IL-33 and ST2 in innate and adaptive airway inflammation

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Abstract

Background: ST2 has been identified in playing an important role in Th2-mediated inflammation and asthma. IL-33 acts as the ligand for ST2; it is a novel cytokine that induces innate Th2/type-2 responses when delivered to the lung. The hierarchy of IL-33 and type-2 cytokines and chemokines in Th2 inflammation in the lung has not been fully elucidated. Furthermore, the role of IL-33 in the adaptive response in allergic mediated airways disease is unclear.

Epithelial cells (ECs) are increasingly recognised as having an immunological role in airway inflammation and asthma, in particular releasing cytokines such as IL-33. Little is known about whether ST2 is expressed on these cells and what function IL-33 responsive ECs may have in Th2 diseases.

Soluble ST2 (sST2) has emerged as a biomarker correlating with disease activity in cardiovascular disease. It is not known if there is a clear association between sST2 and asthma, nor whether measurable IL-33 concentrations are present and if so, their association with disease severity. The influence of smoking and corticosteroid treatment on these parameters has also not been determined.

Aim: To ascertain the levels of systemic sST2 and IL-33 in asthmatic patients. To determine cytokine, chemokine and airway dynamics of IL-33-driven innate airway inflammation. To determine the role of epithelial cells in IL-33-driven innate airway inflammation. To investigate the function of ST2/IL-33 axis in the innate and adaptive responses in allergic airways inflammation and asthma.

Methods and Results: sST2 and IL-33 levels in plasma of never smokers, ex-smokers and smokers were determined by immunoassay before and after a corticosteroid trial. Corticosteroid treatment resulted in increased sST2 levels in all smoking status patient groups; there was no effect attributable to smoking.

Time course and dosage interval experiments were performed in mice treated with intranasal IL-33. IL-5, IL-13, eotaxin/CCL11 and eotaxin2/CCL24 mediated eosinophilic airway inflammation (AI). Treatment of mice with both anti- CCL11 and -CCL24 partially ameliorated the AI. IL-4 gene deficient mice were

protected from IL-33-induced inflammation. BALB/c mice displayed airways hyperreactivity following IL-33 treatment.

Murine, human cell line and primary human ECs were assessed for ST2 expression by immunohistochemistry and fluorescence activated cell sorting (FACS). ST2 expression was clearly demonstrated in the ECs. Subsequently ECs were treated with IL-33 in an *in vitro* setting including in a pseudostratifed epithelium model. ECs produced a range of inflammatory and angiogenic mediators in response to IL-33. In particular IL-33 driven EC-derived VEGF promoted angiogenesis *in vitro*. Intranasal IL-33 induced increased endothelial cells and vascular remodelling *in vivo*.

Experimental allergic airways inflammation (AAI) was generated in BALB/c mice which were co-treated with IL-33 or PBS at allergen sensitisation. IL-33 induced the polarisation of IL-5^{+}IL-4^{-} T cells in the draining lymph nodes and these mice developed more severe inflammation.

AAI was induced in WT, ST2-deficient, IL-4-deficient and ST2/IL-4 deficient mice. These experiments showed IL-4 was necessary for generation of AAI which could not be overcome by ST2-pathway stimulation in an adjuvant-free model.

Conclusions: The data presented further extends the current understanding of the ST2/IL-33 axis in the innate and adaptive aspects of Th2 inflammation in AAI and asthma. In particular the hierarchy of mediators and cells involved in Th2 inflammation, including at the sensitisation phase, have been explored. This identifies ST2/IL-33 as a potential target in the development of biological therapies for asthma.

Table of Contents

List of Tables

List of Figures

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Author's declaration

I declare that, except where explicit reference is made to the contribution of others by the provision of reagents or practical support, that this thesis is the result of my own work. Specifically data in sections 4.2.3 to 4.2.5 and 4.6.1 and 4.6.2 were performed equally and in collaboration with Dr Nick Pitman and presented in Pitman, N (2009), PhD Thesis, University of Glasgow. The remainder of the work presented has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature……

Printed name……..

