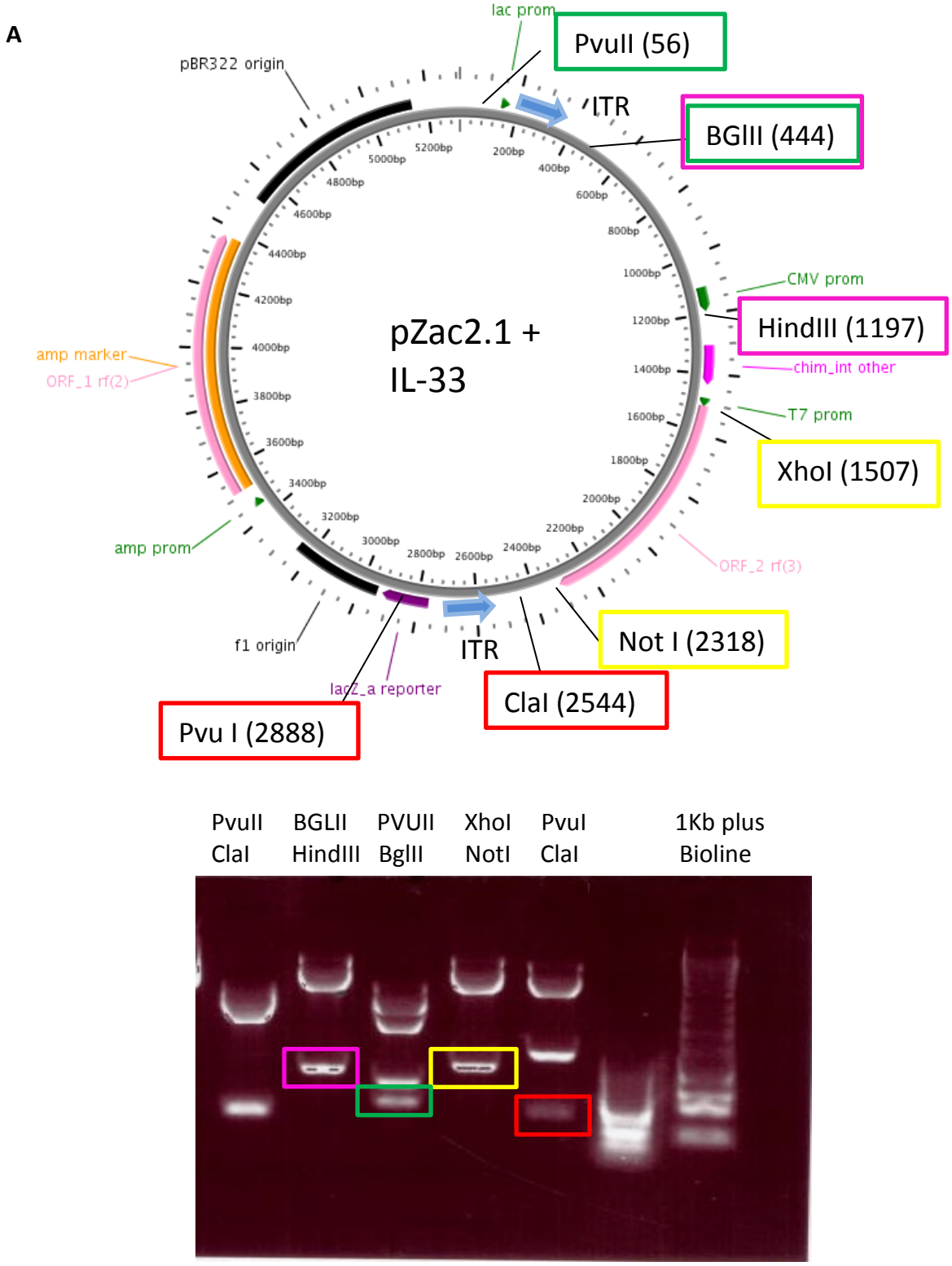

Figure 5.23 plasmid composition for insertion into AAV9

Verification of the plasmid construct. Plasmid map showing the key components to be verified by restriction digest **(A)** and an agarose gel image of restriction digest products **(B)**. Inverted terminal repeats are depicted in red and green, the CMV promoter in pink and the IL-33 insert in yellow.

5.4 Discussion

The aim of this chapter was to investigate the contribution of the IL-33/ST2 signalling pathway to the generation of AAD in response to HDM and Alt and therefore verify our hypothesis that the pathway could be targeted with therapeutics for the treatment of allergic asthma. In order to determine the most appropriate target for therapy, we utilised IL-33$^{-/-}$ and ST2$^{-/-}$ mouse models.

This study demonstrated that cytokine and receptor knockout mouse models responded completely differently to three weeks of HDM exposure. IL-33$^{-/-}$ mice developed almost identical disease to WT control mice while ST2$^{-/-}$ mice did not develop allergic airway disease, suggesting that in this model of AAD, the generation of a disease phenotype is independent of IL-33 (amounts of which were elevated in ST2$^{-/-}$ mice to equivalent levels of WT mice exposed to HDM) but dependent instead on signalling via ST2. In contrast, exposure to three weeks of Alt – an allergen associated with a more severe disease phenotype – caused disease to develop in both IL-33$^{-/-}$ and ST2$^{-/-}$ mice, although classical Th2 cytokines such as IL-4 and IL-5 were significantly decreased in ST2$^{-/-}$ mice compared to both WT and IL-33$^{-/-}$ mice. These data indicate that, compared to HDM, Alt stimulates additional facets of the immune response that are able to overcome the loss of ST2 receptor signalling.

As IL-33 is released into the BAL within an hour of Alt exposure in both patients and murine models (Fux et al., 2014; Snelgrove et al., 2014) the short term effect of removal of this early alarmin system was also analysed. After a single exposure to Alt, both IL-33$^{-/-}$ and ST2$^{-/-}$ mice were protected from airway inflammation and pulmonary eosinophilia that was observed in WT mice. However, after three administrations of Alt over one week, differences in the two knockout models again became apparent; AHR developed only in WT and IL-33$^{-/-}$ mice in response to Alt, indicating that activation of the ST2 receptor is important in the early generation of AHR. In addition, ST2$^{-/-}$mice had significantly lower BAL IL-13 and no increase in lung IL-13 compared to the PBS controls, reinforcing the importance of IL-13 in driving AHR. Investigation of the kinetics of cytokine generation by each genotype over the timecourse of Alt exposure revealed a possible delayed phenotype in ST2$^{-/-}$ mice,

and to some extent in IL-33$^{-/-}$ mice, that has the possibility to reach WT levels if exposure continued for a longer period. We therefore hypothesised that the loss of ST2 would cause a delay in the onset of Th2 and IgE mediated allergic disease that would explain the absence of AHR after a single week of Alt exposure and the presence of disease after three weeks. However, investigation of the capacity of T cells to actively proliferate in response to Alt *in vitro* – used as a surrogate marker to identify antigen specific T cells (Soares et al., 2010) - and the generation of total and Alt specific IgE *in vivo* revealed neither T nor B cell responses were diminished due to a lack of ST2. Investigation of cytokine secretion from mixed lung cell populations showed that despite the equal percentages of T cells with the ability to produce IL-13, secretion of this cytokine from pulmonary cells was vastly lower in ST2$^{-/-}$ mice while IL-33 secretion was comparable. Maximal secretion of IL-33 was reached after 3 exposures to Alt (although an earlier time point was not investigated) while IL-13 secretion was elevated following 9 exposures to Alt. This reiterates the vital role of signalling via ST2 to generate an early IL-13 response from innate cells to initiate AAD. It was therefore concluded that the loss of an early source of ST2 dependent IL-13 from innate cells, such as ILC2s and mast cells, delayed the development of a type 2 immune response and AHR. However, after 3 weeks of Alt exposure, the lack of ST2 was overcome and did not prevent the progression of allergen specific adaptive immune responses resulting in IL-13 production in the lung of ST2$^{-/-}$ mice equivalent to that of WT mice, and subsequent development of AHR.

It was hypothesised that because IL-33, in addition to acting as a secreted cytokine, has an intranuclear role as a transcription factor that ST2$^{-/-}$ mice would have an intermediate phenotype and that IL-33$^{-/-}$ mice would be protected from the development of AAD. However, this was not the case and the reverse was true; ST2$^{-/-}$ mice did not develop HDM induced AAD whereas IL-33$^{-/-}$ mice did. These data indicate that intranuclear IL-33 does not play a role in the onset and progression of AHR. However, as nuclear IL-33 has been linked to the up-regulation of ICAM-1 and VCAM-1 (Choi et al., 2012), this activity may be responsible for the elevated pulmonary and BALF inflammation observed in ST2$^{-/-}$ mice in the absence of traditional Th2 cytokines and AHR.

The data also suggest that ligands other than IL-33 signal through ST2 as loss of IL-33 does not result in the complete loss of HDM induced AAD caused by loss of ST2 signalling. The possibility of additional ligands with the ability to signal through the ST2 receptor has previously been suggested by Martin *et. al*. (2013) following their investigation of the contribution of IL-33/ST2 signalling in a model of experimental arthritis. This group determined that loss of IL-33 had no effect on the development of serum-transfer induced arthritis but the disease was greatly attenuated in a model using $ST2^{-/-}$ mice. However, due to the difference in background strain in the two knockout models the group were unable to conclude definitively that differences were due to additional ligands that might signal via ST2. During the current study both $IL\text{-}33^{-/-}$ and $ST2^{-/-}$ mice were bred on a Balb/c background that allowed the effect of strain difference to be excluded and therefore determined that ST2 has additional roles to IL-33 signalling. Alternatively, the disruption of the ST2 complex in $ST2^{-/-}$ mice may interfere with other innate signalling pathways dependent on the recruitment of MYD88. Previous studies have demonstrated that ST2 plays a role in the activation of both TLR2 and TLR4, both of which are dependent on the adaptor protein MYD88 (Brint et al., 2004; Liu et al., 2010). The authors suggested the sequestration of adaptor proteins by ST2 decreased activation of the TLRs and in $ST2^{-/-}$ knockout mice they were more active. As the $ST2^{-/-}$ mice used in the current study were functional knockouts rather than complete knockouts the effect of MYD88 sequestration or MYD88 dysregulation would be an interesting future investigation as it represents an important difference between $IL\text{-}33^{-/-}$ and $ST2^{-/-}$ models.

Early experiments to identify the ligand for the orphan receptor ST2 determined that no known members of the IL-1 family bound to the receptor. Although two unidentified ligands were able to bind the receptor, neither resulted in any downstream signalling (Gayle et al., 1996; Kumar et al., 1995). The IL-1 family member IL-33 was eventually identified as the ligand for ST2 through computational analysis and further signalling analysis revealed IL-33 binding to ST2 resulted in the expression of Th2 cytokines via MAPK phosphorylation and NF-κβ induction (Schmitz et al., 2005). In the current study, $ST2^{-/-}$ mice were protected from the development of HDM-induced airway

resistance. ST2$^{-/-}$ mice exposed to three weeks of HDM also had no increase in serum IgE, pulmonary IL-4, IL-1α or IL-13. In contrast, both WT and IL-33$^{-/-}$ mice had increased levels of all of these parameters. This indicated that HDM did not lead to the generation of allergen specific immune cells in ST2$^{-/-}$ mice as neither total nor HDM specific IgE was generated, as well as an absence of classical Th2 cytokines. A recently published study demonstrated that *in vitro* IL-1α induced release of IL-33 from epithelial cells in response to HDM. Additionally, the receptor for IL-1α, IL-1R1 was shown to be essential for the development of HDM induced AAD in a murine *in vivo* model (Willart et al., 2012). However, in the current study utilising ST2$^{-/-}$ mice, HDM induced increases of IL-33 in the lung in the absence of elevated IL-1α, raising the possibility of an alternative route of IL-33 production. The involvement of mast cell activation was also investigated by assessing MCPT-1 secreted in the serum. Mast cells express high levels of surface ST2 meaning they can be activated both through the cross-linking of surface IgE and by IL-33 in the absence of IgE (Ho et al., 2007). ST2$^{-/-}$ mice did not have detectable levels of MCPT-1 in the serum after HDM exposure, implying that mast cells are not activated in the absence of ST2 receptor expression.

Despite the complete absence of AHR and classical Th2 cytokines in HDM exposed ST2$^{-/-}$ mice, lung inflammation was not significantly reduced compared to either WT or IL-33$^{-/-}$ mice, possibly due to the activity of intranuclear IL-33 in ST2$^{-/-}$ mice. However, H&E staining of lung sections and differential counts of BAL infiltrates revealed substantial differences between the cytokine and receptor knockout models. Lung sections from HDM exposed WT and IL-33$^{-/-}$ mice showed substantial peri-bronchial and peri-vascular inflammation, while ST2$^{-/-}$ mice had inflammatory cells that were dispersed throughout the parenchyma and therefore may be attracted to the lung by alternative routes. BAL infiltrates in both WT and IL-33$^{-/-}$ mice were composed predominantly of eosinophils, whereas neutrophils were the predominant cell type in the BALF of ST2$^{-/-}$ mice. Neutrophilic infiltration in asthmatic lungs has been associated with a more severe and often steroid resistant disease phenotype (Fahy, 2009). Eosinophils, but not neutrophils, have surface expression of ST2 (Cherry et al., 2008) and neutrophils may therefore play a role in non-ST2 mediated disease,

although HDM may not provide adequate stimulation to activate these cells. It has been demonstrated that only neutrophils isolated from patients with allergen specific IgE release inflammatory mediators, such as elastase, in response to *in vitro* allergen stimulation (Monteseir et al., 2003). Therefore, in this study, neutrophil infiltration in the absence of HDM specific IgE could explain the lack of disease despite elevated neutrophil numbers.

Despite an equal number of cells being recovered from the lungs of HDM exposed WT and ST2$^{-/-}$ mice, IL-13^{+} ILCs and Th2 cell numbers were substantially reduced in ST2$^{-/-}$ mice compared to WT mice. Interestingly, IL-33$^{-/-}$ mice also had reduced IL-13^{+} ILCs, which was represented the only difference between WT and IL-33$^{-/-}$ mice after three weeks of HDM exposure. This is likely because of the role of IL-33 in directly recruiting ILCs to the lung and in their local proliferation. Studies investigating the effect of targeting the IL-33/ST2 pathway in HDM induced AAD in a neonatal ST2$^{-/-}$ mouse model have documented similar results of absent AHR (Saglani et al., 2013). One study has utilised IL-33$^{-/-}$ mice in a model of HDM induced AAD and documented reduced eosinophils and IgE compared to WT control (Llop-Guevara et al., 2014b). However, they did not report effects on lung function, the key parameter of clinical interest. The results relating to sensitisation and inflammation differed from this study and may be due to the time-point of analysis as the study by Llop-Guevara *et. al*. was carried out at 2 weeks while here, outputs were analysed after three weeks of allergen exposure, when AAD is known to be established (Gregory and Lloyd, 2011). The current study has identified a delayed cell inflammatory phenotype after a week of Alt exposure in IL-33$^{-/-}$ mice that may be responsible for the differences between results observed at two weeks compared to three weeks.

In contrast to the non-responsiveness of ST2$^{-/-}$ mice to HDM, exposure to Alt resulted in similar AHR in ST2$^{-/-}$, IL-33$^{-/-}$ and WT exposed mice after three weeks. BAL and lung inflammation were also elevated in response to Alt in ST2$^{-/-}$ mice and in contrast to HDM exposure, IL-33$^{-/-}$ mice that were exposed to Alt had reduced pulmonary inflammation compared to WT mice. Flow cytometric

analysis of lung cells showed a reduction in eosinophils in ST2$^{-/-}$ mice compared to both WT and IL-33$^{-/-}$ mice while neutrophils were raised to the same level in both WT and ST2$^{-/-}$ mice and reduced in IL-33$^{-/-}$. Unlike HDM exposure, inflammatory infiltrates were clustered around the airways and vessels in ST2$^{-/-}$ mice. As with HDM, IL-4, IL-5 and IgE were reduced in ST2$^{-/-}$ mice although the generation of Alt specific IgE was unaffected by the lower level of IL-4. Increased IgE in the absence of ST2 has been described in a model of intestinal nematode infection (Hoshino et al., 1999) although other Th2 parameters, such as IL-4, were unaffected. Interestingly, a study that compared a model of OVA exposure with and without adjuvant sensitisation found IgE was only attenuated in ST2$^{-/-}$ mice in the absence of prior adjuvant treatment (Pitman et al. 2009). As Alt is known to have intrinsic serine protease activity which has been shown to act as an adjuvant in models of allergic airways disease (Porter et al., 2011; Snelgrove et al., 2014), this could provide a mechanistic reason for Alt generating AAD in the absence of ST2 and could be investigated through the utilisation of a serine protease inhibitor in a protocol of Alt induced AAD in ST2$^{-/-}$ mice.

As with HDM, Alt exposure caused similar levels of IL-33 production in the lung of WT and ST2$^{-/-}$ mice. However, unlike HDM, IL-13, MCPT-1 and IL-1α levels were raised from PBS control in ST2$^{-/-}$ mice exposed to Alt. Interestingly, numbers of IL-13^{+} ILCs and T cells were reduced in ST2$^{-/-}$ mice exposed to Alt, in a similar manner to HDM exposure. Despite this reduction in IL-13^{+} cells, increased levels of lung and BAL IL-13 were present in ST2$^{-/-}$ mice following Alt exposure. Lung inflammatory cells from exposed mice cultured *ex vivo* with allergen revealed that significantly less IL-13 was secreted from ST2$^{-/-}$ cells, despite BAL IL-13 levels being comparable between IL-33$^{-/-}$ and ST2$^{-/-}$ mice. Investigation of the contribution of other cell types – such as structural cells and other leukocytes - to the overall level of secreted IL-13 will therefore be an important future study. Analysis of the T cells actively proliferating and producing IL-13 in response to Alt showed this population is not affected by the loss of ST2, which together with the generation of similar levels of Alt specific IgE in the mice, implied that ST2 was not required for the generation of adaptive immune responses against Alt, however ST2 is required for adaptive immunity to develop in response to HDM. The

previous chapter revealed that Alt is a more potent allergen than HDM and therefore potentially overcomes the loss of ST2 by signalling through additional pathways.

Despite the lack of effect on adaptive B and T cells, the current study has shown that early immune responses are affected by the loss of both IL-33 and ST2. After a single exposure to Alt, WT mice had increased lung and BAL inflammation, including an increase in lung eosinophils, while loss of ST2 or IL-33 protected from this initial inflammatory infiltrate. However, after three exposures to Alt over a week, only $ST2^{-/-}$ mice had no increase in BAL inflammation or lung IL-13 above the PBS control and serum MCPT-1 significantly reduced from WT controls. This was accompanied by an absence of AHR in $ST2^{-/-}$ mice while AHR was present in both WT and $IL\text{-}33^{-/-}$ mice. These results from early responses to Alt suggest that if an alternative ligand is able to signal via ST2 in the absence of IL-33 it does not act as rapidly as IL-33 or alternatively, innate signalling pathways dependent on MYD88 may be altered in $ST2^{-/-}$ models and therefore initial innate stimuli will be absent.

Analysis of $IL\text{-}13^{+}$ ILCs after short term Alt exposure also revealed that if an alternative ligand does have the ability to signal via ST2, it does not have the same capacity as IL-33 to induce $IL\text{-}13^{+}$ ILCs, this was also evident after long term Alt and HDM exposure. To date, studies that have investigated the role of IL-33 in the induction of $IL\text{-}13^{+}$ ILCs in AAD have utilised $ST2^{-/-}$ mouse models and shown that IL-33/ST2 signalling is critical in the expansion of this cell population in a model of OVA induced AAD (Barlow et al., 2013). However, there are no reports directly comparing the specific roles of the ligand IL-33 and receptor ST2. As increased Th1 responses have been shown to occur in $ST2^{-/-}$ mice sensitised to OVA (Savinko et al., 2013), the level of IFN-γ was measured in response to short term Alt exposure to investigate the contribution of Th1 responses. However, this cytokine was higher in both WT and $IL\text{-}33^{-/-}$ mice after one week of Alt exposure, indicating that Th1 cells do not play a vital role in the generation of Alt specific immune responses in the absence of ST2; however, flow cytometry for Th1 cells will be required to confirm this.