Abbreviations

$^{-/-}$	knockout
$^{+/+}$	wildtype
7-AAD	7-Amino-actinomycin D
AAI	allergic airway inflammation
AAM	alternatively activated or M2 macrophages
ABC	Avidin-biotin complex
ACQ	Juniper Asthma Control Questionnaire
AEC	Airway epithelial cells
AFO	airflow obstruction
Ag	antigen
AHR	airways hyperresponsiveness
AI	airway inflammation
ALI	air-liquid interface
Alum	aluminium hydroxide
ANOVA	Analysis of variance
APC	antigen presenting cell
ASM	airway smooth muscle
BAL	bronchoalevolar lavage

BALB/c	Bagg Albino
bFGF	basic fibroblast growth factor
BM	basement membrane
BMMC	bone marrow-derived mast cells
bp	base pair
BSA	bovine serum albumin
BTS	British Thoracic Society
CCL	C-C motif ligand
CCR	C-C motif receptor
cDNA	complementary DNA
CD	cluster of differentiation
c-kit	stem cell factor receptor receptor tyrosine kinase
CM	complete medium
CS	corticosteroid
CSR	class switch recombination
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAMP	damage associated molecular patterns
DC	dendritic cell
DEPC	diethyl pyrocarbonate

DLN	draining lymph nodes
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DPX	di-n-butylPhthalate in xylene
DTT	dithiothereitol
e.c	epicutaneous
EC	epithelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacedic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EMTU	epithelial-mesenchymal trophic unit
ERK	extracellular signal-regulated kinase
EP	eosinophil peroxidase
EPC	endothelial progenitor cell
FACS	fluorescence-activated cell sorting
FEV	forced expiratory volume
FCS	foetal calf serum
FSC	Forward scatter

GATA	trans-acting T-cell-specific transcription factor
GINA	Global Initiative for Asthma
GM	growth medium
GM-CSF	granulocyte-macrophage colony-stimulating factor
H&E	haematoxylin and eosin
HBSS	Hank's Balanced Salt Solution
HDAC	histone deacetylase
HDM	house dust mite
HF	heart failure
HMGB	high motility group box
HRP	horseradish peroxidase
HUVEC	Human Umbilical Vein Endothelial cells
IC	intracellular
ICS	inhaled corticosteroid
ICOS	inducible T-cell costimulator
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
IL-1RAcP	IL-1 receptor accessory protein

ILC	Innate lymphoid cell
i.n.	Intranasal
iNKT	invariant NKT
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IP-10	interferon gamma-induced protein 10
IRAK1	IL-1R associated kinase 1
i.v.	intravenous
JNK	c-Jun N-termainal kinase
kDa	kiloDalton
LPS	Lipopolysaccharide
LVEF	Left ventricular ejection fraction
LXA4	Lipoxin AX4
MBP	Major basic protein
MC	Mast cell
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
MMP	matrix metalloproteinase
MI	Myocardial infarction

mRNA	messenger ribonucleic acid
MTT	3,[4,5-dimethykthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MyD88	myeloid differentiation primary response protein 88
NBF	neutral buffered formalin
NF-kB	nuclear factor-kB
NHBE	normal Human Bronchial Epithelial
NK	natural killer
NKT	natural killer T
NLRP3	nod-like receptor family pyrin domain containing 3
NO	nitric oxide
NRAD	National Review of Asthma Deaths
NSTEMI	non-ST elevation myocardial infarction
OCT	optimum cutting temperature medium
OVA	ovalbumin
PAMP	pathogen associated molecular pattern
RANTES	regulated on activation, normal T cell expressed and secreted
PAS	Periodic Acid-Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PEF	Peak Expiratory Flow
Penh	enhanced pause
pHBE	primary human bronchial epithelial cells
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
RAG	recombination-activating gene
RNA	ribonucleic acid
RT	room temperature
Sca	stem cell antigen
SEM	standard errors of the mean
SIGN	Scottish Intercollegiate Guideline Network
SLE	systemic lupus erythematosus
SSC	side scatter
ST2	Shoji Tominaga 2
sST2	soluble sST2
ST2L	longer ST2
STAT	signal transducer and activator of transcription
STEMI	ST elevation myocardial infarction
TAE	tris-acetate-EDTA