Despite the reduced BAL IL-13 and absent lung tissue IL-13 after short term Alt exposure in $ST2^{-/-}$ mice, the population of T cells producing IL-13 and those actively proliferating – as determined by Ki67 expression - in response to Alt stimulation *in vitro* was increased in $ST2^{-/-}$ mice compared to WT and $IL\text{-}33^{-/-}$ mice, revealing a difference between the intracellular generation and the ability to secrete IL-13 in $ST2^{-/-}$ mice. An interesting further experiment would be the exclusion of PMA/Ionomycin stimulation after culture *in vitro* with Alt to determine the number of cells that produce IL-13 in response to allergen stimulation alone. Isolation and culture of individual cell types – including T cells and ILCs – from WT and knockout mice will also be of interest as we have hypothesised that the dramatic reduction of IL-13 secretion seen in $ST2^{-/-}$ mixed cell cultures may be due to the loss of IL-13 secretion from innate sources – such as ILCs and mast cells, however, the contribution from T cells alone may be comparable between genotypes. Analysis of the generation of antibodies over a course of Alt exposure revealed the loss of IL-33 or ST2 did not affect or delay the generation of total and Alt specific immunoglobulins. The generation of IgG2a in response to Alt in WT mice indicated a Th1 type response might have occurred in WT mice that was absent when the IL-33/ST2 pathway was disrupted. Further analysis of the involvement of Th1 cytokines and cells over the time-course of Alt sensitisation would be of interest to identify any further potential targets for the treatment of severe asthma. Analysis of the kinetics of cytokine generation in response to Alt exposure *in vivo* revealed a different pattern of cytokine generation in $ST2^{-/-}$ mice compared to WT. The patterns of pulmonary production of IL-13 and IL-1α suggested that production might be delayed in an $ST2^{-/-}$ model as levels continued to rise over the timecourse of Alt exposure whereas levels decreased in WT mice compared to exposure for one week. A longer course of exposure may therefore result in comparable levels in WT and $ST2^{-/-}$ mice. This will need to be considered in the development of future therapeutics against ST2 in models of severe asthma with fungal sensitisation as short term treatment may have initial beneficial results but could later be overcome by signalling of non-ST2 dependent pathways.

The final part of this study focused on the generation of an adeno-associated virus (AAV) containing IL-33 that could be used to mimic the increased expression of IL-33 observed in asthmatic lungs (Préfontaine et al., 2009, 2010; Saglani et al., 2013). Due to the emerging role of IL-33 in the homeostatic control of obesity and the anti-inflammatory control of atherosclerosis (Brestoff et al., 2014; Miller et al., 2008) the need for tissue specific investigation of IL-33 dysregulation is becoming more apparent. The importance of investigating individual tissue or cell types in the pathogenesis of disease has recently been highlighted through the study of eosinophils in inflammatory disease. The location of eosinophils within the body determines the function of the cell as eosinophils located within adipose tissue have homeostatic roles while those located within the airway submucosa are associated with inflammation and a severe asthma phenotype (Desai et al., 2013; Lloyd and Saglani, 2013; Wu et al., 2011). Global knockout of these cells, or mediators such as IL-5, could therefore have a detrimental effect on homeostatic regulation of inflammation as well as limiting asthma symptoms. Determining the tissue specific effects of IL-33 was therefore an important next step for this investigation. *In vitro* transfection of macrophages with the proposed IL-33 insert revealed that the full length protein was expressed and could be detected by a standard IL-33 ELISA.

Pulmonary IL-33 expression is known to differ between mice and humans as murine IL-33 is expressed in alveolar epithelial cells (Hardman et al., 2013) while human IL-33 expression is contained to bronchial epithelial cells (Préfontaine et al., 2010). The release of IL-33 from both cell types has been documented as HDM exposure of human bronchial epithelial cells (Llop-Guevara et al., 2014) and Alt exposure of murine alveolar epithelial cells (Snelgrove et al., 2014) results in IL-33 secretion. In order to determine the role of bronchial IL-33 to inhaled allergen and therefore mimic the response occurring in humans it was decided that IL-33 overexpression in bronchial epithelial cells would be the most appropriate murine model. A library of AAV serotypes encoding GFP was screened to determine the best serotype for infection of bronchial epithelial cells and AAV9 was identified as an appropriate serotype. In this study, administration of AAV9 via an intranasal route ensured only the lung was targeted for infection with bronchial epithelial cells showing the most

transfection. Future work will now focus on the effects of IL-33 overexpression in response to allergen exposure.

5.4.1 Conclusion

Overall, the experiments in this chapter showed differing roles of IL-33 and its receptor ST2 in the generation of AAD. This is likely due to either the presence of an alternative ligand with the ability to signal through ST2 or dysregulation of intracellular signalling proteins, such as MYD88. This study also revealed the generation of antigen specific IgE was unaffected by the loss of ST2 in response to Alt, but ST2 was critical for the generation IgE in response to HDM. Further investigation into the activation of pulmonary resident cells, such as dendritic cells, by both HDM and Alt is therefore an important factor for future studies, with a focus on the adjuvant effects of Alt on antigen uptake, presentation and trafficking to lymph nodes. This will determine the cells and mediators responsible for the generation of B and T cells in response to Alt in the absence of classical innate inflammation, such as the early influx of ILC2s, and will therefore provide further clues to the potency of Alt over HDM and potential targets for severe, therapy resistant disease.

This chapter has demonstrated the importance of ST2 in the generation of AAD in response to HDM, although this protection was not apparent with Alt following three weeks of exposure, despite reduced levels of a number of Th2 parameters in $ST2^{-/-}$ mice. This chapter, along with previous chapters have highlighted the association of lung IL-13 with AHR and the development of allergic disease. The next chapter will therefore focus on the potential therapeutic value of an α-ST2 and an α-IL-13 antibody in the prevention and treatment of AAD.

Chapter 6 –ST2 and IL-13 as potential therapeutic targets for Alternaria induced allergic airways disease

6.1 Introduction

The previous chapter highlighted that HDM induced AAD was dependent on ST2, but not IL-33. In addition, ST2 was important for disease inception of Alt induced AAD. Concurrent with the first two chapters, the importance of the association between elevated IL-13 levels in the lung and AHR was emphasised. Whether deficient IL-13 resulted naturally during development, by the use of steroids or in $ST2^{-/-}$ mice after HDM exposure, it was clear that AHR did not develop when pulmonary IL-13 levels were not elevated. The aim of this chapter was to further investigate these two key findings by using an α-ST2 antibody designed to neutralise the binding of IL-33 to ST2 and an α-IL-13 antibody that prevented the binding of IL-13 to both IL-13Rα1 and IL-13Rα2.

The majority of asthmatics have symptoms that can be managed using inhaled steroids and β_2 agonists. However, a small proportion have severe symptoms that do not respond to steroids, even in high doses, and have difficult to manage symptoms (Barnes and Adcock, 2009). A number of treatment options for severe asthmatics are currently available including anti-IgE antibody therapy such as Omalizumab (Schulman, 2001), non-steroidal immunosuppressives including sodium cromoglycate and allergen specific immunotherapy. In addition, targeting of IL-13 in mouse models (Tomlinson et al., 2010) and human trials (Brightling et al., 2014; Corren et al., 2010, 2011; Wenzel et al., 2013) as well as ST2 in the mouse (Kearley et al., 2009; Lee et al., 2014; Yin et al., 2012) have highlighted these targets as alternative treatments for asthmatic disease.

To date, therapeutic targeting of ST2 has not been carried out in humans, although mouse models of α-ST2 or sST2 treatment indicate such treatments may be beneficial. Our lab previously published that signalling through ST2 was responsible for the symptoms during the resolution phase of OVA induced AAD, when disease had been established but exposure to allergen was ceased. Treatment of mice with α-ST2 antibody during the resolution of OVA induced AAD caused a significant reduction of airway resistance and inflammation one week after the final OVA exposure (Kearley et al., 2009). As this antibody is no longer available we aimed to test a similar antibody generated by Janssen

Pharmaceuticals, Inc. (Fursov et al., 2011) initially in a model of OVA induced AAD to compare with and confirm the published results and subsequently in a short term model of Alt exposure to identify the therapeutic potential in a model of severe asthma with fungal sensitisation. These experiments were conducted in collaboration with Janssen Phamaceuticals inc. following a pre-determined protocol developed by the company to test the effectiveness of the antibody.

IL-13 has been long regarded as a key component in driving airway hyper-responsiveness associated with allergic asthma (Wills-Karp et al., 1998). This has led to a number of studies investigating its potential as a therapy for asthma (Corren et al., 2010; Tomlinson et al., 2010; Wenzel et al., 2013). Both the cytokine and the receptor IL-4Rα (a receptor shared between IL-13 and IL-4) have been targeted as therapeutics with varying outcomes. Corren *et. al* (2010) utilised a blocking antibody against IL-4Rα and demonstrated no differences between placebo and antibody treated groups, however an improvement in overall symptom scores in a subset of patients treated with the antibody who had initial higher baseline scores was documented. Wenzel *et. al*. (2013) also used an antibody against IL-4Rα in a trial of patients with asthma characterised by high sputum eosinophil levels despite medium-high doses of inhaled glucocorticoids. This study demonstrated a significant improvement in lung function parameters, reduced asthma exacerbations and reduced markers of Th2 inflammation.

Two recent clinical trials of α-IL-13 antibodies have focussed on targeting the cytokine in patients with severe asthma (De Boever et al., 2014; Brightling et al., 2014). De Boever *et. al* (2014) utilised a monoclonal α-IL-13 antibody that specifically targeted and neutralised the cytokine. Initial trials were undertaken in a population of mild asthmatics to determine the safety and an appropriate dose (Hodsman et al., 2013). This showed an improvement in some parameters of inflammation in mild to moderate asthmatics. However, subsequently a randomised trial over three months in severe asthmatics with steroid resistant disease did not show significant improvement in scores from an Asthma Control Questionnaire or in FEV_1. The authors suggested a number of possibilities

for the lack of effect including an inappropriate dosing regimen, that high dose steroids had reduced the IL-13 component of disease, possible immunogenicity – as some patient samples were positive for anti-drug antibodies - and the fact that previous antibodies against the receptor also targeted IL-4 signalling. Tralokinumab is another α-IL-13 antibody that has been recently trailed in a group of severe asthmatics. This trial included a long term assessment of asthma exacerbations over a year and identified a subset of patients with reduced exacerbations. These patients were classified as having high serum levels of DP44 or periostin at the initiation of the trial – both are mediators regulated by IL-13 (Takayama et al., 2006; Yong Zhang et al., 2014). The results from clinical trials may differ from the expected outcome from mechanistic mouse studies as the majority of murine studies have utilised IL-13 or IL-13R knockout mice or preventative α-IL-13 Ab blocking before the onset of AAD (Tomlinson et al., 2010; Wills-Karp et al., 1998b) and therapeutic treatment with α-IL-13 Ab may not be as effective. The α-IL-13 antibody used in the experiments presented in this chapter (UCB CA154_582 mAb A) was generated by UCB Celltech to directly interact with IL-13 and prevent binding to both IL-13Rα1 and IL-13Rα2 (Berry et al., 2009). This will therefore allow the involvement of IL-13, in the absence of IL-4 contribution or steroid intervention, to be examined in a model of Alt induced AAD.

6.1.1 Hypothesis

Use of an α-ST2 antibody will result in the ablation of OVA induced AAD and can be used as a therapeutic in HDM and Alt induced AAD models.

Blocking IL-13 will not prevent the onset of Alt induced AAD in a similar manner to HDM exposure.

6.1.2 Aims

1. To test a new α-ST2 antibody for potential therapeutic use in a murine model of OVA induced AAD.
2. To determine if α-ST2 treatment resulted in reduced inflammation in response to Alt exposure that was comparable to $ST2^{-/-}$ mouse models.
3. To ascertain the effect of preventative and therapeutic blocking of IL-13 during Alt exposure.

6.2 Methods

In protocol A, 6-8 week old Balb/c females were injected i.p with OVA and alum or PBS and alum at day 0 and 12. Mice were then exposed to 50mg/ml aerosolised OVA for 20 minutes per day for 6 days. As a positive control, 4 OVA exposed mice were assessed for resistance and inflammation 24 hours post last OVA exposure. Mice were injected i.p on days 25, 27 and 29 with PBS, 2mg/kg α-ST2, 10mg/kg α-ST2 or 10mg/kg Isotype control. AHR was assessed 24 hours after the last antibody injection.

In protocol B, 6-8 week old Balb/c females were injected i.p with OVA and alum or PBS and alum at day 0 and 12. Mice were then exposed to 50mg/ml aerosolised OVA for 20 minutes per day for 6 days. Mice were injected i.p on days 18, 20 and 22 with PBS, 2mg/kg α-ST2, 10mg/kg α-ST2 antibody or 10mg/kg Isotype control (ISO). AHR was assessed 24 hours post last OVA exposure.

In protocol C, 6-8 week old Balb/c females were injected i.p with 10mg/kg α-ST2 or isotype control on day 1 and 2. These mice and an $ST2^{-/-}$ control group were exposed intranasally to 25µg Alt using recovery anaesthesia with inhaled isoflurane. Mice were culled 12 hours post Alt exposure. This timepoint was suggested by Janssen Pharmaceuticals, Inc. as suitable for assessment of peak antibody activity.

In protocol D, 6-8 week old Balb/c females were injected sub cutaneously (sub-cut) with 2mg/kg α-ST2 or isotype control on day 1 to investigate if an alternative dosing route altered the effect of the antibody. These mice and an $ST2^{-/-}$ control group were exposed intranasally to 20µg Alt using recovery anaesthesia with inhaled isoflurane. Mice were culled 12 hours after the last Alt exposure.

In protocol E, 6-8 week old Balb/c females were dosed intranasally with 10µg Alt three times per week for three weeks using recovery anaesthesia with inhaled isoflurane. Throughout allergen exposure, mice were dosed i.p with 10mg/kg αIL-13 antibody, 2mg/kg αIL-13 antibody or isotype control twice weekly. Mice were culled 18 hours after the last Alt dose.

In protocol F, mice were exposed to Alt as in protocol E. After three weeks of allergen exposure, mice were dosed i.p with 10mg/kg αIL-13 antibody, 2mg/kg αIL-13 antibody or isotype control twice weekly. Mice were culled 18 hours after the last dose of Alt.

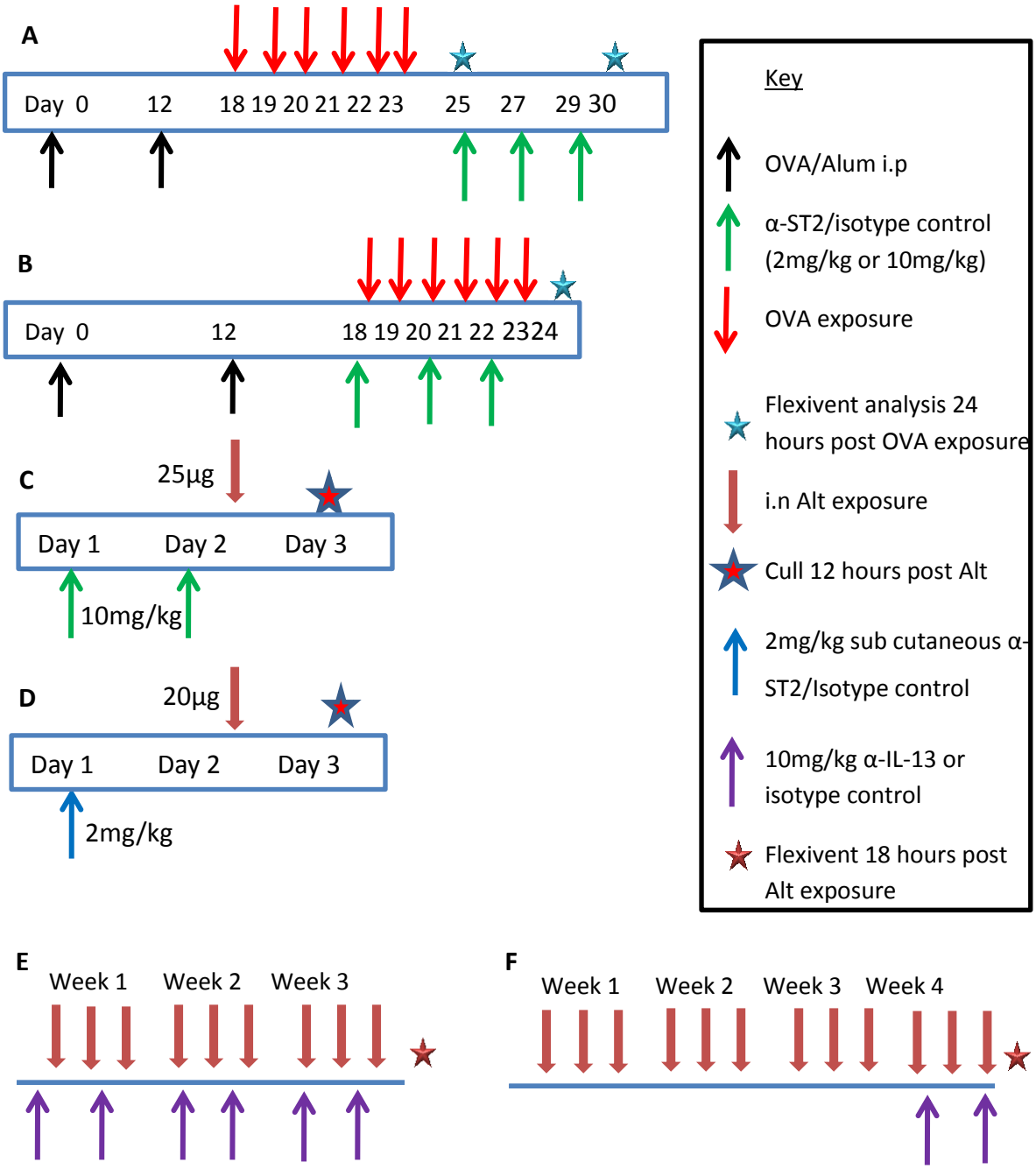

Figure 6.1 Chapter 6 methods

All mice were 6-8 week old females. Mice were injected i.p with OVA/Alum or PBS/Alum then injected i.p with α-ST2 or isotype three times during 6 days of OVA inhalation **(A)**. Mice were sensitised and exposed to OVA before injection of i.p α-ST2 **(B)**. WT mice were injected i.p twice before WT and $ST2^{-/-}$ were exposed once to ALT **(C)**. WT mice were injected subcutaneously before both WT and $ST2^{-/-}$ mice were exposed once to ALT **(D)**. Mice were exposed to Alt three times per week for three weeks and injected with α-IL-13 throughout **(E)**. Mice were exposed to Alt three times per week for 4 weeks and injected with α-IL-13 twice in the final week **(F)**.

6.3.1 anti-ST2 Ab treatment administered after OVA exposure did not affect AHR

To assess the effect of α-ST2 Ab treatment as a therapeutic treatment for OVA induced AAD a published protocol was replicated in which another α-ST2 Ab, when administered after OVA challenge, resulted in enhanced resolution of OVA induced AAD including a significant reduction in airway resistance (Kearley et al., 2009). Therapeutic α-ST2 Ab was administered for one week after OVA exposure (Figure 6.2 A). To assess if the isotype control caused any difference to the AHR or lung inflammation a PBS injected control was compared to the ISO injected control, as no difference was seen, data was not included.

AHR was assessed after the OVA exposure period to determine that AHR had been induced in response to OVA sensitisation and challenge. Resistance, compliance and inflammatory counts were increased from the PBS controls (Fig 6.2 B-E). α-ST2 Ab treatment throughout the resolution period did not reduce airway resistance in comparison to the ISO control at 100mg/ml MCh (Fig 6.3 A, B). BAL and lung inflammatory counts were also unaltered between treatment groups in OVA/Alum treated mice (Fig 6.3 C, D). Levels of total and OVA specific IgE were unaffected by the α-ST2 Ab (Fig 6.3 E, F).

Lungs were removed and sent to Janssen Pharmaceuticals, Inc. for analysis of Th2 cytokines by Luminex. IL-1α, IL-4 and IL-5 all remained elevated above the PBS/Alum injected control with no difference between antibody treatment groups (Fig 6.4 A-C). Interestingly, despite elevated airway resistance in OVA sensitised mice, no IL-13 was detectable above the PBS/Alum control (Fig 6.4 D).

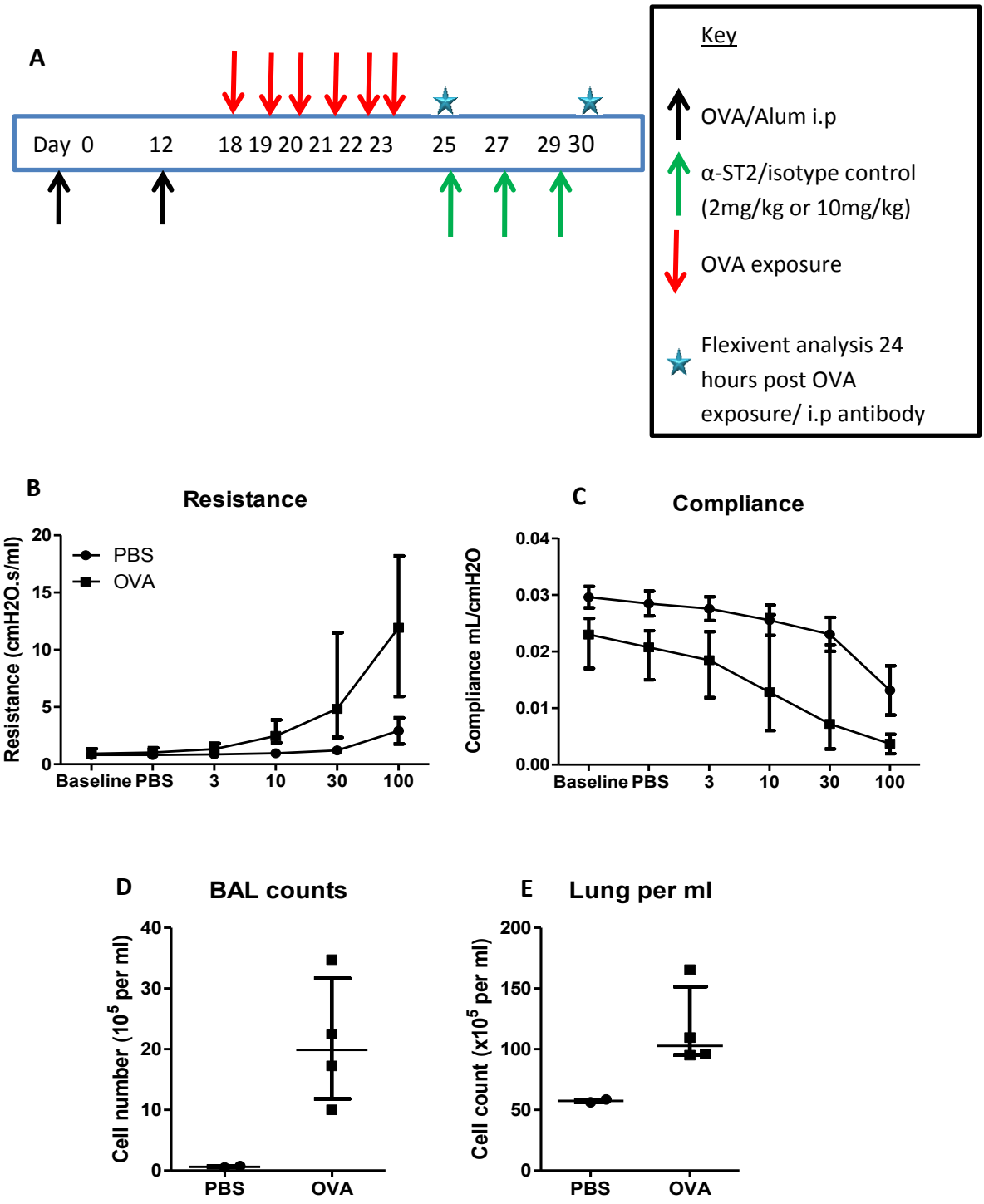

Figure 6.2 Positive control for OVA sensitisation.

Mice injected with PBS/Alum or OVA/Alum and exposed to aerosolised OVA **(A)**. Airway resistance over increasing concentrations of methacholine **(B)**. Compliance over increasing concentrations of methacholine **(C).** BAL **(D)** and lung **(E)** cell counts. Black circles represent mice sensitised with PBS OVA, black squares were dosed i.p with PBS. n= 2-4 mice per group. Data displayed as median and interquartile range**.**

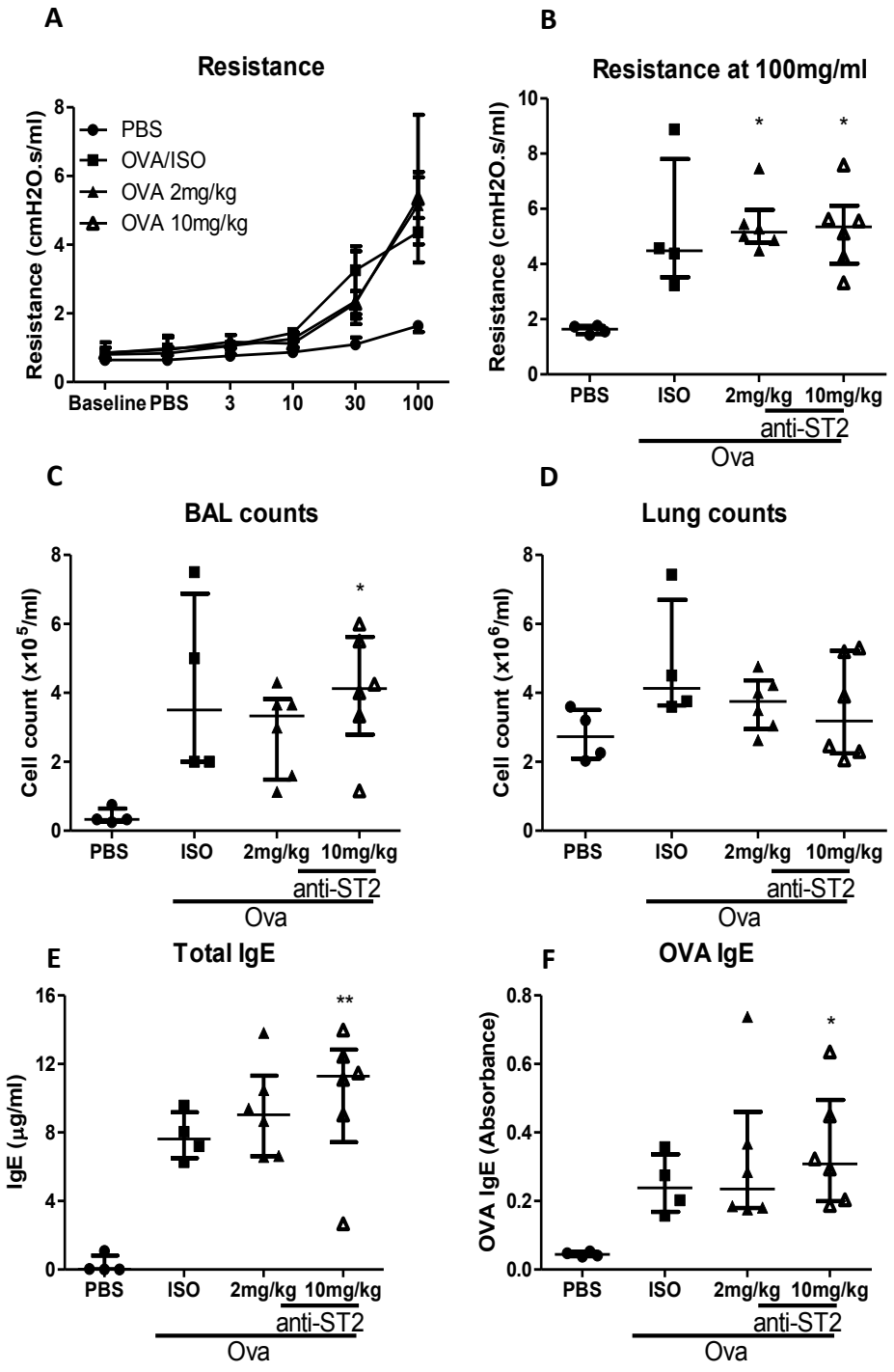

Figure 6.3 α-ST2 after OVA exposure does not affect airway resistance

Mice injected with PBS/Alum or OVA/Alum and exposed to aerosolised OVA before treatment with α-ST2, PBS or isotype. Airway resistance over increasing concentrations of methacholine **(A)**. Airway resistance at 100mg/ml methacholine **(B)**, BAL **(C)** and lung **(D)** cell counts. Total serum IgE **(E)** and Alt specific IgE **(F)**. Black circles represent mice sensitised with PBS OVA, black squares were dosed with 10mg/kg isotype control, triangles with 2mg/kg α-ST2 and hollow triangles with 10mg/kg α-ST2. n= 4-6 mice per group. * $p<0.05$ and * $p<0.01$ from PBS control as assessed by Kruskal-Wallis with Dunns post-test. Data displayed as median and interquartile range.

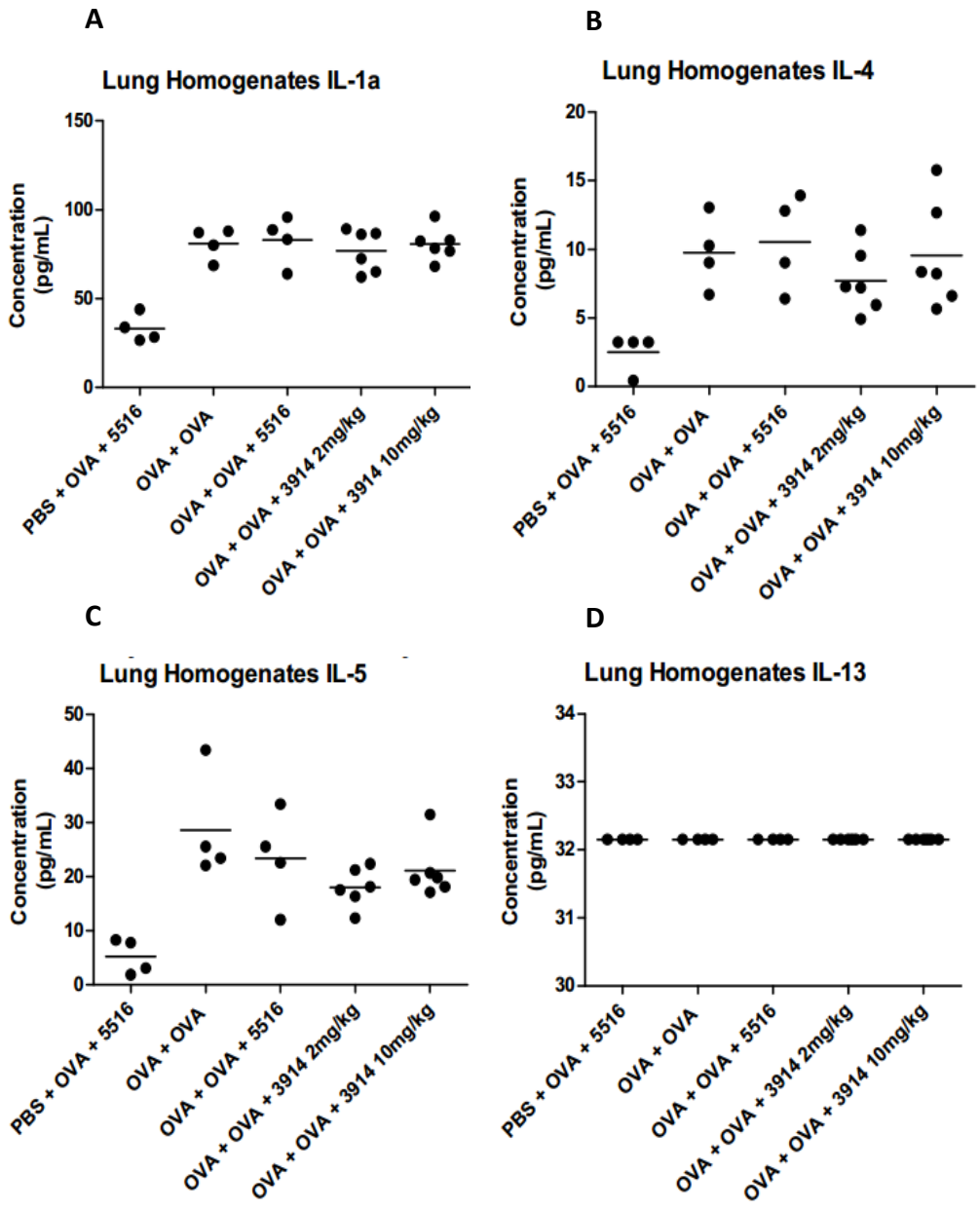

Figure 6.4 Th2 cytokines are not affected by α-ST2 treatment after OVA exposure

Mice sensitised with PBS or OVA and exposed to aerosolised OVA with or without α-ST2 treatment. Lungs were collected and in collaboration with J and J, Luminex analysis was performed. IL-1α **(A)**, IL-4 **(B)**, IL-5 **(C)** and IL-13 **(D)** as pg/ml lung homogenate. Data displayed as mean. 5516 is the isotype control, 3914 is α-ST2.

6.3.2 α-ST2 Ab reduced ST2 expression on ILCs, while IL-13$^+$ ILCs were unaffected

Lung and BAL cells were analysed by flow cytometry to determine the populations remaining in the lung a week after final OVA exposure and to determine whether α-ST2 Ab treatment had any effect on the recruitment or clearance of cells. ILCs were significantly increased in the lungs of OVA/Alum injected mice in comparison to PBS/Alum controls. Treatment with α-ST2 Ab during the resolution phase of disease did not significantly reduced the number of total ILCs in the lung (Fig 6.5 A). IL-13$^+$ ILC populations were equally induced in all mice exposed to OVA irrespective of antibody treatment (Fig 6.5 B). Even though IL-13$^+$ ILCs were unaffected, α-ST2 Ab treatment caused a significant down-regulation of ST2$^+$ ILCs and surface expression of ST2 (Fig 6.5 C, D).

As with IL-13$^+$ ILCs, IL-13$^+$ T cells in the lung were not significantly reduced by treatment with α-ST2 Ab and no change to T cell trafficking to the BAL result from the blocking of ST2 (Fig 6.6 A, B). In contrast to ILCs, OVA sensitisation did not cause a significant increase to the population of ST2$^+$ T cells in the lung although α-ST2 Ab treatment reduced the population to the same as the PBS control (Fig 6.6 C, D).

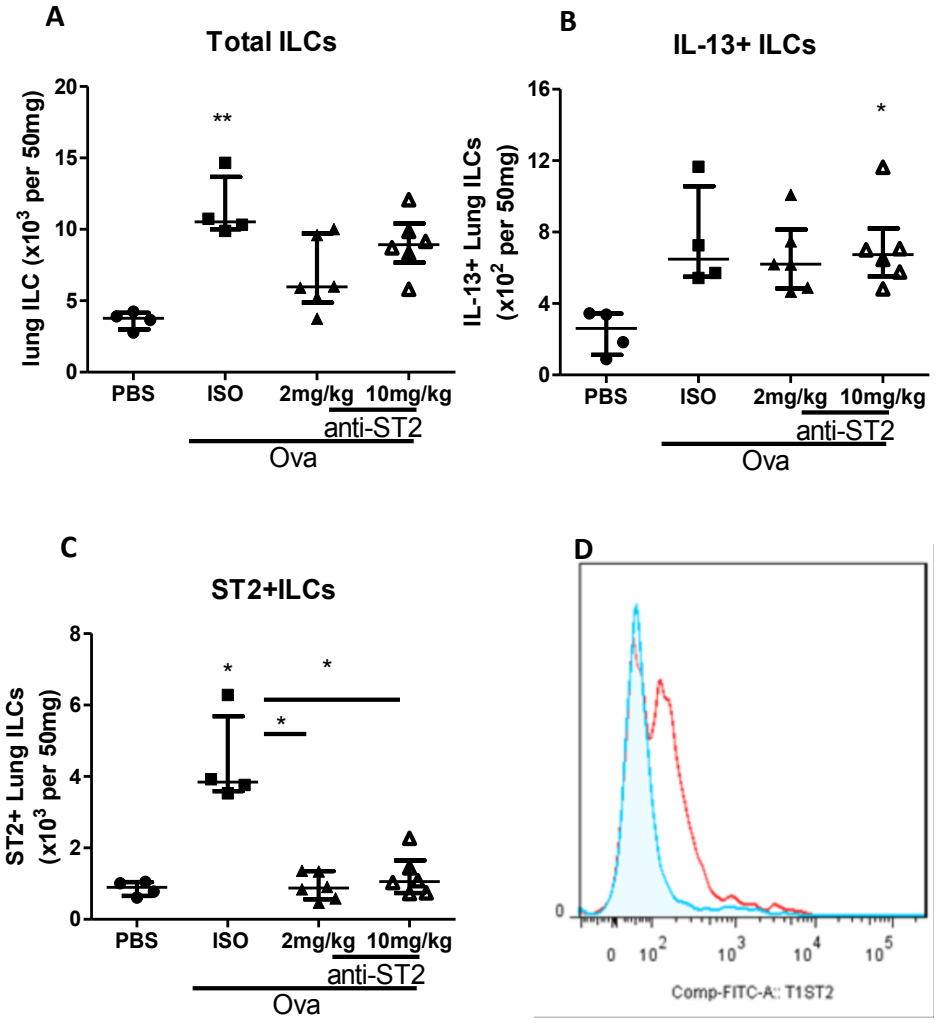

Figure 6.5 α-ST2 reduced the expression of ST2 on ILCs, IL-13 production was unaffected

Mice sensitised with PBS or OVA and exposed to aerosolised OVA before treatment with α-ST2, PBS or isotype. Lung cells were isolated and prepared for flow cytometric analysis. Total ILCs **(A)**, IL-13+ ILCs **(B)**, ST2+ ILCs **(C)** and a representative histogram of ST2 expression on ILCs – Iso is shown in red and 10mg/kg α-ST2 in blue **(D)**. Black circles represent mice sensitised with PBS OVA, black squares were dosed with 10mg/kg isotype control, triangles with 2mg/kg α-ST2 and hollow triangles with 10mg/kg α-ST2. n= 4-6 mice per group. * $p<0.05$ and ** $p<0.01$ from PBS control or as indicated by bars as assessed by Kruskal-Wallis with Dunns post-test. Data displayed as median and interquartile range.

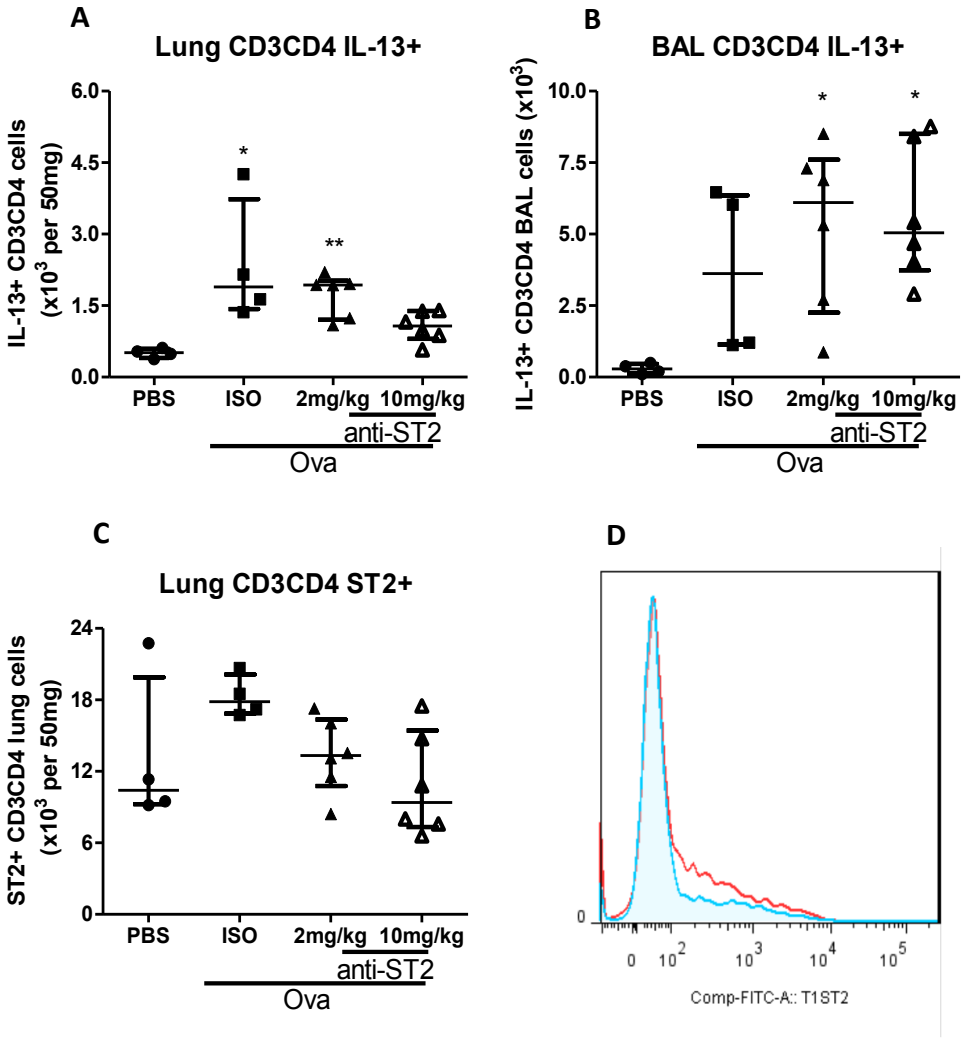

Figure 6.6 α-ST2 reduces the expression of ST2 on T cells

Mice injected with PBS/Alum or OVA/alum and exposed to aerosolised OVA before treatment with α-ST2, PBS or isotype. Lung and BAL cells were isolated and prepared for flow cytometric analysis. IL-13+ T cells in the lung **(A)** and BAL **(B)**. ST2+ T cells **(C)** and a representative histogram of ST2 expression on lung T cells ILCs – Iso is shown in red and 10mg/kg α-ST2 in blue **(D)**. Black circles represent mice sensitised with PBS OVA, black squares were dosed i.p with 10mg/kg isotype control, triangles with 2mg/kg α-ST2 and hollow triangles with 10mg/kg α-ST2. n= 4-6 mice per group. * $p<0.05$ and ** $p<0.01$ from PBS control as assessed by Kruskal-Wallis with Dunns post-test. Data displayed as median and interquartile range.