T-bet	T-box transcription factor
TBST	tris buffered saline
TEER	Trans-epithelial electrical resistance
TG2	transglutaminase 2
TGF	transforming growth factor
TCR	T cell receptor
Th	T helper cell
TIR	Toll-IL-1R
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
TRAF	TNF receptor associated factor
TReg	regulatory T cells
TSLP	thymic stromal lymphoprotein
UA	uric acid
UK	United Kingdom
UV	ultraviolet
vWF	von-Willebrand factor
VEGF	vascular endothelial growth factor

1 Introduction

1.1 Asthma

1.1.1 Clinical asthma and epidemiology

Asthma is a chronic inflammatory condition of the airways. It is defined clinically by reversible airflow obstruction (AFO) and results in symptoms of wheeze, breathlessness and cough. The cardinal pathophysiological features are airway hyperresponsiveness (AHR) and airway inflammation (AI), the latter giving rise to mucus hypersecretion. Furthermore, structural changes and lung remodelling are now recognised as increasingly important features[1].

The global burden of asthma is staggering with as many as 300 million people worldwide estimated to suffer from asthma according to The Global Initiative for Asthma 2014 (GINA)(www.ginasthma.org) and the worldwide incidence is increasing (World Health Organisation, www.who.int/en/). As well as the effect on the individual, huge healthcare cost and economic impact ensures it is a serious global healthcare issue. In the United Kingdom (UK) alone, 5.4 million people (www.asthma.org.uk) are currently receiving treatment for asthma. The National Review of Asthma Deaths (NRAD) attributes approximately 1200 deaths each year in the UK to asthma, a figure which has not changed in many years (www.rcplondon.ac.uk); 90% of these are potentially preventable. In addition to understanding how life-threatening conditions arise, healthcare policies are targeted to improve the quality of life for people with this long term condition. Locally, the Scottish Government considers asthma one of the 4 main health challenges facing children today (www.scotland.gov.uk).

Susceptibility to the development of asthma is controlled by genetic and environmental factors. Risk factors include family history, premature or low birthweight, viral infection in early childhood, maternal smoking and sex (male for development of asthma in children and female for persistence of asthma into adulthood). There is up to a 30 fold difference in prevalence between countries with a trend towards an increase in developed nations. Socioeconomic factors may play a role with the incidence of asthma highest amongst low-income populations (www.isaac.auckland.ac.nz/).

Despite efforts to raise awareness and improve education amongst patients and healthcare providers, suboptimal control is widespread. One contributing factor to this is that only recently has asthma been recognised as a heterogeneous condition and a number of asthma phenotypes exist within the spectrum of chronic airways disease; therefore, some phenotypes may not be appropriately treated[2].

The most common classification of asthma is based on the level of asthma control. Other clinical descriptions are used such as brittle, refractory, late-onset or steroid-resistant phenotypes. Classification can also be based on pathological type: atopic (extrinsic) or non-atopic (intrinsic) being one historical distinction [3]. Atopic asthma is defined by the presence of elevated serum immunoglobulin (Ig)-E antibody to common inhalant allergens and accounts for approximately 55-80% of cases [4]. Atopic asthma has a genetic predisposition and tends to develop at a younger age[3]. Targeted treatment to selected patients in this phenotypic group with anti-IgE therapy has been very successful [5,6]. However, only one third of individuals with atopy and raised IgE have asthma [1]. Furthermore, the pathological changes seen in atopic and non-atopic asthma are similar and these relationships are not fully understood [7].

Other phenotypes can be considered on the basis of the predominant inflammatory cellular infiltrate such as eosinophilic or neutrophilic asthma[8,9], or other physiological variables such exhaled nitric oxide concentration [10]. All of these serve as biomarkers and have been evaluated as the basis for directed treatment. Overall, asthma comprises multiple and incompletely understood clinical phenotypes and clinico-pathological correlates. Thus efforts to understand these phenotypes on a basic and cellular level are paramount with the ultimate aim of targeted treatments and promoting the concept of individualised care.

1.1.2 Management of asthma

The mainstay of asthma management is aimed at suppressing chronic airway inflammation, predominantly by using inhaled corticosteroids (ICS), and relieving acute bronchoconstriction with inhaled bronchodilators. Guidelines produced by the British Thoracic Society (BTS) and Scottish Intercollegiate Guideline network

(SIGN) aid physicians in the management of acute and chronic asthma based on a step-wise approach (www.sign.ac.uk).

Corticosteroid (CS) therapy is the most effective anti-asthma treatment currently available and its mode of action switches off gene transcription for multiple inflammatory pathways; the molecular basis of these mechanisms is still unresolved [11]. One view is that CS recruit the nuclear enzyme histone deacetylase 2 (HDAC2) to hyperacetylated inflammatory genes leading to deacetylation and reduced inflammation [11]. Clinically, CS resistance in severe asthmatics has been linked to impairment in the function of (HDAC2) [12].

The main limitation of steroids is their side effects although this is somewhat mitigated by using inhaled preparations. However, a proportion of asthmatics, around 5%, require regular oral steroids yet despite this they often remain symptomatic [13]. The economic impact of this small proportion is substantial [14]. Other asthmatic patients are relatively resistant to steroid effects and this is particularly the case for smokers [15]; more concerningly, worsening of neutrophilic asthma with CS has been described [16]. A number of other immune modulating drugs (cyclosporine, tacrolimus, methotrexate, azathioprine) have been trialled with varying degrees of success, but all carry a significant side effect profile [17]. Omalizumab is a monoclonal antibody against IgE that is the only licensed treatment for severe asthma. Thus there is a significant unmet clinical need for novel therapies, which in turn requires research and suitable disease models.

Other non-biological treatments currently available for asthma include: anticholinergics; leukotrine receptor antagonists; cromoglycates (mast cell stabilisers); and theophylline (a bronchodilator).

1.1.3 Immune responses in asthma

A complex interaction of genetic and environmental factors is thought to give rise to asthma. In atopic asthma, this interaction somehow drives the immune response to direct an active response resulting in inflammation to an otherwise innocuous antigen. Since the Mossman classification of adaptive T helper (Th) cells as Th1 (mainly Interferon-γ [IFN-γ] producing) or Th2 (mainly type-2

cytokine producing) [18], this immune response has been described as predominantly Th2 driven. Further sub-classification is now suggested based on the degree of Th2-inflammation, dividing into Th2-low and Th2-high phenotypes [19]. A multitude of inflammatory and immune cells and mediators have been described in the development and persistence of asthma. The following sections outline in more detail the evidence for some of these and their role in asthma, whilst acknowledging there is obviously cross-talk between cells and the importance of their individual roles is debated.

1.1.3.1 Adaptive immune system in asthma

Adaptive immune responses are antigen-specific and exhibit memory. Specificity is achieved by the random recombination of genes in the T-cell receptor (TCR) and the somatic hypermutation of the variable region of immunoglobulin genes that produce antibodies.

1.1.3.1.1 T lymphocytes

There are increased numbers of cluster of differentiation 4 (CD4) T cells in the airways of asthmatic patients. These are predominantly Th2 cells as opposed to predominating Th1 cells found in the normal airway [20]. These Th2 cells secrete Th2 cytokines; for example interleukin (IL)-4, IL-5 and IL-13 [21]. However, they are not the only cells that contribute to Th2 or type-2 cytokine production and inflammation.