6.3.3 Preventative anti-ST2 Ab caused no reduction in AHR and no effect on inflammation

As therapeutic treatment of OVA induced AAD was unaffected by α-ST2 Ab treatment, a preventative dosing regimen was used to assess the effect of α-ST2 Ab dosing after sensitisation for the duration of the aerosolised OVA challenge phase (Fig 6.7 A). Injection with α-ST2 Ab resulted in a partial reduction in AHR at 100mg/ml MCh from 10 in ISO control mice to 6 in 10mg/kg α-ST2 Ab injected mice, although significance was not reached (Fig 6.7 B, C). Airway compliance was unaffected by α-ST2 Ab treatment (Fig 6.7 C, D).

BAL and lung inflammation were increased in response to OVA exposure and this was unaffected by α-ST2 Ab (Fig 6.8 A, B). Serum was analysed for both total and OVA specific IgE to determine sensitisation. All mice had been exposed to aerosolised OVA and only those pre-sensitised with OVA/Alum had elevated IgE. The level of IgE and OVA specific IgE was unaffected by α-ST2 Ab in comparison to ISO (Fig 6.8 C, D).

After assessing AHR, lungs were snap frozen and sent to Janssen Pharmaceuticals, Inc for analysis of cytokines by Luminex. Th2 cytokines analysed included IL-1α, IL-4, IL-5 and IL-13. All were increased in OVA/Alum injected mice in comparison to PBS/Alum control mice, but α-ST2 Ab treatment during OVA exposure did not reduce cytokine levels in comparison to ISO treated mice (Fig 6.9 A-D).

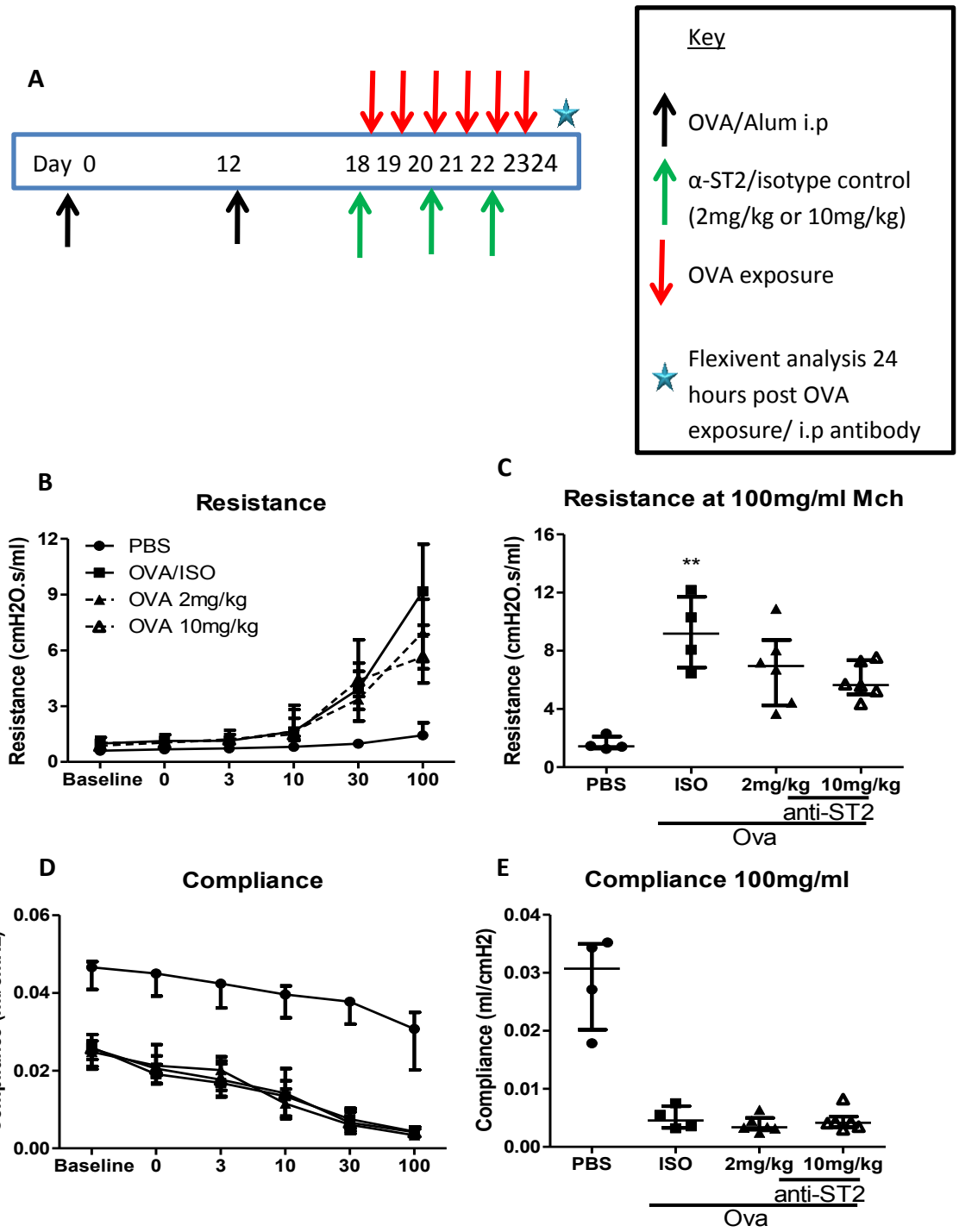

Figure 6.7 α-ST2 during OVA exposure partially down-regulates airway resistance.

Mice were sensitised with PBS or OVA and exposed to aerosolised OVA with or without α-ST2 treatment **(A)**. Airway resistance over increasing concentrations of methacholine **(B)**. Resistance at 100mg/ml Methacholine **(C)**. Compliance over increasing concentrations of methacholine **(D)**. Compliance at 100mg/ml methacholine **(E).** Black circles represent mice sensitised with PBS OVA, black squares were dosed i.p with 10mg/kg isotype control, triangles with 2mg/kg α-ST2 and hollow triangles with 10mg/kg α-ST2. n= 4-6 mice per group. ** $p<0.01$ from PBS control as assessed by Kruskal-Wallis with Dunns post-test. Data displayed as median and interquartile range.

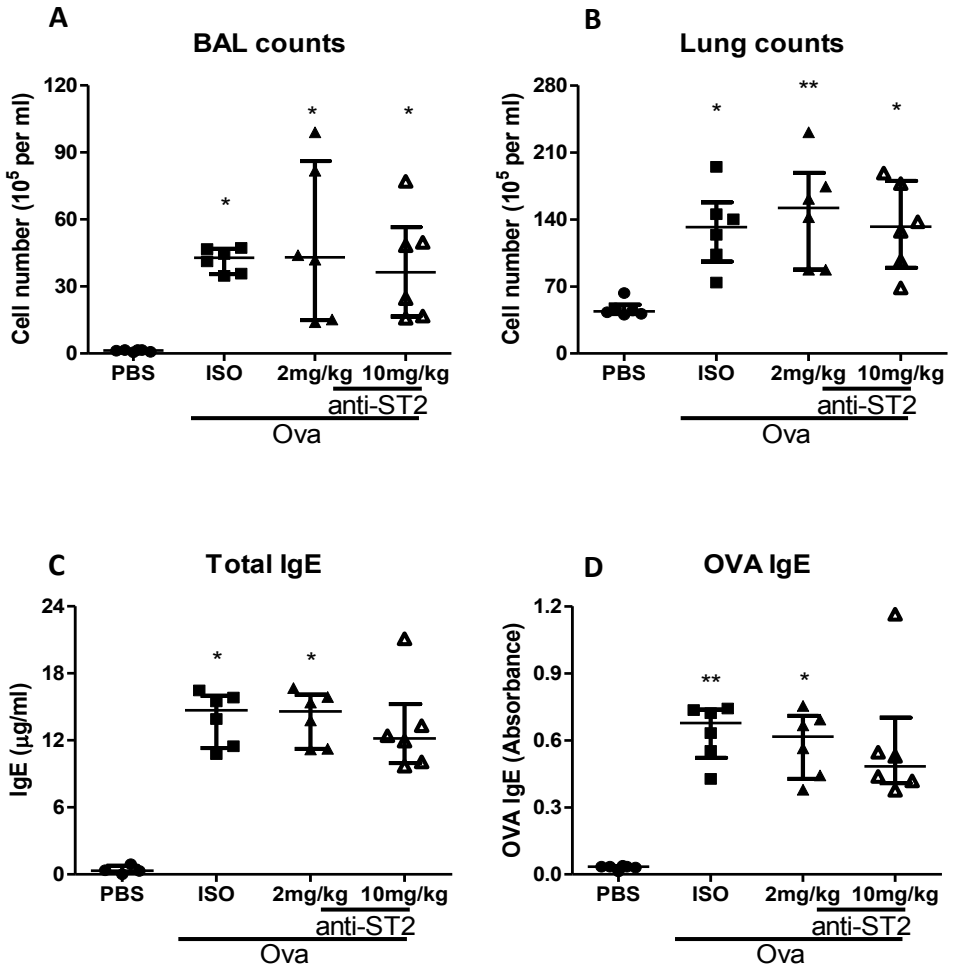

Figure 6.8 α-ST2 during OVA exposure does not affect inflammation or OVA induced IgE

Mice sensitised with PBS or OVA and exposed to aerosolised OVA with or without α-ST2 treatment. BAL **(A)** and lung **(B)** cell counts. Total serum IgE **(C)** and Alt specific IgE **(D)**. Black circles represent mice sensitised with PBS OVA, black squares were dosed i.p with 10mg/kg isotype control, triangles with 2mg/kg α-ST2 and hollow triangles with 10mg/kg α-ST2. n= 4-6 mice per group. * $p<0.05$ and ** $p<0.01$ from PBS control as assessed by Kruskal-Wallis with Dunns post-test. Data displayed as median and interquartile range.

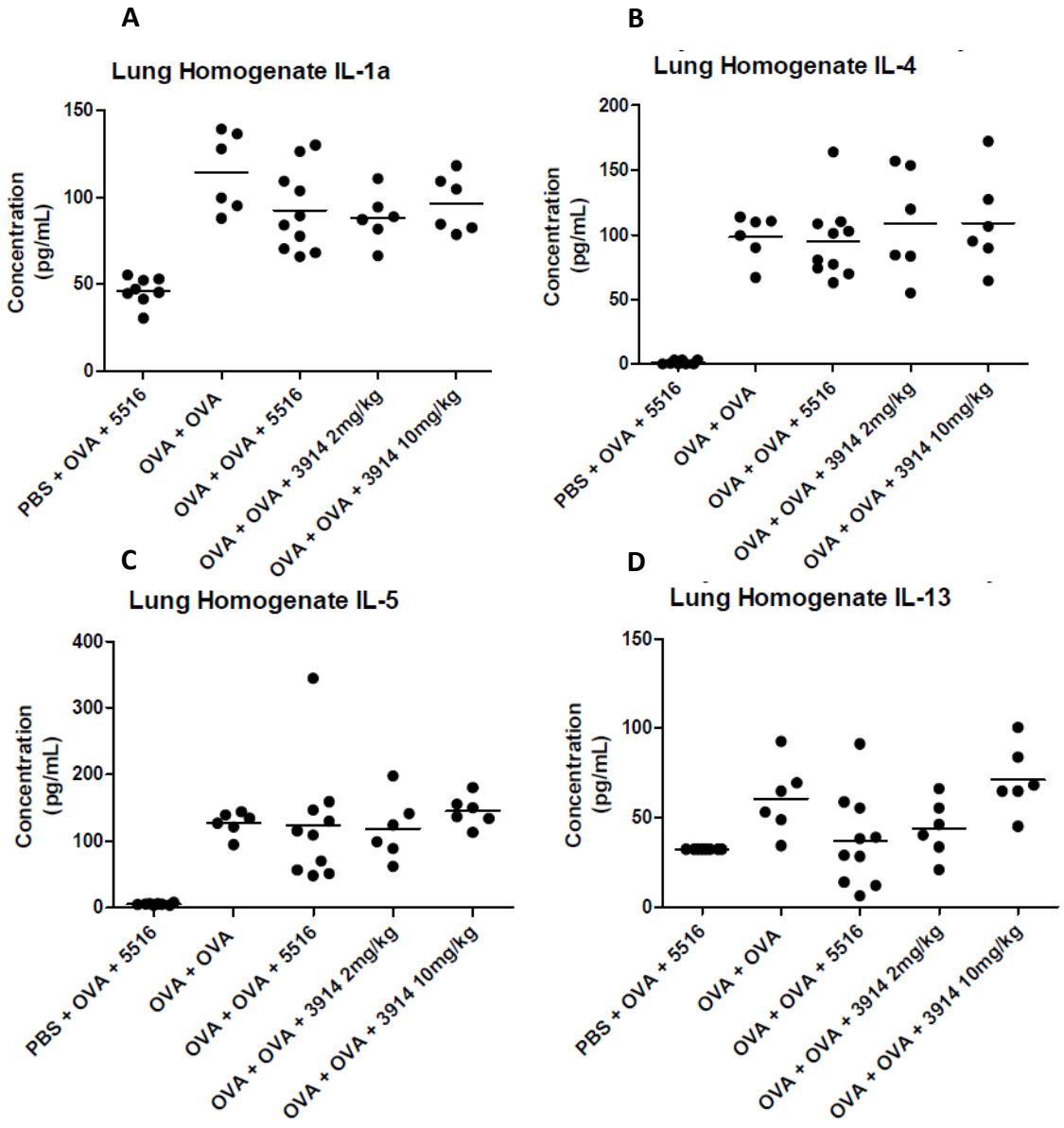

Figure 6.9 Th2 cytokines are not affected by α-ST2 treatment throughout OVA exposure

Mice sensitised with PBS or OVA and exposed to aerosolised OVA with or without α-ST2 treatment. Lungs were collected and in collaboration with Janssen Pharmaceuticals inc., Luminex analysis was performed. IL-1α **(A)**, IL-4 **(B)**, IL-5 **(C)** and IL-13 **(D)** as pg/ml lung homogenate. Data displayed as mean. 5516 is the isotype control, 3914 is α-ST2.

6.3.4 α-ST2 Ab administered in a prevention regimen does not affect Alt induced inflammation

As the previous chapter highlighted, $ST2^{-/-}$ mice had reduced lung inflammation after short term Alt exposure. To ascertain whether blocking ST2 with an Ab before Alt exposure using a prevention regimen had a similar effect, a short term Alt protocol with preventative α-ST2 treatment was carried out (Figure 6.10 A). Preventative treatment with α-ST2 Ab did not reduce BAL or lung inflammation after a single exposure to Alt while $ST2^{-/-}$ mice had significantly lower BAL inflammation in comparison to both ISO and α-ST2 treated mice (Fig 6.10 B, C). The number of neutrophils and eosinophils in the BAL were also investigated by flow cytometry. Neutrophils and eosinophils were significantly reduced from ISO control in $ST2^{-/-}$ mice while α-ST2 Ab caused no reduction (fig 6.10 D, E).

In order to determine whether the route of administration of the antibody affected the outcome of inflammation in response to Alt, it was injected subcutaneously (sub-cut) instead of intra-peritoneally (Fig 6.11 A). Preventative treatment with sub-cut α-ST2 did not reduce total BAL inflammation (6.11 B); however, there was a reduction in eosinophils after a single dose of Alt (Fig 6.11 C). Sub-cut α-ST2 Ab did not affect the population of lung IL-13^{+} ILCs in comparison to ISO, while $ST2^{-/-}$ mice had a reduction in IL-13^{+} ILCs (Fig 6.11 D). No significant reduction in the surface expression of ST2 on ILCs resulted from sub-cut α-ST2 Ab treatment (Fig 6.11 E-H).

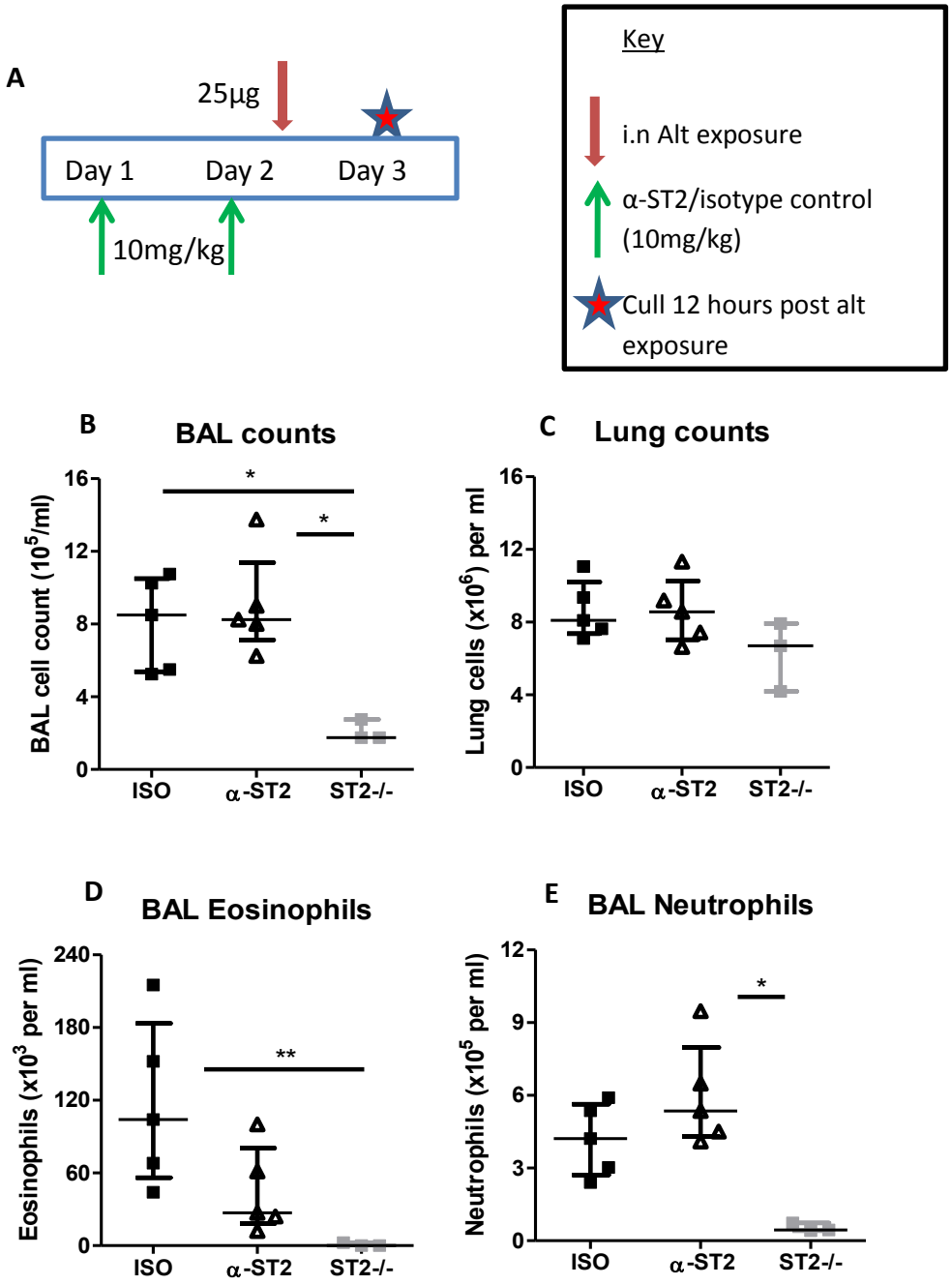

Figure 6.10 Preventative i.p α-ST2 with single dose Alt

Mice were dosed with i.p 10mg/kg α-ST2 or isotype control before a single dose of Alt **(A)**. BAL **(B)** and lung counts **(C)**. BAL was analysed by facs for neutrophils ($GR1^{high}CD11b^{+}$) **(D)** and eosinophils ($Siglecf^{+}CD11c^{-}$) **(E)**. Black squares were dosed i.p with ISO and hollow triangles with 10mg/kg α-ST2, grey squares represent $ST2^{-/-}$ mice. n= 3-5 mice per group. * $p<0.05$ and ** $p<0.01$ by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