Studies in animal models further demonstrate a role for Th2 cells in asthma. Transfer of antigen-specific T cells into antigen-naïve mice induces AI and AHR [22]. In models of allergic airways inflammation (AAI), transfer of CD4 cells into recombination-activating gene (RAG) deficient mice deficient in an adaptive immune response, restores the ability of these mice to develop AI and AHR when sensitised to allergen [23]. In support of this, CD4 cell depletion in wild-type mice impairs the induction of AI and AHR [24]. However, this effect is not sustained in models of chronic inflammation and there is no effect on subsequent remodelling [25].

Further evidence for involvement of CD4 cells in asthma comes from increased understand of their molecular signalling. T-box transcription factor (T-bet) is a key Th1 lymphocyte transcription factor and its expression is reduced in the airway cells of asthmatics. Experimentally, mice gene-deficient in T-bet show spontaneous features of Th2 inflammation and remodelling [26]. Similarly, trans-acting T-cell-specific transcription factor (GATA) binding protein 3 (GATA3), the key Th2 lymphocyte transcription factor was increased in airway cells of asthmatics [27] and blockade of this molecule in mice prevented the development of AHR and AI [28].

Other Th subsets have been implicated in asthma and these likely reflect the potential phenotypic difference previously mentioned. The detection of IFNγ in the bronchoalveolar lavage (BAL) T cells has led to the possibility that Th1 cells may have a role in some phenotypes of asthma [29]. Novel Th subsets including Th17 cells which secrete interleukin (IL)-17 and may be particularly important in the neutrophilic asthma phenotype [30] and Th9 cells, which secrete IL-9, have been linked to asthma [31].

$CD4^+CD25^+$ regulatory T cells (TReg) can suppress both Th1 and Th2 development and function [32,33]. Some investigators have found reduced numbers or function of TRegs in asthmatics [34]. One study of T cell phenotypes found that $CD4^+CD25^+$ cells with correlated Foxp3 messenger ribonucleic acid (mRNA) expression in 2% of blood and 6% of BAL cells in asthmatic patients suggesting a role for Tregs. These cells were increased in patients treated with CS suggesting that CS effects are in part mediated by Treg cells secreting IL-10 [34]. Experimentally, $CD4^+CD25^+$ cells were seen to reverse AI and moreover prevent the development of airway remodelling, suggesting there may be substantial gain from investigating the mechanisms of these cells [35].

Another subset of CD4 T cells, the invariant natural killer T (iNKT) cell might account for a significant proportion of the $CD4^+$ cells described in asthmatic airways as these cells also produce the Th2 cytokines IL-4 and IL-13. Approximately 60% of CD4 T cells noted in the bronchial biopsies from asthmatic patients were iNKT cells [36]. However, this was not confirmed in a further study so the presence and role of these cells remains uncertain [37].

CD8 cells are present in the asthmatic airway in patients with more severe disease and irreversible airflow obstruction. These cells include cytotoxic T cell (Tc)1 or Tc2 and may reflect a change in phenotype skewing towards a type-1 response at this end of the clinical severity spectrum [21].

1.1.3.1.2 Th2 inflammation

In addition to the canonical Th2 cytokines IL-4 and IL-5, additional cytokines that can be produced by Th2 cells have been described and include IL-13, IL-6 and IL-9. Th2 cytokines can also be produced by many other cells. In the following section these cytokines that contribute to the resulting Th2 or type-2 inflammation in this distinct cytokine pattern will discussed regardless of the cellular source.

Type 2 cytokine concentrations are increased in sputum [38] as well as in airway lavage from asthmatics [39]. Similarly type-2 cytokine mRNA expression is increased in asthmatic bronchial biopsies [40]. Genome-wide searches have provided evidence of linkage of asthma to the 5q31-33 region, which contains the Th2 cytokine cluster (IL-4, IL-13, IL-5, and IL-9) [41].

Originally described as a B cell growth factor, and for its ability to initiate class-switching from IgM to the Th2 isotypes IgG1 and IgE in mice, IL-4 was subsequently described in relation to Th2 development [42,43] and involved in eosinophil chemotaxis. B lymphocytes and IgE will be further discussed in section 1.1.3.1.3 and Th2 development in section 1.1.3.1.3.