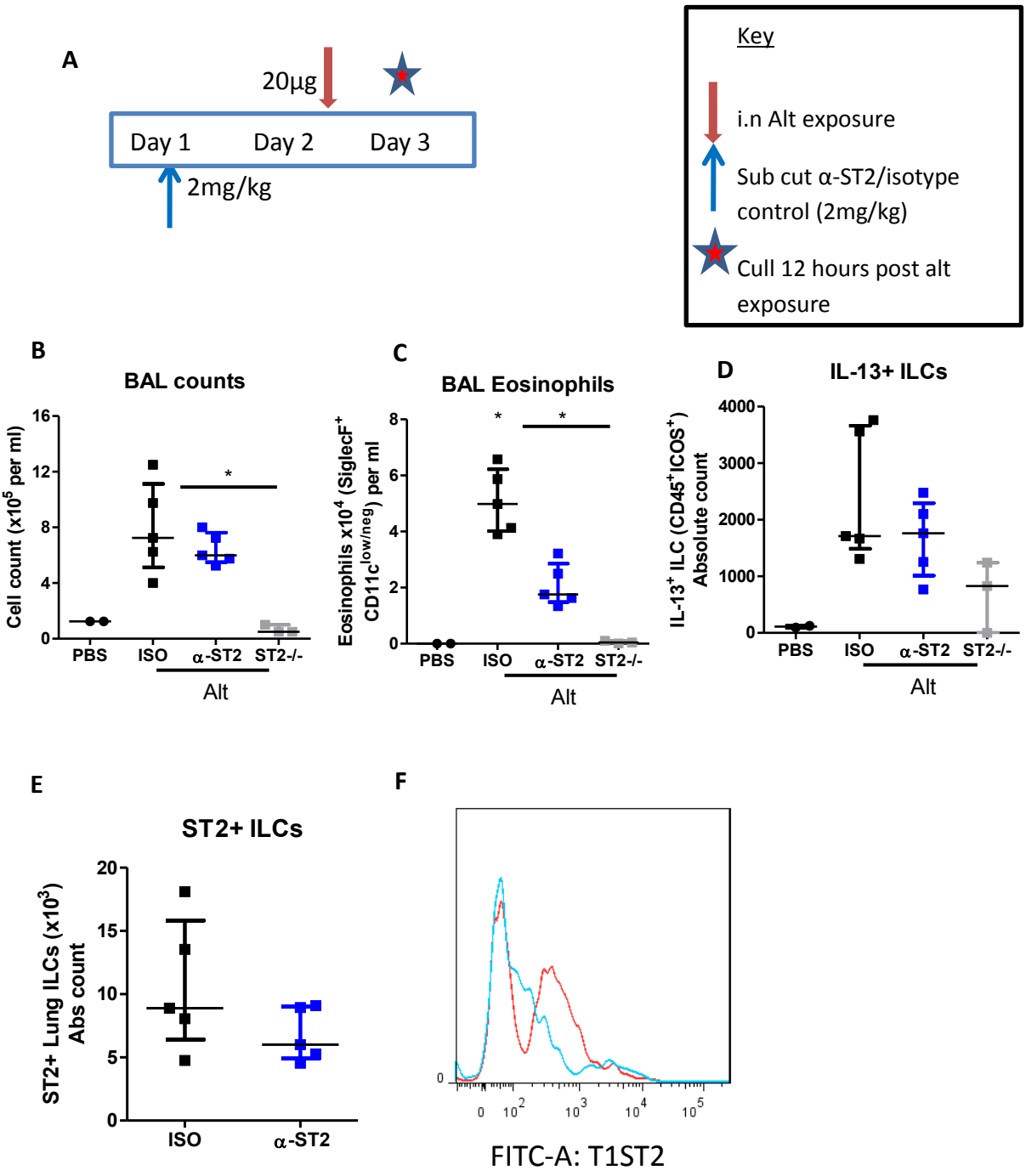

Figure 6.11 Preventative sub cutaneous α-ST2 with single dose Alt

Mice were dosed with sub cutaneous 2mg/kg α-ST2 or isotype control before a single dose of Alt **(A)**. BAL counts **(B)** BAL eosinophil counts **(C)**. Lung IL-13+ ILCs ($Lin^-CD45^+ICOS^+$) as analysed by flow cytometry **(D)**. $ST2^+$ ILCs **(E)** and a representative histogram ILCs – Iso is shown in red and 2mg/kg α-ST2 in blue **(F)**. Black squares were dosed i.p with ISO and blue squares with 2mg/kg α-ST2, grey squares represent $ST2^{-/-}$ mice. n= 3-5 mice per group. * $p<0.05$ by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

6.3.5 Alt induced AHR is IL-13 dependent

Our lab has previously published HDM induced AHR in adult mice is dependent on IL-13 (Tomlinson et al., 2010). Throughout the previous two chapters it was determined that Alt sensitisation resulted in a more severe disease phenotype characterised by higher IL-13 levels and steroid resistance and that Alt induced AAD was not dependent on IL-33 or ST2. To determine whether Alt sensitisation was dependent on IL-13 in a similar manner to HDM exposure, a neutralising antibody from UCB (UCB CA154_582) was administered throughout Alt exposure (Fig 6.12 A).

Treatment with αIL-13 Ab completely abolished Alt induced AHR. Airway resistance at 100mg/ml MCh was similar to PBS controls (fig 6.12 B, C). In contrast, although BAL and lung inflammation were reduced in αIL-13 Ab treated mice compared to Alt ISO dosed mice, inflammation remained elevated above PBS controls (Fig 6.12 D, E).

Cytokines in the lung and serum IgE were analysed by ELISA to elucidate the effects of blocking IL-13. Lung IL-13 levels were not increased above PBS level when αIL-13 Ab was administered during Alt exposure (Fig 6.13 A). IL-33 and IL-1α levels were reduced following αIL-13 Ab in Alt exposed mice compared to ISO controls although neither were reduced to the PBS control levels (Fig 6.13 B, C). Alt specific IgE was unaffected and similar to Alt/ISO mice, indicating mice still become sensitised to Alt in the absence of IL-13 (Fig 6.13 D)

Both lung and BAL cells were analysed by flow cytometry to establish the effect of blocking IL-13 on the composition of the inflammatory infiltrate. Eosinophils were reduced in αIL-13 treated mice in comparison to Alt/ISO mice, although the reduction was only partial (Fig 6.14 A). Despite the complete down regulation of IL-13 in the lung homogenate, the number of IL-13^{+} T cells in the lung was not altered by α-IL-13 treatment (Fig 6.14 B). In contrast to this, total ILCs and IL-13^{+} ILCs in the lung and BAL were substantially reduced by blocking IL-13 in comparison to ISO treated mice, although all populations were elevated from the PBS control (Fig 6.14 C-F).

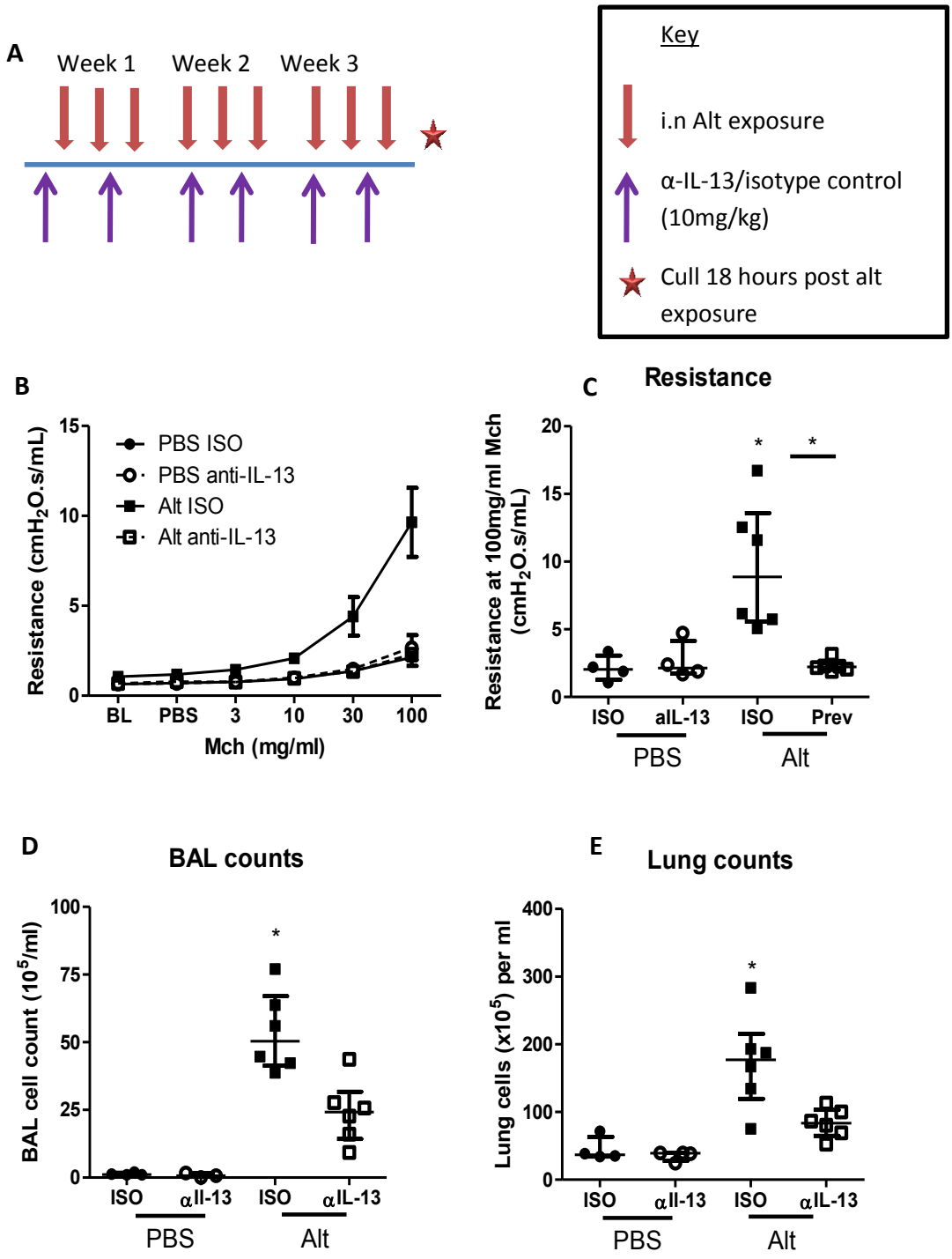

Figure 6.12 Airway resistance and inflammation in response to Alt and αIL-13

Adult mice were dosed i.p with αIL-13 or isotype control throughout Alt exposure before measure of airway resistance and collection of BAL and lung **(A)**. Airway resistance over increasing concentrations of methacholine **(B)** and resistance at 100mg/ml Methacholine **(C)**. BAL **(D)** and lung **(E)** inflammatory counts. n= 4-6 mice per group. * $p<0.05$ from relevant PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

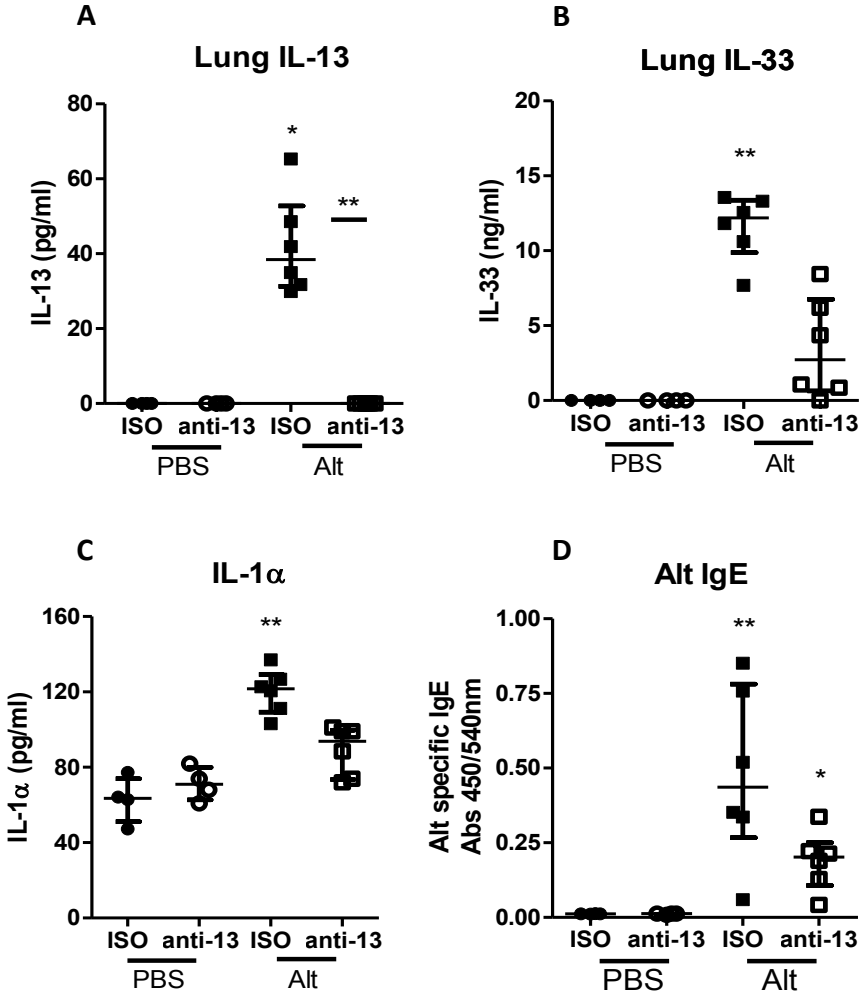

Figure 6.13 inflammatory cytokines and IgE in response to Alt and αIL-13

Adult mice were dosed i.p with αIL-13 or isotype control throughout Alt exposure before collection of blood and lung. Lung IL-13 **(A)**, IL-1α **(B)** and IL-33 levels after Alt exposure **(C)** as concentration per ml lung homogenate (50mg/ml). Serum Alt specific IgE as absorbance **(D)**. n= 4-6 mice per group. * $p<0.05$ and ** $p<0.01$ from relevant PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

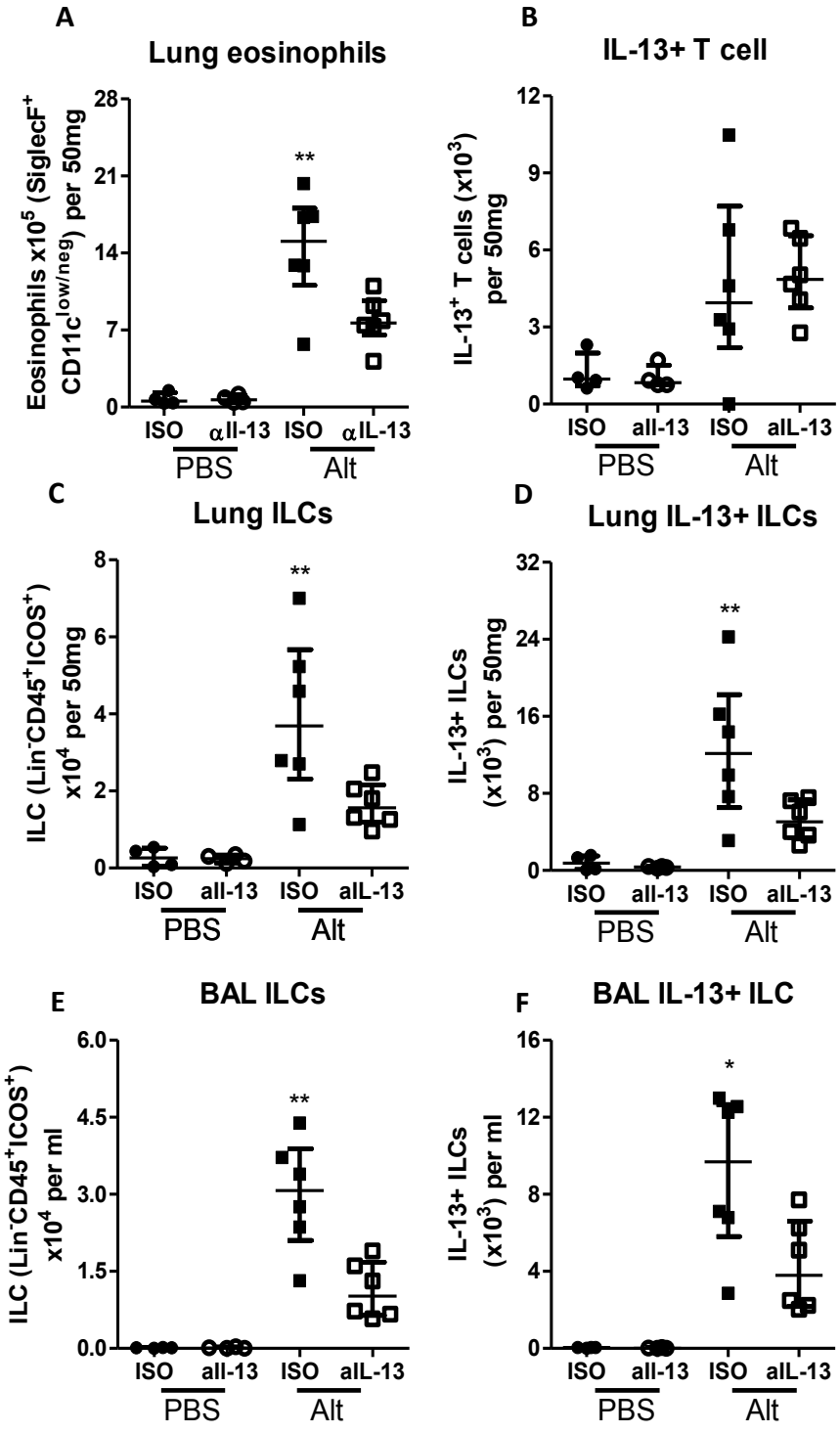

Figure 6.14 BAL and lung inflammatory cells in response to Alt and αIL-13

Adult mice were dosed i.p with αIL-13 or isotype control throughout Alt exposure before collection and preparation of BAL and lung cells for facs analysis. Eosinophils **(A)**, IL-13$^+$ T cells (CD3CD4$^+$) **(B)**, ILCs (lin$^-$CD45$^+$ICOS$^+$) **(C)** and IL-13$^+$ ILCs **(D)** per 50mg lung tissue. ILCs **(E)** and IL-13$^+$ ILCs **(F)** per ml BAL. n= 4-6 mice per group. * $p<0.05$ and ** $p<0.01$ from relevant PBS control or as indicated by a bar * $p<0.05$ and ** $p<0.01$ from relevant PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

6.3.6 α-IL-13 Ab did not affect AHR when administered in a therapeutic regimen, after AAD was established.

Although effective, administration of αIL-13 Ab in a preventative regimen would not be a practical to treat severe asthma in patients, as treatment would have to begin before the onset of disease. To determine if therapeutic treatment with αIL-13 Ab would reduce AHR, dosing was started after Alt induced AAD was established (Fig 6.15 A). In contrast to preventative dosing with αIL-13 Ab, therapeutic dosing did not reduce AHR. BAL and lung inflammatory counts were also not reduced from Alt ISO controls (Fig 6.15 B-E).

Unlike preventative α-IL-13, therapeutic dosing did not completely abolish lung IL-13, although it was reduced in comparison to ISO treated mice (6.16 A). Therapeutic α-IL-13 Ab did not cause a significant down-regulation of IL-1α, IL-33 or serum IgE (Fig 6.16 C, D).

Analysis of lung and BAL inflammatory infiltrates by flow cytometry showed therapeutic αIL-13 Ab dosing caused no reduction in the number of lung eosinophils, IL-13^+ T cells, ILCs or IL-13^+ ILCs compared to Alt ISO treated mice (Fig 6.17 A-D). Total ILCs and IL-13^+ ILCs in the BAL remained significantly elevated in therapeutic αIL-13 treated mice from PBS control (Fig 6.17 E, F).