Interleukin-4 can be secreted by Th2 cells, mast cells, basophils, eosinophils and alveolar macrophages [44]. In mice, administration of IL-4 directly into the airway results in features of AI and AHR; moreover, in IL-4 receptor (IL-4Rα) knockout mice, this effect was lost [45]. However, as this cytokine shares a receptor subunit with IL-13, the lack of response could be attributed to the loss of IL-13 effects. In the allergic airways model, IL-4 knockout ($^{-/-}$) mice had reduced but not abolished AI and AHR compared to their wildtype ($^{+/+}$) comparators [46]; the effect on AHR in has been replicated by some but not by all studies [47]. Transfer of IL-4$^{-/-}$ or IL-4 $^{+/+}$ Th2 cells into IL-4$^{-/-}$ showed IL-4 was required for eosinophilic inflammation but not for AHR [47].

These findings have led to clinical trials directed at antagonising IL-4 with anti-IL-4 monoclonal antibodies or a soluble recombinant receptor but also by binding targeting the IL-4Rα subunit with the protein Pitrakinra and the monoclonal antibody dubilumab. The latter of these has been the most promising in advanced clinical trials with improved success likely as the target inhibits both IL-4 and IL-13 activity [48,49].

As partial redundancy in the function of IL-4 was found in IL-13, focus of Th2 cytokine involvement in asthma has been directed towards the role of IL-13 as a key regulator in pathogenesis of asthma. As noted above, IL-13 binds to the α-chain of the IL-4 receptor. IL-I3 is located on chromosome 5q31 and has been linked to asthma in genetic linkage studies. Specifically, the IL-13 R110A genetic polymorphism has been linked to asthma or allergy [50]. IL-13 is present in the sputum[51] and BAL of asthmatics [52].

IL-13 shares some functionality with IL-4; overexpression or administration of IL-13 induces asthma-like phenotype namely, AI, mucus hypersecretion, chemokine induction (e.g. CCL11/eotaxin), airway fibrosis and AHR by increasing contractility of airway smooth muscle (ASM) [53,54]. In comparison, neutralisation of IL-13 abrogates these features as well as reducing associated airway remodelling [54]. A number of downstream pathways for IL-13 effects are described: via signal transducer and activator of transcription (STAT)-6 dependent effect on epithelial cells for AHR and mucus production driven by IL-13 [53]; via chitin from epithelial cells or macrophages [55]; via or in association IL-5/eotaxin(CCL11)-dependent eosinophil recruitment [56]; or IL-5 /eotaxin(CCL11)-independent AHR [57]. IL-13 is produced in large quantities by CD4 Th2 lymphocytes and innate lymphoid cells (ILC), and lesser amounts by natural killer cells, mast cells and basophils [54,58].

Human clinical trials examining the role of IL-13 antagonists in the treatment of asthma and related allergic diseases are in development. Along with the Pitrakinra trials detailed above, monoclonal antibody trials are in phase I and II clinical trials. Beneficial effects are seen in particular on bronchial hyperresponsiveness. Indeed, Lebrikizumab in a phase II trial (MILLY) showed more improvement in lung function of a subgroup of patients with high levels of periostin, an extracellular matrix protein produced by bronchial epithelial cells,

indicating a further biomarker that can aid individualised targeted treatments [59]. Other Phase II trials have shown benefit and Phase III trials are underway (Table 1.1).

Interleukin-5 is inextricably linked to eosinophils and controls their growth, differentiation and activation [60]. In animal studies, over-expression of IL-5 leads to pathological changes characteristic of asthma [61] and deficiency of IL-5 minimises eosinophilia and AHR [62].

IL-5 mRNA is found at increased levels in cells from the sputum, bronchial biopsies as well as elevated concentrations of IL-5 in the serum of asthmatic patients; this correlates with disease severity [63-65]. Inhalation of IL-5 results in sputum eosinophilia and AHR [66]. The primary source of IL-5 in the airways includes Th2 cells, ILC, eosinophils and mast cells [58,67].