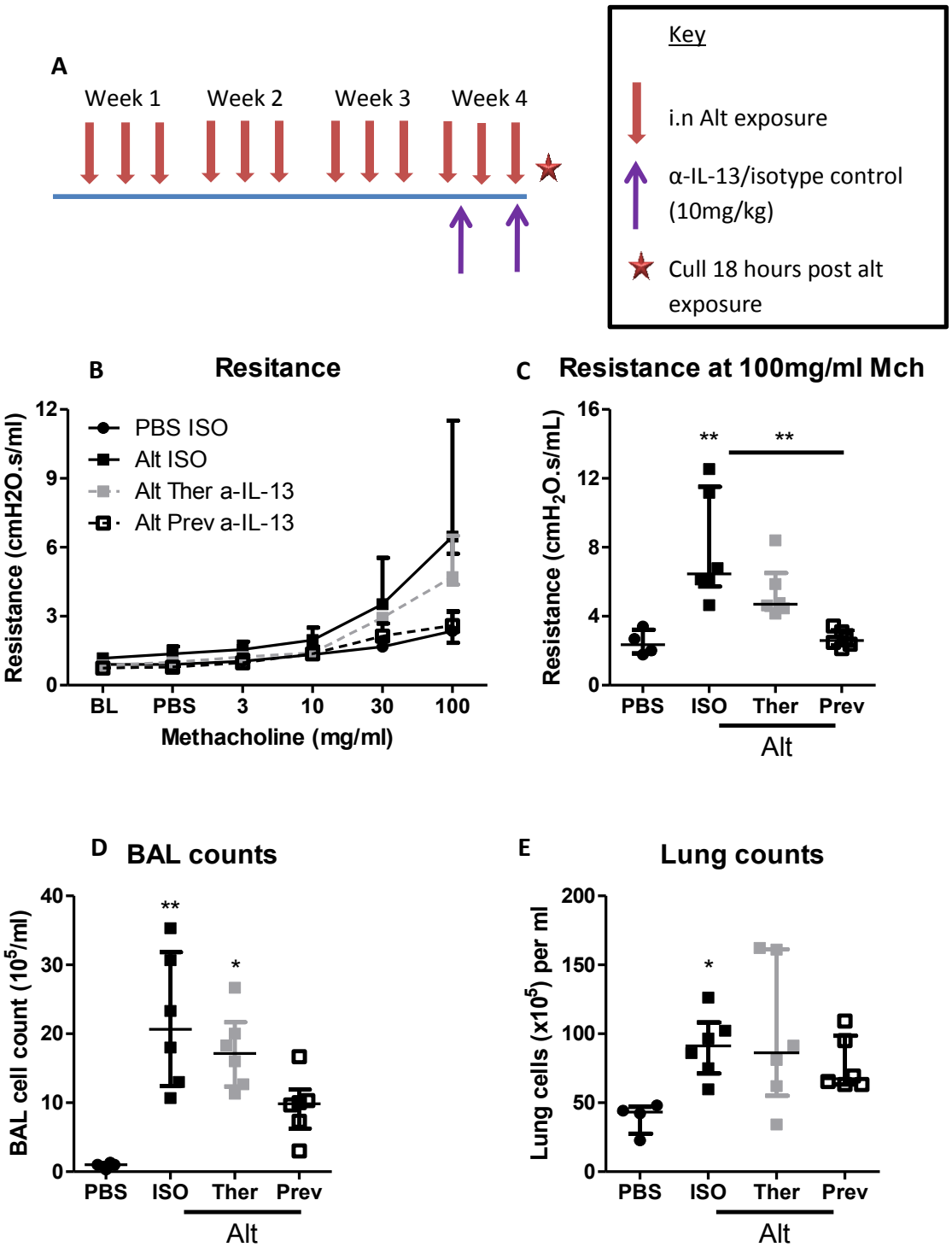

Figure 6.15 Airway resistance and inflammation in response to therapeutic αIL-13

Adult mice were exposed to Alt over three weeks before commencement of twice weekly i.p αIL-13 dosing (Ther) or dosed with αIL-13 throughout Alt exposure (Prev). Airway resistance over increasing concentrations of methacholine in mice exposed to Alt **(A)** and resistance at 100mg/ml Methacholine **(B)**. BAL **(C)** and lung **(D)** inflammatory counts. n= 4-6 mice per group. * p<0.05 and ** p<0.01 from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

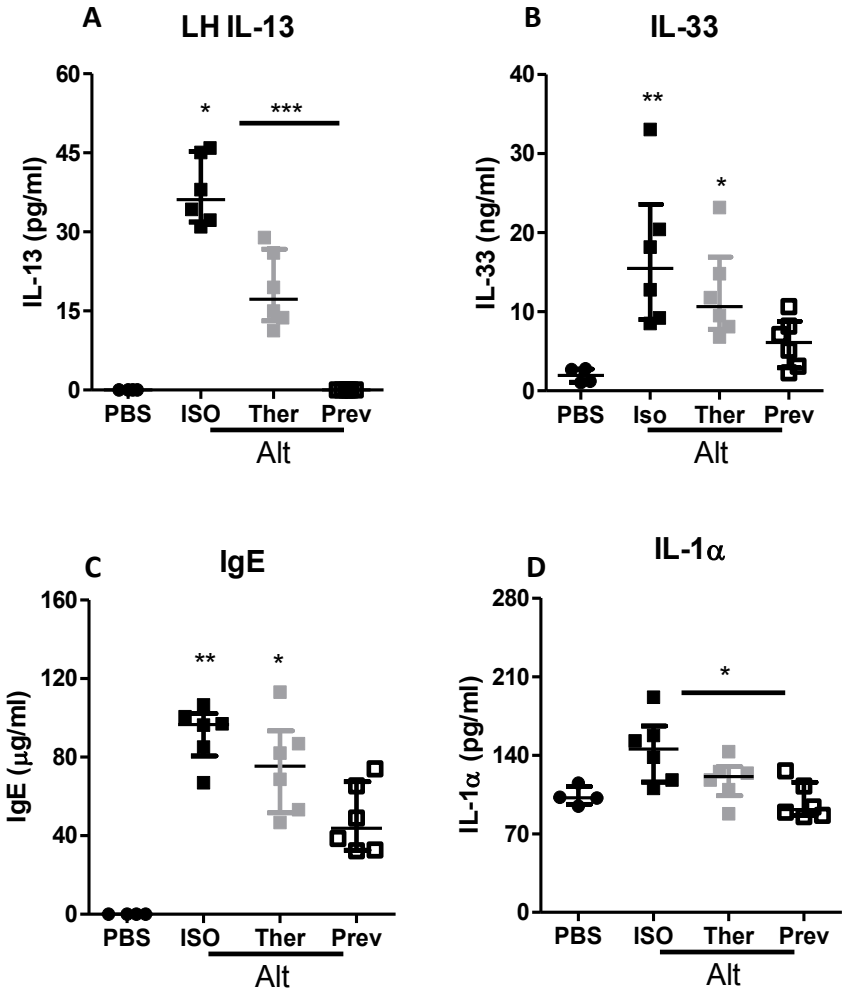

Figure 6.16 Inflammatory cytokines and IgE in response to therapeutic αIL-13

Adult mice were exposed to Alt over three weeks before commencement of twice weekly i.p αIL-13 dosing (Ther) or dosed with αIL-13 throughout Alt exposure (Prev). Lungs and blood were collected and processed for ELISA analysis. Lung IL-13 **(A)**, IL-33 **(B)** and IL-1α **(C)** and serum IgE **(D)** levels after Alt exposure as concentration per ml lung homogenate (50mg/ml). Alt specific IgE as µg/ml serum **(D)**. n= 4-6 mice per group. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from PBS control or as indicated by a bar by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

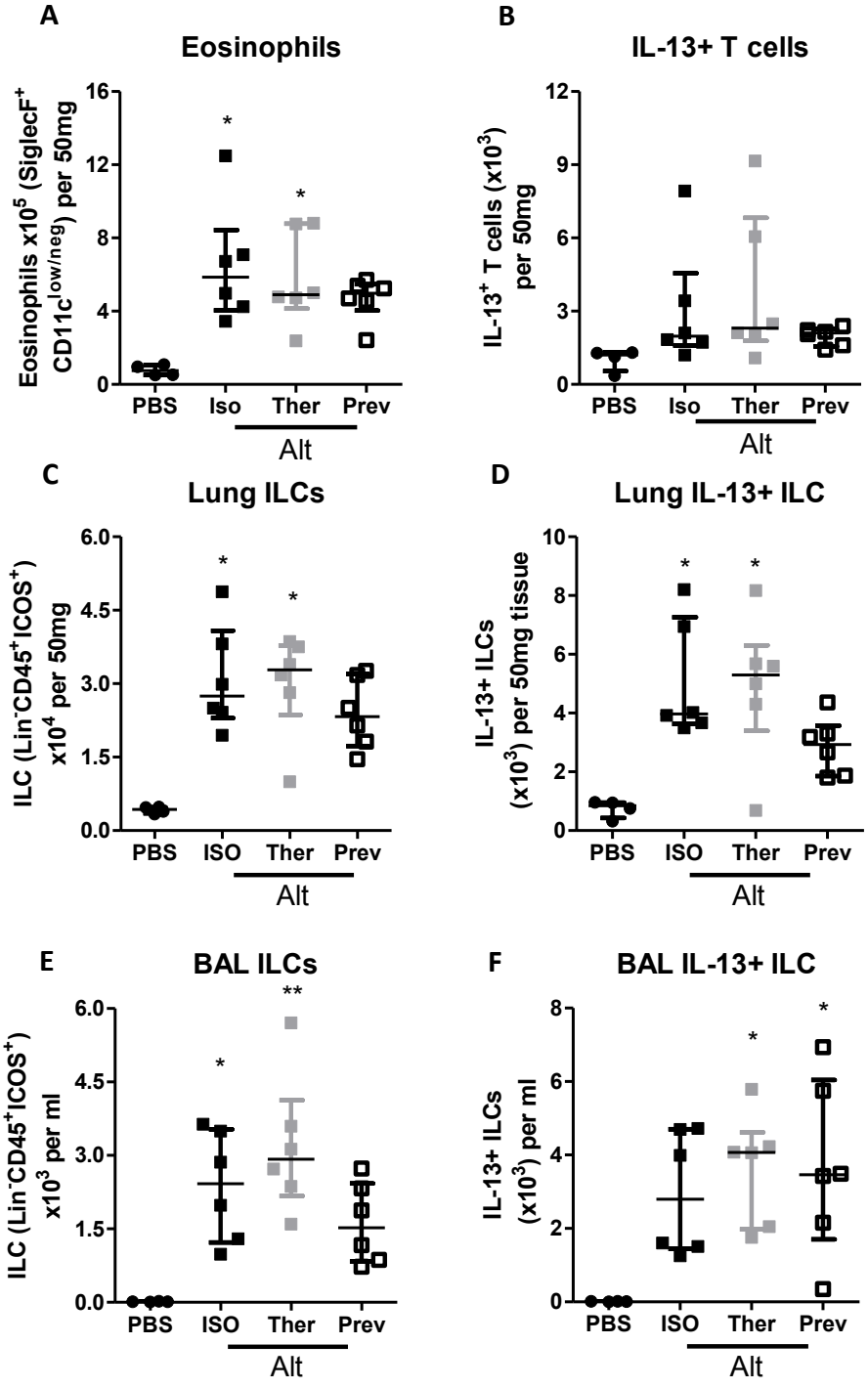

Figure 6.17 Inflammatory BAL and lung cells in response to therapeutic αIL-13

Adult mice were exposed to Alt over three weeks before commencement of twice weekly i.p αIL-13 dosing (Ther) or dosed with αIL-13 throughout Alt exposure (Prev) before collection and preparation of BAL and lung cells for facs analysis. Eosinophils (A), IL-13$^+$ T cells (CD3CD4$^+$) (B), ILCs (lin$^-$ CD45$^+$ICOS$^+$) (C) and IL-13$^+$ ILCs (D) per 50mg lung tissue. ILCs (E) and IL-13$^+$ ILCs (F) per ml BAL. n= 4-6 mice per group. *p<0.05 and ** p<0.01 from PBS control by Kruskal-Wallis with Dunn's multiple comparison test. Data displayed as median and interquartile range.

6.4 Discussion

The aim of this chapter was to investigate the therapeutic potential of an α-ST2 and α-IL-13 antibody in the treatment and prevention of allergic airways disease. We aimed to assess the effectiveness of a new α-ST2 antibody in a model of OVA induced AAD to provide a direct comparison to work carried out in our lab previously with a new antibody. A further aim of this chapter was to assess the contribution of IL-13 to Alt induced AAD as previous chapters highlighted a direct association between IL-13 and AHR.

This study demonstrated that the new version of α-ST2 Ab (CNTO3914), generated by Janssen Pharmaceuticals, Inc. (Fursov et al., 2011), was not as effective as a previously available antibody (3E10 mAb) in a model of resolution of OVA induced AAD (Kearley et al., 2009). The majority of the hallmarks of AAD were unaffected by the i.p injection of CNTO3914 throughout the resolution phase of disease. This included no effect on AHR, pulmonary inflammation, serum IgE, inflammatory cytokines and IL-13^{+} ILCs. However, CNTO3914 did result in a trend towards the reduction of IL-13^{+} T cells although repeat experiments will be required to confirm this finding. The antibody also had no effect on the pulmonary inflammation, Th2 cytokines or IgE levels. Due to findings in the previous chapter that the loss of ST2 protects from early inflammation after short term exposure to Alt, experiments with single exposure to Alt were conducted alongside ST2$^{-/-}$ mice as controls for a direct comparison of the antibody's ability to block ST2 signalling. Intraperitoneal injection of 10mg/kg CNTO3914 had no effect on lung or BAL inflammation and did not affect eosinophils or neutrophils, unlike the findings in ST2$^{-/-}$ mice. In contrast, when 2mg/kg CNTO3914 was injected subcutaneously BAL eosinophils were significantly reduced compared to WT ISO controls, although not to the same extent as in ST2$^{-/-}$ mice. Further assessment of the effectiveness of antibody dosing sub-cut will be required to determine if this antibody will be useful for the treatment of AAD. In contrast to the previously untested α-ST2, the αIL-13 antibody utilised during this study had established efficacy and had been previously used in a model of HDM induced AAD (Tomlinson et al., 2010). In the current

study, i.p injection of αIL-13 Ab throughout Alt exposure for three weeks was used as a preventative protocol. Blocking IL-13 completely ablated Alt induced AHR and caused a reduction in lung and BAL inflammatory infiltrates, although not to the level of PBS control. The antibody caused the complete ablation of lung IL-13 levels and reduced the levels of tissue IL-33. Despite the absence of AHR, Alt specific IgE and sensitisation still occurred in response to Alt exposure. Eosinophils, IL-13^{+} T cells and IL-13^{+} ILCs were also elevated. In order to investigate blocking IL-13 in Alt induced AAD in a more clinically relevant model a therapeutic blocking experiment was conducted. αIL-13 Ab was administered after three weeks of Alt exposure, after AAD had been established. Therapeutic blocking of IL-13 resulted in a partial, but non-significant, reduction in AHR and caused no reduction in BAL or lung inflammation. Unlike preventative dosing, lung IL-13 was only partially down-regulated by therapeutic αIL-13 and there was no effect on inflammation in the lung or BAL.

The role of the ST2 receptor during OVA exposure has been a controversial topic as contradictory papers have been published on the subject. Mangan *et. al*. (2007) utilised adoptive transfer of ST2 deficient T cells, specific for OVA, to conclude that loss of ST2 on antigen specific T cells led to the worsening of allergic airway disease. This group demonstrated that ST2 deficient T cells secreted similar levels of Th2 cytokines to WT cells with the exception of IL-5 which was increased by ST2$^{-/-}$ cells, leading to increased eosinophil populations in the lung and a subsequent worsening of airway resistance (Mangan et al., 2007). Due to this group targeting T cells specifically, the role of ST2 on other cell types – such as mast cells and eosinophils – was not taken into account and as specific targeting of T cells is an unlikely therapeutic avenue for human treatment, these results may not reflect a clinically relevant scenario. However, utilisation of a global ST2$^{-/-}$ mouse model also revealed no reduction in pulmonary inflammation from WT mice in an OVA exposure protocol (Hoshino et al., 1999). In contrast to this, experiments conducted by an alternative group utilising global ST2$^{-/-}$ mice demonstrated a reduction in total BAL inflammation and reduced eosinophils in the airways of OVA exposed ST2$^{-/-}$ mice compared to WT. It was later speculated that the level of i.p sensitisation with OVA/alum prior to aerosolised OVA exposure was responsible for the different

outcomes observed as mice sensitised twice developed similar disease to WT mice but mice sensitised only once with OVA/alum showed reduced airway inflammation from WT mice (Oboki et al., 2010). However, the background strain of the $ST2^{-/-}$ models also differed in these experiments and may have been responsible for the observed differences. The role of background strain in the effect of $ST2^{-/-}$ mice during OVA sensitisation is supported by Barlow *et. al*. (2012). This group utilised $ST2^{-/-}$ mice crossed to a Balb/c background and OVA-induced airway resistance was completely ablated in $ST2^{-/-}$ mice, despite two injections of OVA/alum prior to OVA exposure.

The development of an α-ST2 antibody (3E10 mAb) allowed the identification of the surface receptor specifically on Th2 cells (Löhning et al., 1998) and was subsequently used to investigate the role of the receptor due to the ST2 blocking nature of the antibody. α-ST2 administration prior to OVA exposure in a Th2 transfer model prevented the accumulation of eosinophils and secretion of Th2 cytokines. This antibody was further investigated in a model of OVA induced AAD and was found to reduce IgE, eosinophils and IL-5 compared to an isotype control (Coyle et al., 1999), and later in the clearance of $ST2^{+}$ T cells in the resolution phase of OVA induced AAD (Kearley et al., 2009). As the 3E10 mAb clone of α-ST2 is no longer available, the current study aimed to test an alternative antibody generated by Janssen Pharmaceuticals, Inc. (CNTO3914) (Fursov et al., 2011) in a protocol identical to that used by Kearley *et. al*. (2009). As evident from this study, CNTO3914 did not result in the same ablation of AHR after OVA exposure as 3E10 mAb. In addition, treatment with i.p CNTO3914 throughout OVA exposure did not result in a significant reduction in airway resistance, inflammation or inflammatory cytokines. Interestingly, flow cytometric analysis of pulmonary cells isolated from mice treated with CNTO3914 during resolution of OVA AAD showed ST2 was significantly reduced in ILC populations, this is most likely due to down-regulation of ST2 in response to antibody binding as CNTO3914 and the FITC-α-ST2 antibody used for facs analysis bind to different domains of the receptor and therefore interference in binding of the FITC-conjugated antibody is unlikely. Another interesting observation from this study was the lack of lung IL-13 despite the elevated AHR one week after last exposure to OVA. Throughout the previous chapters,

IL-13 was closely associated with airway resistance; however, the final allergen exposure before analysis was maximum 18 hours prior to culling. This may indicate that persistent AHR in the absence of continued allergen exposure is not IL-13 dependent. Interestingly, examination of the interaction between IL-4, IL-5 and Il-13 in a model of OVA induced AAD revealed that IL-5 was responsible for AHR in the absence of IL-13 (Webb et al., 2000). In the current study of OVA induced AAD, IL-5 was elevated in the absence of IL-13 one week following OVA exposure, providing a possible explanation for the increased AHR.

Characterisation studies of CNTO3914 showed inhibition of AHR and a reduction in BAL inflammation in a model of IL-33 induced AAD (Duffy et al., 2013). Due to the lack of effect in a model of OVA exposure, subsequent experiments to determine the potential of CNTO3914 for the treatment of AAD focused on a short term Alt exposure protocol – known to be a potent source of epithelial IL-33 release (Snelgrove et al., 2014). Pre-treatment of WT mice with i.p CNTO3914 had no effect on the level of BAL inflammation or eosinophil infiltration to the lung. However, when the antibody was injected subcutaneously prior to Alt exposure, a two-fold reduction in the population of lung eosinophils occurred, while $ST2^{-/-}$ control mice had a significantly lower population. Investigation of the route of administration on the systemic concentration of injected substances has been carried out in the context of insulin delivery (Kelley et al., 1996). This group determined that i.p injection resulted in rapid and higher systemic insulin concentrations while subcutaneous injection resulted in a more persistent, steady level of insulin after injection. The mode of delivery may therefore be an important aspect of the ability of α-ST2 to bind to surface receptors and this will need to be investigated further. An interesting future experiment would be the repetition of OVA exposure experiments with a sub-cut route of α-ST2 Ab administration. To date, no human trials have been conducted to assess the effect of ST2 blocking, or introduction of sST2 as a decoy receptor, in the context of any disease type despite promising results from studies conducted in mice (Kearley et al., 2009; Lee et al., 2014; Yin et al., 2012). Both serum IL-33 and sST2 levels are

known to be increased in asthmatic patients and may therefore act as biomarkers to identify patients likely to benefit from future therapies to block the pathway (Azazi et al., 2014).

Unlike the previously untested α-ST2, the αIL-13 antibody (UCB CA154_582) used throughout this study is well established and has proved successful in eliminating HDM induced AAD if administered at the instigation of HDM exposure, but did not reduce AHR if treatment was administered therapeutically once disease had been established (Tomlinson et al., 2010). As previous chapters in this study had identified the increased pathology associated with Alt exposure in comparison to HDM exposure, highlighted by the steroid resistant nature of Alt induced disease, this study aimed to determine the role of IL-13 in the pathogenesis of severe, Alt induce AAD. Due to the increased pathology associated with Alt induced AAD it was hypothesised that blocking IL-13 may not be adequate for the prevention of Alt induced AAD as the intrinsic serine protease activity of the allergen may induce a number of additional mediators to be released in addition to IL-33 and subsequent IL-13. However, preventative treatment with αIL-13 during Alt exposure did result in the complete ablation of airway resistance and reductions in inflammatory cell infiltrates and cytokines. Interestingly, blocking IL-13 resulted in the down-regulation of lung IL-33, an effect that has not been demonstrated under any other circumstance throughout this study. As IL-33 is widely accepted as inducing IL-13^{+} immune cells it would not be expected that neutralisation of IL-13 would have an effect on the level of IL-33, although further investigation of the relationship between IL-13 and IL-33 would be of interest, for example determining if recombinant IL-13 administration *in vitro* or *in vivo* causes IL-33 expression in cells such as epithelial or immune cells. Neutralising IL-13 did not prevent the generation of Alt specific IgE or IL-13 producing T cells although the presence of these cells obviously did not result in the generation of AHR, as any IL-13 released from these cells would be neutralised.