In terms of biological therapy, as with IL-4 and IL-13, there have been a number of clinical trials targeting IL-5. Early trials have shown these agents (mepolizumab, resilizumab) are effective in reducing eosinophilia but were not associated with a reduction of symptoms [68]. More recently, three trials of Mepolizumab in patients with a particular asthma phenotype consisting of eosinophilia and frequent exacerbations, demonstrated clinical improvements along with a reduction in sputum and blood eosinophilia and a reduction in exhaled nitric oxide (NO); an easily measurable biomarker used in the DREAM trial [69,70]. Other promising Phase III studies involving antibodies in these selected patients have just been published providing an opportunity for future licensed therapy (Table 1.1).

Interleukin-9 and the IL-9 producing T cell subset Th9 play a role in the promotion and regulation of asthma [31]. IL-9 promotes the production of epithelial growth factor, the proliferation of goblet cells and increased mucus production. IL-9 mRNA is expressed more highly in bronchial biopsy studies in asthmatics and levels correlated with AHR [71]. *In vivo*, overexpression of IL-9 results in features of asthma developing in mice [72]. These findings have led to the development of clinical trials antagonising IL-9. These recent trials have shown a trend towards efficacy but further trials to confirm this are awaited [73]. More recently, a link between IL-9, mast cell function and fibrosis has been

described in a chronic inflammation model suggesting that extended therapeutic inhibition of IL-9 may be necessary to observe significant changes[74].

Target	Biological Drug	Clinical trial stage	Outcome
Omalizumab	Binds free IgE	Approved; in use	↓Exacerbation, symptoms. ↑quality of life
Pascolizumab	Blocks IL-4	Phase II	No benefit
Altrakincept	Soluble IL-4R	Phase II	No benefit
Pitrakinra	Inhibits IL-4Rα binding	Phase II	↓Bronchoprovocation
Dupilumab	Inhibits IL-4Rα binding	Phase II/III	↓Exacerbation
Tralokinumab	Blocks IL-13	Phase I/ II	↓Eosinophilia
Anrukinzumab	Blocks IL-13	Phase II	↓Decrease late asthmatic response
Lebrikizumab	Blocks IL-13	Phase II	↑ Lung function if high periostin (MILLY)
		Phase III	Awaited (NCT-01868061,1867125, 01875003, 01545440 (LUTE), 01545453(VERSE)
Bendralizumb	Blocks binding to IL-5Rα	Phase I/II	↓Blood eosinophilia
Reslizumab	Blocks IL-5	Phase II	↓Sputum eosinophilia and improve lung function (primary outcome exacerbations no benefit)
		Phase III	Awaited
Mepolizumab	Blocks IL-5	Phase II	↓Eosinophilia, steroid use, exacerbations (DREAM)
		Phase III (Sept 2014)	↓Exacerbations (MENSA trial) and steroid use (SIRIUS trial)
MEDI-528	Blocks IL-9	Phase II	↓Exercise related bronchoconstriction
Infliximab	Blocks TNFα	Phase II	↓Exacerbations
Etanercept	Soluble TNFα receptor	Phase II	↓AHR and ↑quality of life. Safety issues
Golimumab	Blocks TNFα	Phase II	Terminated-safety issues
Lumiliximab	Binds CD23	Phase I	Safety study

Table 1.1: Biological drugs used in the treatment of asthma

1.1.3.1.3 B lymphocytes and IgE

The serum substance first described in relation to allergy and development of type 1 hypersensitivity reactions was subsequently identified as IgE [75].The finding of elevated concentrations in the serum of asthmatics led to research into its role in asthma [76]. The role of B cells in asthma has not been studied as extensively as that of T cells but their ability to produce IgE means they occupy a place in asthma pathophysiology.

In sensitised individuals, the majority of IgE is bound to the surface of mast cells and basophils by the high affinity IgE receptor FcεRI [77]. Allergen crosslinking of this bound IgE leads to receptor mast cell or basophil degranulation with release of preformed mediators and the initiation of an rapid inflammatory cascade [77]. Most asthmatics have increased levels of circulating IgE and this correlates with severity of disease [78].