In concordance with the effects of IL-13 neutralisation after the establishment of HDM induced AAD (Saglani et al., 2013; Tomlinson et al., 2010), therapeutic treatment with IL-13 did not significantly

down-regulate Alt induced airway resistance. In contrast to preventative IL-13 treatment, therapeutic treatment in the final week of Alt exposure did not completely reduce lung IL-13. Investigation of a longer period of αIL-13 as a therapeutic therapy during Alt exposure would therefore be of interest to determine if the lack of AHR down-regulation was due to residual IL-13 not bound by the antibody, or if neutralising IL-13 after the initiation of AAD would not be effective due to the role of alternative cytokines such as IL-5 in the continuation of AHR. Results from this study into the effect of α-ST2 in the resolution of OVA AAD suggests that IL-13 does not have a role in the continuation of airway resistance as resistance was significantly elevated in the absence of IL-13, this could provide a mechanistic explanation for the lack of success documented in human clinical trial of αIL-13 treatment.

The association of IL-13 with numerous features of allergic asthma is well documented (Wills-Karp et al., 1998b) and the treatment of asthmatic patients with αIL-13 therapy has therefore been an attractive treatment option investigated by a number of groups. Both IL-13 and the IL-4 receptor – shared by both IL-13 and IL-4 – have been experimentally targeted with variable results (De Boever et al., 2014; Brightling et al., 2014; Corren et al., 2010; Wenzel et al., 2013). Corren *et. al* (2010) and Wenzel *et. al*. (2013) both utilised a blocking antibody against IL-4α; Corren *et. al*. demonstrated improvements only in a subset of patients with initial higher baseline scores in an Asthma Control Questionnaire while Wenzel *et. al*. (2013) demonstrated a significant improvement in lung function parameters after initially selecting for patients with asthma characterised by high sputum eosinophil levels. Both studies demonstrated the need to extensively phonotype patients prior to treatment to identify subsets likely to respond to IL-13 blocking as a therapeutic treatment. A recent trial of the αIL-13 Ab Tralokinumab in severe asthma patients further demonstrated this need as surrogate biomarkers were identified to determine a subgroup of patients most likely to respond to αIL-13 Ab therapy (Brightling et al., 2014). Patients with high serum levels of periostin or DPP4 at commencement of the trial were more likely to benefit from improvements to FEV_1 and have fewer asthma exacerbations. Both DPP4 and periostin are induced by IL-13 (Takayama et al., 2006; Yong

Zhang et al., 2014) and thus provide biomarkers for patients likely to respond to αIL-13 Ab therapy. In the current study, fungal exposure was identified as a potent inducer of IL-13 associated with steroid resistance. Sensitisation to fungal allergens could therefore act as a potential defining factor to identify asthmatic patients who may benefit from αIL-13 Ab therapy. Interestingly, despite the documented improvement in airway function and quality of life scores in certain subsets of patients treated with αIL-13 Ab therapy, no data on the effects of inflammation have been reported. As with the effects of steroids documented in chapter 4 of this study, a disconnect exists between the effect of therapies on airway resistance and pulmonary inflammation and cytokines. While both eosinophil numbers and BAL IL-13 levels were reported in patients with severe asthma undergoing high dose steroid treatments there is no corresponding data available for the comparisons of results from the studies of αIL-13 Ab in clinical trials. In this study, preventative αIL-13 Ab therapy caused complete ablation of Alt induced airway resistance while pulmonary inflammation and the development of antigen specific IgE was unaffected. This suggests allergic airways disease could develop if therapy was removed due to the presence of antigen responsive T cells and IgE. Corresponding cellular and cytokine data from future clinical trials will therefore be or great interest.

Overall, results from initial trials of asthmatic patients in combination with results from the current study demonstrate that IL-13 has a vital role in the onset of allergic airways disease but it may not be integral to the continuation and persistence of disease and therefore targeting the cytokine in established disease may not be effective. However, if effective biomarkers are used to identify patients with high IL-13 levels and therefore IL-13 driven pathology, treatment may result in improvements to severe asthma and may provide an option for the control of severe, steroid resistant disease.

6.4.1 Conclusion

This chapter described the action of a new α-ST2 antibody, developed by Janssen Pharmaceuticals, Inc., in the context of allergic airways disease. It was determined that the antibody was not as effective as a previously available version of an α-ST2 antibody as it did not result in prevention of OVA induced AAD and was ineffective during the resolution of disease. The new antibody also had no effect prior to a single dose of Alt. However, subcutaneous injection of the antibody did cause a partial reduction in the population of lung eosinophils and therefore route of injection on the effectiveness of the antibody needs further investigation. It was also determined that severe asthma, generated through Alt exposure, was IL-13 dependent and treatment with preventative αIL-13 Ab ablated Alt induced airway resistance, however, did not prevent the development of Alt specific IgE or IL-13+ T cells and ILCs. In contrast, IL-13 blocking after the induction of disease did not cause a reduction in airway resistance or inflammation. This provides a possible mechanism for the observed lack of effect of IL-13 blocking in the majority of asthmatics in recent clinical trials.

Chapter 7 – Discussion

7.1 Summary of findings and impact of project

The overall objective of this project was to investigate molecular mechanisms underlying risk factors associated with the development and severity of asthma and, where possible, to identify the effects of intervention with novel therapeutics. Environmental risk factors examined included age at first exposure to an allergen, from early life through to adulthood, and the effects of the type of allergen to which mice were exposed. Genetic risk factors that were manipulated focused on two genes that have been highlighted as important in asthma pathogenesis through GWAS associations; IL-33 and ST2. The final aim was to investigate the therapeutic potential of manipulating ST2/IL-33 signalling as a possible candidate for disease intervention.

7.1.1 Pitfalls of inappropriate experimental models

As asthma is predominantly a childhood onset disease, with 95% of all cases developing in early life (Masoli et al., 2004), age is an essential aspect to factor into models that investigate mechanisms underlying disease. However, the majority of experimental models are undertaken in adult mice. To the best of our knowledge, this study is the first to demonstrate the age-dependent development of allergic disease by employing a HDM exposure protocol at sequential points during development. This allowed the identification of a period between day 14 of life and day 21 of life in which mice were non-responsive to HDM exposure; characterised by a lack of airway resistance, IgE and IL-13 generated in response to HDM exposure beginning during this early life time point and continuing for three weeks. The importance of carefully considering age has been mirrored in cohort studies of children. Several long-term birth cohorts have followed children through to adolescence and shown different clinical phenotypes that can be distinguished by age of onset of wheeze (Henderson et al., 2008; Taussig et al., 2003). When lung function is related to the clinical phenotype, persistent wheeze that was characterised by early onset in the first three years of life and early allergic

sensitisation then continues to progress to asthma is associated with an early and irreversible loss in lung function. In contrast, late onset wheeze that develops after the age of three is not associated with atopy or loss of lung function. It could be hypothesised that the early life model that includes allergen exposure starting at day 3 of life mimics persistent childhood wheeze, while first allergen exposure at day 14-21 is more representative of the late-onset phenotype. Since we have shown contrasting allergic immune responses determined by age at first allergen exposure in our murine model, it may be possible to translate the mechanistic differences to the clinical phenotypes to help determine management and use of potential disease modifying therapies in the future.

A phenotype that did not respond to allergen has previously been described by Al-Garawi *et. al*. (2011) and was attributed to the naturally occurring regulatory environment during early life, characterised by increased levels of IL-10 and TGFβ in neonatal lungs. The current study did not identify a substantial reduction in levels of these cytokines even when allergen exposure was commenced at the same age – day 14 of life. An important point highlighted by the current data is the need for accurate nomenclature when describing early life models. Murine studies that utilise young mice varying from day 3 to day 21 have used numerous terms including 'neonate', 'infant', 'pre-weanling' and 'weanling'. However, this has resulted in confusion in the interpretation of the data, since there are no uniform age-related definitions of these terms. It has become apparent from this work that the best way to describe age effects is to refer to the actual age or allergen exposure is commenced.

One profound difference identified in the pulmonary immune environment during early life was the number of IL-13$^+$ ILCs and T cells in the lungs of allergen naïve mice, as described in chapter 3. 3 day old mice had a higher number of IL-13$^+$ T cells in the lung than IL-13$^+$ ILCs and both cell numbers dropped to a low level during the period of allergen non-responsiveness and by day 28 mice had a larger population of IL-13$^+$ ILCs. Interestingly, to date, murine studies that have investigated the role of ILCs in the inception of AAD have only included adult models, whereby allergen exposure is

commenced in 6-8 week old mice (Barlow et al., 2012; Halim et al., 2012, 2014) These data imply innate responses involving the induction of ILCs are sufficient to initiate allergic responses. However, it can be argued that these data actually represent a very specific adult-onset phenotype of asthma, characterised by eosinophilia, but a relative absence of atopy. In contrast, when we have investigated an age-appropriate model, it is apparent that in very early life (day 3 of life) the pulmonary immune environment in naïve mice had a dominance of IL-13^{+} T cells, and thus allergen exposure at this early age may result in a different immune phenotype.

Any immunotherapies designed to target specific cell populations would therefore have to take into account the alteration of dominant IL-13 producing populations with age. In collaboration with Gollwitzer *et. al*. we were able to link our findings of age and allergen induced responses with observations made in their lab regarding the development of inducible regulatory cells (Helios^{-} Tregs) in response to the developing pulmonary microbiome during early life (Gollwitzer et al., 2014). Taken together, these findings offer a possibility for intervention in babies by manipulation of the pulmonary microbiome. Manipulation of the colonising microbiome as a therapy for allergic airways disease has previously been described in an adult model of pulmonary exposure to *Escherichia coli*, which was shown to act by inhibiting in situ Th2 cytokine production, resulting in suppressed AHR. Taken together, this therapy could be optimised by targeting patients in early life during the time in which Helios^{-} Tregs emerge with bacteria from the Bacteroidetes phylum, shown to be responsible for the generation of Helios^{-} Tregs (Gollwitzer et al., 2014). Manipulation of the microbiome has also been successful for the treatment of antibiotic induced *Clostridium difficile* (C. diff) infection where the overuse of antibiotics almost completely abolishes the natural bowel colonising bacteria (Petrof and Khoruts, 2014). Therapies ranging from faecal transplants from healthy donors to a more refined administration of a mixture of bacterial strains cultured from a faecal sample have proved successful for the treatment of C diff. The response of pulmonary IL-13^{+} ILCs to the developing microbiome will also be an interesting future research area as data in chapter 3 indicated both ILC2 and Th2 cell numbers were at a nadir in the day 14-21 period when allergic

responses failed to develop. Data from studies of intestinal homeostasis in neonatal mice have shown that RORγt^{+} ILC3s develop alongside the colonisation of the intestine with the microbiome, however, they also develop in germ free models and therefore differ from induced Tregs (Gollwitzer et al., 2014) which are hypothesised to develop as a pre-emptive protective population (Sawa et al., 2010). The translational potential for alteration of the pulmonary microbiome will require the investigation of equivalent cell populations of ILCs, T cells and Tregs in humans throughout development.

The period of non-responsiveness during early life was susceptible to intervention by the addition of rIL-33 in the first week of allergen exposure. Introducing IL-33 into the pulmonary immune environment overcame the lack of response to HDM exposure. Recently, IL-33 release has been documented in the lungs of asthmatic patients undergoing experimental rhinoviral infection while infection of non-asthmatic patients did not result in elevated IL-33 release (Jackson et al., 2014a). A recent study following a cohort of children at high risk of developing asthma showed that sensitisation to aeroallergens in early life, followed by viral infection was associated with the risk of developing viral wheeze, a common predisposer to the development of asthma in later life (Jackson et al., 2012). This could provide a mechanistic reason for the association between viral infections in early life to the increased development of allergic asthma (Jackson et al., 2012; Kusel et al., 2007). Alteration of the pulmonary immune environment during this critical window could therefore be responsible for the susceptibility to future disease. Previously published reports have shown that allergic disease develops in the absence of adaptive cells in adult mice in response to papain exposure (Halim et al., 2012; Oboki et al., 2010). In the study presented here, SCID and WT mice were utilised from day 14 of life to determine whether, as with adults, innate immune cells were sufficient to overcome the natural resistance to the development of AAD in early life. SCID mice developed similar levels of AHR to WT mice and a similar population of IL-13^{+} ILCs were attracted to the lungs in response to cytokine stimulation. As this experiment was conducted with recombinant IL-33 it may not represent a realistic model for allergic airway disease as IL-33 release into the BAL is

not detectable in response to HDM exposure (Snelgrove et al., 2014), however, could provide mechanistic evidence for the association of Alt sensitisation and viral infection in early life – both shown to be potent inducers of IL-33 (Jackson et al., 2014a; Snelgrove et al., 2014).

In addition to the contribution of altered pulmonary inflammatory cell populations during development, the genetic changes occurring in the lungs from day 3 to day 28 were also investigated to determine the effect of developmental changes on gene expression. Initially, the aim of this experiment was to isolate IL-13^{+} T cells and investigate the gene expression in this cell subset during immune maturation in allergen naïve mice. However, the isolation of sufficient quality RNA from these cells was not successful. Instead, RNA was extracted from whole lung and RNAseq was undertaken to detect changes in pulmonary gene expression during development. Initial analysis focused on genes that were altered at day 14 compared to both day 3 and day 28 of life to identify any up or down-regulation during this period, which could be responsible for non-responsiveness to HDM exposure. This highlighted two genes of interest that may contribute, including CD302 – which encodes DCL-1 - and Angp2 – which encodes angiopoientin. Both gene products have the potential to be involved in the generation of immune cells in response to the developing microbiome at this age, as described by Gollwitzer *et. al*. (2014), as angiopoientin is associated with lymphocyte trafficking (Roviezzo et al., 2005) and DCL-1 is a phagocytic receptor expressed on a variety of antigen presenting cells (Kato et al., 2007). However, this was a preliminary analysis of the data and confirmation using different analytical techniques is currently underway in the lab. The ultimate goal is to identify a molecule or group of molecules that result in the change in immunophenotype in early life from being extremely susceptible to allergic responses to almost complete protection, with a partial return of allergic responses in later life. It is apparent that disease modification or alteration in the natural history of asthma can only be achieved with intervention in the very early preschool years (Jackson et al., 2014b). Identification of potential molecules responsible for the changing immune phenotype in early life may be novel candidates to achieve this.

Another factor that is known to be dependent on age is gender differences associated with the prevalence and severity of asthma. Up to the age of 16, males have twice the prevalence of asthma compared to females (Schaubel et al., 1996; Venn et al., 1998), while in adulthood, females have a greater risk of developing asthma. Asthma in adult females is also characterised by a more severe disease phenotype (van den Berge et al., 2009; Strachan et al., 1996). The current study utilised mixed male and female pups for neonatal experiments and no difference was observed in any disease parameter that was affected by sex (data not shown). However, due to convenience of housing up to 5 adult females per cage compared to one male per cage, all adult experiments were conducted on females only. This may therefore represent a more severe disease phenotype associated with female asthma in adulthood and a comparison of disease severity between murine sexes would be an interesting future study. 7.1.2 Technical challenges of neonatal experiments

Overall, the importance of using age-appropriate disease models has been demonstrated by both chapters 3 and 4 of this study, as the maturing immune system and potential differences in smooth muscle responsiveness of developing lungs need to be factored in to investigations of the childhood associated disease. However, a number of disadvantages associated with the use of neonatal mice make these experiments more technically challenging for research. For example, differences in litter size mean some litters grow at a slower rate than others do and by age 3 weeks when analysis was undertaken the weight and therefore size of neonatal mice could vary substantially, and this may affect the baseline values of lung function experiments. The differences in litter size also make the distribution of treatment groups difficult. Neonatal mice cannot be ear tagged and superficial markings are often licked off by the mother. Fostering of pups from large litters to mothers with smaller litters is one possibility; however, this is not always successful and sometimes results in the death of fostered pups. The size of the pup also makes both intranasal and intraperitoneal administration of drugs and allergens difficult. Small volumes have to be used which means pre-

made solutions may not be adequate to give a high enough concentration. Intraperitoneal dosing also has to utilise small doses, as the skin over the abdomen is tight with no fat layer, meaning liquids often leak out of the injection site when the needle is removed, making it difficult to administer exact amounts of liquid. The extracted tissues – such as serum and lung tissue - are also substantially smaller than adult equivalents, meaning assays need to be prioritised for best use of small samples.

7.1.3 The contribution of allergen type to disease manifestation

Direct comparison of immune responses to inhaled allergen in adults compared to early life revealed the hyper-responsive nature of neonatal murine lungs when allergen exposure began at day 3 of life. Adult mice exposed to Alt - a fungal allergen associated with a severe asthma phenotype – developed significantly higher AHR and lung IL-13 levels compared to HDM exposure. In contrast, neonatal mice developed similar levels of AHR following exposure to either HDM or Alt. In addition, both airway resistance and pulmonary IL-13 levels were substantially increased in neonates compared to adult mice with both allergens. This led to the conclusion that neonatal mice have an exaggerated response to HDM exposure compared to adult mice and therefore the increased immunogenicity associated with Alt did not lead to increased airway resistance in neonates as it did in adults. These results correspond to observations documented by Hopp *et. al*. (1985) in which paediatric subjects reacted to methacholine with increased bronchial responses when compared to adult subjects undergoing the same exposure. As methacholine targets smooth muscle directly this implicates smooth muscle function as a possible determinant of differences observed between ages. Functional differences between paediatric and adult smooth muscle is an interesting avenue for investigation as paediatric patients with wheeze have been shown to have abnormal lung function in the absence of increased smooth muscle (O'Reilly et al., 2013), while adolescents with asthma have

abnormal lung function in addition to increased smooth muscle mass (Regamey et al., 2008), indicating differences in the function rather than quantity of pulmonary smooth muscle. Smooth muscle is known to release cytokines including IL-6 and GM-CSF in response to stimulation with cytokines such as IFNγ (Johnson and Knox, 1997b). The responses of paediatric vs. adult smooth muscle cells in response to inflammation generated by allergen exposure would potentially identify age appropriate targets for the treatment of paediatric asthma. Initial experiments would most likely focus on a purified population of cultured human airway smooth muscle cells (HASM) isolated from both paediatric and adult bronchial biopsies, as described by Fayon (2011), and analysis of secreted inflammatory mediators in response to allergen or cytokine stimulation.

A further key finding of this study was the increased disease severity associated with exposure to the fungal allergen Alt in comparison to HDM in both adults and neonates, highlighted by the steroid resistant AHR in neonatal mice exposed to Alt. A publication from our lab (currently in press – JACI 2015) links this finding to patient data from children with severe therapy resistant asthma (STRA). Patients were assessed for fungal sensitisation by skin prick test or specific IgE for Alt, *Aspergillus fumigatus,* or *Cladosporium herbarum*, and those that were sensitised to fungal allergens were characterised as having severe asthma with fungal sensitisation (SAFS). Compared to patients with STRA without fungal sensitisation, SAFS patients had significantly worse disease characterised by earlier disease onset, higher serum IgE, higher BAL IL-33 and significantly more SAFS children were prescribed oral steroids for symptom control. Interestingly, no difference was observed in lung function between children sensitised to fungal allergens and those who were not, this corresponds to neonatal mouse data generated in the current study. Mechanistic studies carried out in neonatal mice showed Alt sensitisation is characterised by increased lung IL-33, BALF IL-13 and lung infiltrating IL-13^{+} ILCs. This study further emphasised IL-33 as a steroid resistant cytokine (Saglani et al., 2013) as levels of IL-33 were increased in the lung tissue of HDM exposed mice despite a complete ablation of AHR in response to a preventative steroid regimen. This study further

highlighted the IL-33/ST2 pathway as a possible target for the therapeutic intervention of severe asthma.

7.1.4 Mechanistic differences in manipulation of cytokine compared to receptor

In addition to the contribution of age on the development and severity of AAD, genetic susceptibility was also investigated. GWAS of patients with childhood onset asthma identified SNPs in the gene regions of both IL-33 and ST2 being associated with asthma (Moffatt et al., 2010; Ramasamy et al., 2012; Torgerson et al., 2011). Investigation using knockout models of the cytokine and its receptor with exposure of HDM and Alt identified striking differences between the role of the cytokine and the role of the receptor in the initiation of HDM induced AAD. The absence of IL-33 had no effect on the development of disease in response to HDM exposure, while the absence of a functioning receptor for IL-33 (ST2) prevented the development of HDM induced allergic airways disease. This result suggests there may be an alternative ligand that is able to signal through the ST2 receptor in addition to IL-33. Similar findings in a model of arthritis had previously also led to this conclusion (Martin et al., 2013), but had the limitation of different background strains of receptor and ligand knock-outs. As both IL-33$^{-/-}$ and ST2$^{-/-}$ mice used in the current study were on a Balb/c background, the data are more robust. The possible involvement of additional ligands signalling through ST2 have significant implications for the development of therapeutics for allergic asthma, since targeting IL-33 may not have the expected effect. However, due to the lack of appropriate reagents to block IL-33, the therapeutic potential of blocking the cytokine once disease has been established was not possible. This study suggests targeting ST2, certainly for disease prevention and in the context of HDM, induced disease may be more appropriate. However, the effectiveness of blocking ST2 in a more severe asthma phenotype, especially in adult onset disease is questionable as AAD induced by Alt persisted in adult ST2$^{-/-}$ mice.

Investigation of the contribution of ST2 to the early development of Alt induced AHR utilised an adult ST2$^{-/-}$ mouse model. Alt induced disease differed in an ST2$^{-/-}$ model compared to WT mice as Alt responsive T cells and Alt specific IgE developed without the early influx of innate cells, such as ILCs and eosinophils, in response to initial allergen exposure. ST2$^{-/-}$ mice had a slower onset of the AAD phenotype – as evident by the lack of airway resistance and lung IL-13 after a week of Alt exposure, which was evident in both WT and IL-33$^{-/-}$ mice. However, after three weeks of exposure to Alt in ST2$^{-/-}$ mice, AHR was similar to WT and IL-33$^{-/-}$ mice. Overall, this showed that Alt driven AAD did not require the involvement of cells immediately recruited to the lungs of WT mice, such as ILCs and eosinophils, for the development of antigen responsive Th2 cells, antigen specific IgE and the subsequent onset of AHR. As antigen specific IgE and T cells develop in response to Alt but not HDM, investigation of pulmonary resident cells at the barrier of environment and host – such as dendritic cells – to determine differing responses to HDM and Alt exposure may provide therapeutic targets for disease intervention. Of particular interest would be the effect of the serine protease activity of Alt (Snelgrove et al., 2014) on dendritic cells and the subsequent transport and interaction of these cells with immature B and T cells leading to the development of antigen specific cells. The targeting of serine protease in asthma is an active area of research (Guay et al., 2006; Lin et al., 2014) and a recent study has identified epistasis between SNPs in the region of TSLP and inhibitor Kazal-type 5 (SPINK5), a serine protease inhibitor, in childhood asthma (Biagini Myers et al., 2014). The authors discovered that children with SNPs in SPINK5 and no SNPs in TSLP were associated with a higher risk of asthma while children with SNPs in SPINK5 and TSLP had a protective phenotype. This study highlights the potential importance of serine protease inhibitors in the pathogenesis of asthma and the need for in-depth analysis of GWAS as individual SNPs may not be significantly associated with disease, however, when considered together with a biologically relevant gene an association may be found. A further factor to consider in the slower onset of AAD in ST2$^{-/-}$ mice is the involvement of MYD88 dysregulation in the ST2$^{-/-}$ model as disruption of this molecule has the potential to interfere with innate signalling pathways including TLR signalling. Published data shows the sequestration of

MYD88 by the intracellular region of the ST2 receptor affects the activation of both TLR4 and TLR2 signalling (Brint et al., 2004; Liu et al., 2010). However, previous work in the lab utilising a MYD88$^{-/-}$ mouse model revealed no difference in HDM response to WT mice (unpublished data). Investigation of the kinetics of inflammation and cytokine production in WT and ST2$^{-/-}$ mice in response to an increasing length of Alt exposure has highlighted the need for a longer protocol to be investigated. In WT mice, BAL inflammation, BAL IL-13 and IL-13^{+} ILCs were highest after one week of Alt exposure and began to decline after three weeks of exposure. In contrast, levels of BAL IL-13, IL-13^{+} ILCs and BAL inflammation continued to rise over the three weeks of Alt exposure in ST2$^{-/-}$ mice with levels remaining below those observed in WT mice. A longer timecourse of Alt exposure would therefore determine if blocking of ST2 reduced inflammation or simply delayed the onset of inflammatory mediators, an important consideration for the effectiveness of ST2 blocking in clinical trials of severe therapy resistant asthma.

Results from this study suggest pulmonary IL-13 levels are closely linked to the level of AHR in both adults and neonates. IL-13 was therefore investigated further in a model of severe, Alt induced AAD in adult mice. Preventative treatment with αIL-13 Ab during Alt exposure completely prevented the development of AHR. One potential avenue of investigation is the release of IL-13 by mast cells in response to the cross-linking of surface IgE by allergen (Toru et al., 1998). Blocking either ST2 or IL-13 resulted in the development of Alt specific IgE although only the blocking of IL-13 prevented the development of AHR. The non-ST2 dependent pathways leading to the secretion of IL-13, such as IgE cross-linking of mast cells, would therefore be interesting for future investigation. Evidence for the importance of mast cells in the generation of AHR in our ST2$^{-/-}$ model was apparent from the levels of MCPT-1 associated with AHR. ST2$^{-/-}$ mice exposed to HDM had no detectable MCPT-1 in the serum, in contrast, ST2$^{-/-}$ mice exposed to Alt for three weeks had similar levels of serum MCPT-1 to both WT and IL-33$^{-/-}$ mice. Studies of the involvement of mast cells in the pathogenesis of asthma have revealed the important contribution of these cells to both the initiation and continuation of disease. Increased mast cell numbers in the smooth muscle of asthmatics is associated with increased AHR

(Brightling et al., 2002) and the ability of the cells to produce IL-13 both in response to IL-33 signalling and the cross linking of surface IgE (Ho et al., 2007) make them an interesting future target for investigation in this study. A number of targets have been proposed for the inhibition of mast cells and their secreted mediators, however no therapeutics specific to the inhibition of mast cells are currently available (Harvima et al., 2014). The use of the monoclonal antibody to IgE, Omalizumab, in clinical trials has shown the importance of IgE cross-linking to the pathogenesis of severe asthma. Blocking IgE binding to mast cell surface receptors results in fewer exacerbations in patients with severe asthma (Busse et al., 2001). As fungal sensitisation is associated with high levels of serum IgE and difficult to manage symptoms, the potential for anti-IgE therapy for these patients is promising. However, the levels of IgE in patients with fungal sensitisation are often outside the recommended levels for Omalizumab treatment (30–1500 kUA/l). The first published case study reporting the effects of anti-IgE therapy on SAFS combined Omalizumab with an anti-fungal drug, Itraconazole, and demonstrated clinical improvements to the patient (Pizzimenti et al., 2013). However, data in press from our lab (JACI 2015) has shown paediatric patients with SAFS do not respond better to Omalizumab than severe asthmatics without fungal sensitisation, despite higher baseline IgE levels. Further, large scale trials are therefore required to assess the effectiveness of blocking IgE function in patients with difficult to control SAFS.

Despite the efficacy of blocking IL-13 prior to Alt exposure in the prevention of AHR, targeting IL-13 after the establishment of Alt induced AAD did not cause a significant reduction in airway resistance. Clinically, this is very important because treatment of patients will only occur after disease is established. Therefore, a therapeutic experimental protocol is a more realistic representation of the clinical translatability of targeting IL-13. Despite two injections of αIL-13 Ab after the establishment of disease, pulmonary IL-13 was still elevated. This suggests a longer course of αIL-13 may be required to fully block the effects of the cytokine. However, clinical trials of αIL-13 in the treatment of patients with severe, therapy resistant asthma have recently been conducted and after twelve weeks of antibody administration, no improvement in airway function or the number of

exacerbations was seen (De Boever et al., 2014). Taken together with results from the current study, the targeting of IL-13 after the establishment of AAD may not be an effective treatment option. However, two additional studies into the potential of blocking IL-13 in asthma have identified beneficial effects in a subset of patients. Corren *et. al*. (2011) identified clinical benefits in patients with a high 'Th2' phenotype, identified by high periostin – a biomarker of high IL-13. A further clinical trial assessing a longer period of αIL-13 Ab treatment of severe asthmatics also found a subset of patients – identified as periostin or DP44 high - responded to treatment with a significantly reduced incidence of asthma exacerbations (Brightling et al., 2014). Taken together, these results indicate that future investigations of the effectiveness of blocking IL-13 after the onset of disease should focus on a longer period of treatment and thorough phenotyping of patients for markers of elevated IL-13 to identify those likely to respond to IL-13 blocking treatments.

The current study shows blocking IL-13 in a prevention regimen, prior to Alt exposure in an adult model of Alt sensitisation prevents the development of AHR, but this was not apparent when IL-33 was blocked in IL-33$^{-/-}$ mice or in ST2$^{-/-}$ mice. However, the importance of age appropriate models has also been highlighted in this thesis and neonatal ST2$^{-/-}$ mice exposed to Alt were shown to have a partial reduction in AHR in response to Alt exposure compared to WT mice (Paper in press – JACI 2015).

7.2 Future work

7.2.1 Further investigation of RNAseq results

Initial analysis of RNAseq results from whole lung tissue at different ages has focussed on genes differentially expressed between day 14 of life in comparison to both day 3 and day 28 of life in order to compare ages at which AAD develops and the age period in which mice are non-responsive. However, the same gene at different expression levels may not be responsible for the altered

responsiveness and therefore further analysis of genes at each age is required. In addition, in depth analysis of the identified genes of interest are required. Both Angpt2 and CD302 are increased at day 14 when there is an absence of immune responses to HDM so the most logical experiments to conduct would involve blocking or knocking out the protein/gene and observing the effect on the non-responsive phase between ages 14 and 21. Additional analysis would focus on the generation of Helios$^-$ Tregs when these genes are targeted as emergence of these cells in response to a developing pulmonary microbiome have been shown to be responsible for the period of non-responsiveness to HDM during early life.

7.2.2 Overexpression of epithelial IL-33

During this study, an AAV containing a plasmid encoding full length IL-33 was generated, however, due to time constraints the effects of IL-33 overexpression in the pulmonary epithelium could not be investigated. GWAS of asthmatic patients has identified SNPs in the gene region of IL-33, although the effect on the protein expression is not known (Moffatt et al., 2010). Despite this study showing absence of IL-33 has no effect on the generation of allergen induced allergic disease, it may be that increased IL-33 in the bronchial epithelium leads to the development or worsening of allergic disease as direct release of IL-33 from the epithelium is proposed to be responsible for the enhanced disease phenotype associated with Alt exposure (Snelgrove et al., 2014) and the current study shows rIL-33 is sufficient to induce airway resistance and inflammation in WT and SCID mice from day 14 of life.

Experiments with the generated AAV would first focus on the timeline of expression to determine how rapidly the protein is expressed in the epithelium to investigate the suitability of the AAV for use in neonatal models. The effect of increased bronchial IL-33 on the non-responsive phase between the ages of day 14 and day 21 will also be investigated as the cytokine has the potential to alter the balance of pulmonary immune cells in the lung leading to overcoming the non-responsive phase. Another avenue of investigation will be the threshold level of allergen exposure to induce inflammation and airway resistance as increased epithelial IL-33 may lead to the generation of AAD

in response to lower or less frequent allergen exposure. However, due to the differential expression patterns of IL-33 observed between mice and human tissues the clinical translatability may not be directly relatable to humans. Ideally, an antibody with the ability to block IL-33 would be utilised to test the therapeutic effect although none are currently available.

7.2.3 Identification of possible alternative ligands with the ability to signal through ST2

Experiments in chapter 5 identified the likely existence of an additional ligand with the ability to signal through ST2. Traditional methods to identify receptor binders – such as receptor pull down assays - failed to identify IL-33 as the ligand for ST2 (Gayle et al., 1996; Kumar et al., 1995), the identification of further ligands by these methods will also prove difficult. The first stage will be providing definitive proof of an additional ligand via *in vitro* methods. There are two main possibilities for the source of an alternative ligand for ST2; either an inflammatory mediator in the pulmonary environment generated in response to HDM exposure or from the allergen preparation itself. The DerP2 component of HDM is known to have a similar structure to MD-2; the region of TLR4 responsible for binding LPS. Signalling assays have demonstrated MD-2 can cause TLR-4 activation therefore acting as an auto-adjuvant with the potential to directly activate pulmonary structural and immune cells (Trompette et al., 2009). It is therefore possible that HDM contains a component with a structure similar to either an ST2 binding ligand or a component of the ST2 signalling complex with the ability to trigger downstream signalling pathways via molecular mimicry.

One option for the detection of alternative ligands is the utilisation of an ST2 reporter assay as described by Schmitz *et. al*. (2005) through the transfection of HEK293 cells with two vector constructs encoding GFP-NFκβ and ST2 (Schmitz et al., 2005). BAL or tissue homogenates from IL-33$^{-/-}$ mice could then be used to stimulate the transfected cells before assessing for GFP expression and therefore identifying stimulation of the receptor construct. Any signalling through ST2, indicated by a GFP signal, would therefore be generated by a ligand other than IL-33. A further option is the employment of an IL-33 reporter cell system that has recently been developed by Zhao *et al.* (2015).

This group have demonstrated that the internalisation of IL-33 bound ST2 is dependent on the action of glycogen synthase kinase 3β (GSK3β), resulting in the downstream signalling from ST2. In order to determine this, a cell system was developed to express fluorescently labelled ST2 surface receptors which were shown to internalise upon IL-33 binding, a response which was easily visualised by fluorescent microscopy. The utilisation of this cell system would therefore allow us to test the binding capacity of both HDM extract and tissues isolated from IL-33$^{-/-}$ mice initially and then individual molecules. Regardless of the alternative ligand source – endogenous or from the allergen – identification will provide further targets for the development of therapies with the potential to halt the development of allergic airways disease. These therapies could either be used in combination with an α-IL-33 or α-ST2 antibody therapy, dependent on the outcome of receptor binding assays.

7.2.4 Further characterisation of antibody therapies in murine models

Despite the lack of effect on AHR and inflammation observed in OVA exposure models with preventative and therapeutic α-ST2 antibody treatments, subsequent experiments with an alternative dosing route showed a promising prevention of eosinophilic inflammation. It would therefore be beneficial to further test the antibody using a sub-cutaneous dosing rather than i.p - which was used throughout the OVA exposure protocols.

The need for further characterisation of α-IL-13 therapy for the treatment and prevention of severe allergic asthma have also been highlighted. As the two dose therapeutic dosing regimen used in this study did not result in the complete ablation of IL-13 in lung homogenate – as seen with preventative antibody dosing – it was unclear whether the lack of effect on AHR and inflammation was due to the already established allergic airways disease or an inadequate dosing regimen to completely block IL-13. It will therefore be beneficial to extend the period of therapeutic α-IL-13

antibody treatment after the establishment of disease to investigate the reproducibility of improved airway function observed in clinical trials of long term antibody therapy (Brightling et al., 2014).

Despite complete ablation of airway resistance observed in a model of preventative α-IL-13 antibody treatment, both IL-13^{+} T cells and specific IgE were generated in response to Alt exposure. An interesting future experiment would therefore be the investigation of withdrawal of therapy with either continuous exposure to allergen or a one off exposure to Alt. This would determine if α-IL-13 antibody treatment would be required for the lifespan of the patient once started as it does not seem to affect the development of antigen specific immune responses and the patient would therefore be primed to mount a response to allergen exposure.

7.3 Final conclusions

Overall, the studies conducted in this thesis investigated a number of suspected predisposing factors for the generation of allergic airways disease and provided mechanistic explanations for some associations. This study highlighted the need for appropriate experimental models for the investigation of potential therapeutics. In particular, the need to investigate the effect of age on the impact of allergic disease manifestation proved vital. In addition to an enhanced development of AAD in neonatal mice, a natural period of non-responsiveness to allergen exposure was identified. In collaboration with another lab, we were able to link our observations with the development of airway microbiome dependent Tregs in the lung, providing an additional target area for future therapeutics.

The necessity of models using the most appropriate allergen was also demonstrated by the differences observed between Alt and HDM sensitisation. Investigation of Alt compared to HDM induced disease correlated to results in children with SAFS, whereby fungal sensitisation resulted in

more severe, steroid resistant disease, and highlighted a number of potential therapeutic targets with the use of mechanistic mouse models.

Differences between cytokine and receptor knockout models were also highlighted as ST2 and IL-33 knockouts behaved dramatically differently in response to HDM exposure. Therefore, any conclusions determined by the use of $ST2^{-/-}$ models in studies of allergic disease could not be translated to the targeting of IL-33 and any therapeutics designed to target IL-33 would most likely not have the desired effect.

Finally, the importance of clinically relevant models to test potential future therapeutics was highlighted. Although targeting IL-13 completely prevented the development of AAD in response to a potently pathogenic allergen Alt, blocking IL-13 once the disease was established was not beneficial. Future studies of novel therapeutics must therefore include comparisons of the effect of blocking using prevention and therapeutic regimens in order to determine the clinical applicability.

Overall, this study has provided mechanistic evidence for changing disease phenotypes determined by age and the effects of immune maturation, and the potential mechanisms explaining increased disease severity associated with fungal sensitisation. ST2 has been identified as an important target for future therapies targeting the IL-33/ST2 signalling pathway.

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Appendix I – Abstracts related to PhD Thesis

Age of first allergen exposure is critical in determining the development of allergic airway disease.

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The immunological landscape of adult and neonatal lungs are different, while adult lungs are immunologically mature and able to mount both innate and adaptive immune responses, neonatal lungs are developing and undergoing immune maturation. Failure of development of allergic airway disease (AAD) following inhaled house dust mite (HDM) commencing at 14 days of age in neonatal mice has been described. However, we have documented robust AAD in neonatal mice when HDM exposure is commenced from day 3 of life. We hypothesised that age at first allergen exposure is critical in determining the development of allergic immune responses and aimed to investigate disease onset when allergen exposure is commenced at different ages. Intranasal HDM or saline was administered intermittently for three weeks, with the first dose administered at day 3, 7, 14, 21, 28 and 42 of life. Assessments of airway hyperresponsiveness (AHR) to methacholine, inflammation in lung and broncho-alveolar lavage, cytokine mediators of AAD in the lungs, and serum Ig E was measured. HDM exposure at day 3 or 7 of life resulted in the development of AHR, eosinophilic inflammation and increased Th2 cytokines (IL-4 and IL-13) in the lung, with elevated serum IgE. Allergen exposure from day 14 or 21 of life did not result in the development of AAD, with absent AHR and serum IgE along withTh2 cytokines remaining at control levels. The development of AAD in response to HDM exposure was restored when allergen was administered from day 28 and 42 of life. This study demonstrates the critical role of immune maturation and lung development in determining the development of allergic immune responses and the development of neonatal AAD.

Poster presentation at the 11th ERS Lung Science Conference, Estoril, Portugal. Awarded best poster presentation.

Oral presentation at the 11th EAACI Immunology Winter School 2013, Austria.

Paediatric house dust mite induced allergic airways disease is ST2, but not IL-33, dependent.

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Asthma is characterised by reversible airway obstruction, pulmonary inflammation and remodelling. The inflammatory cytokine IL-33 has been strongly implicated in the pathogenesis of asthma. IL-33 is known to act as a chemoattractant for IL-13 secreting T cells and also cause the proliferation of type 2 innate lymphoid cells (ILCs). Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) in both IL-33 and its receptor, ST2, in patients with childhood onset asthma compared to healthy controls. We aimed to investigate the role that IL-33 and ST2 play in a paediatric model of HDM-induced allergic airways disease in neonatal mice. Mice were exposed from day 3 of life to intermittent intranasal house dust mite (HDM) for 3 weeks. Airway hyperresponsiveness (AHR) to methacholine, characterisation of IL-13^{+} immune cells by flow cytometry and quantification of cytokines in lung homogenate was carried out. Surprisingly, IL-33$^{-/-}$ and ST2$^{-/-}$ mice showed strikingly different outcomes to HDM sensitisation. IL-33$^{-/-}$ neonates showed responses to HDM sensitisation comparable to WT mice; with increased AHR, BAL inflammation, lung IL-13, IL-13^{+} ILCs and serum IgE levels. In contrast, HDM treated ST2$^{-/-}$ mice did not develop AHR and had significantly lower BAL inflammatory infiltrates, lung IL-13, IL-13^{+} ILCs and IgE levels, while lung IL-33 levels were elevated to the level of a WT control. We have identified opposing disease outcomes in ST2$^{-/-}$ and IL-33$^{-/-}$ models of allergic airways disease (AAD) that indicate ST2, rather than IL-33 is essential for the onset of neonatal HDM induced AAD. In support of this, by utilising airway epithelial cells, bronchial biopsies and BAL from children with severe asthma, mild asthma and non-allergic controls, we have demonstrated that differential expression of ST2 and its accessory proteins are altered in severe asthma whilst IL-33 remains similar between groups.

Oral presentation at the 12th EAACI Immunology Winter School 2013, Romania.

Appendix II – Publications

Castanhinha, S.*, **Sherburn R.***, Walker S, Gupta A., Bossley C.J., Grychtol R., Maglione M, Koo S., Fleming L., Gregory L., Snelgrove R., Bush A., Lloyd C.M., Saglani S. Paediatric severe asthma with fungal sensitisation is mediated by steroid resistant IL-33. In Press – JACI 2015

Gollwitzer, E.S., Saglani, S., Trompette, A., Yadava, K., **Sherburn, R**., McCoy, K.D., Nicod, L.P., Lloyd, C.M., and Marsland, B.J. (2014). Lung microbiota promotes tolerance to allergens in neonates via PD-L1. Nat. Med. *20*, 642–647.

Saglani, S., Lui, S., Ullmann, N., Campbell, G.A., **Sherburn, R.T**., Mathie, S.A., Denney, L., Bossley, C.J., Oates, T., Walker, S.A., et al. (2013). IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma. J. Allergy Clin. Immunol. *132*, 676–685.e13.