In murine models of allergic airway inflammation, IgE appears to be a requirement for AHR and eosinophilia [79]. However, others have found that the requirement for IgE was not obligatory[80] but it is likely that the choice of model and mouse strains used have influenced the findings and this may have relevance to different asthma phenotypes.

Anti-IgE therapy has unambiguously confirmed the role of IgE in the pathogenesis of allergic asthma. Omalizumab is currently the only anti-IgE biologic approved for the treatment of moderate-to-severe persistent asthma in adults and children 12 years and older, whose symptoms are inadequately controlled with inhaled CS. Omalizumab is a recombinant DNA-derived humanised monoclonal antibody to the Fc portion of the IgE antibody that prevents the binding of IgE to FcεRI, thus blocking FcεRI cross-linking and reducing the release of inflammatory mediators in response to allergen exposure. Three large randomised control trials have shown a significant decrease in the incidence of asthma exacerbations as well as in hospitalisations and ICS usage in asthmatics given omalizumab [81-83]. In addition, significant improvements in quality of life measures were seen[5] (Table 1.1).

In allergy, the respiratory tract mucosa is a major source of IgE production [77]. IgE synthesis as well as class switch recombination (CSR) of antibody class to IgE in B cells can occur [84]. Non-atopic asthmatics can also synthesise IgE in the bronchial mucosa but the specificity of this IgE antibody is unclear [84]. Nethertheless, trials of omalizumab in non-atopic asthma are ongoing and should answer some questions about IgE in these particular asthmatics with early results showing a possible benefit [85].

In the absence of IL-4, IL-13 promotes IgE class switching [54]. However, in animal models it has been shown that IL-4/13 may not actually be essential in IgE generation under certain conditions [86].

In addition to IgE production, B lymphocytes may have further roles including production of other immunoglobulin isotypes and cytokines as well as in antigen presentation. To consider this, in experiments using B cell deficient mice with impaired IgE production, no requirement was found for B cells in the development of eosinophilia or AHR in an ovalbumin model of AAI [87]. However, a requirement for B cells in the development of AHR is unclear with contradictory results identifying a role for B cells in AHR in other AAI models [88] [89]. Different sensitisation protocols used in these models suggests this can influence the development of AHR.

1.1.3.1.4 Allergen sensitisation

There is growing interest in the early allergen sensitisation phase of allergic disease. As antigen priming is likely to occur early in life, a therapeutic strategy at this stage seems implausible. However, the possibility to intervene before development of allergy is still intriguing. With the increasing development of biomarkers, it is entirely possible that sensitisation could be detected in children, for example by measuring increased CD23 (low affinity IgE receptors) or serum IgE concentrations [77]. Furthermore, our increased understanding in this area could help to define therapeutic targets and inform immunotherapy strategies aimed at inducing immunological tolerance [90].

Many factors affect the likelihood of developing clinically significant sensitisation: type of allergen, allergen concentration, pattern of allergen

contact; co-exposure to other agents including endotoxins, chitin, environmental pollutants; and host genotype [91].

Defects in epithelial barrier function, including the inherited *FLG* mutation which encodes for filaggrin, a protein involved in barrier function, are associated with increased risk of developing asthma presumably by increasing exposure to allergens [92].

As the allergen enters the tissue either via a disrupted epithelium or using endogenous protease activity, it is taken up by a local antigen presenting cell (APC), most likely a dendritic cell. Activated dendritic cells (DC) migrate to regional lymph nodes or local mucosa where peptides derived from the processed allergen are presented in the molecular context of major histocompatibility complex (MHC) class II molecules to naïve T cells. In the presence of IL-4, these cells develop characteristics of Th2 cells, which go on to produce Th2 cytokines and stimulate B cell CSR [93]. Professional APCs require the ability to process and present MHC class 2 restricted peptide and to provide second-signal co-stimulation and initiate responses in antigen-naïve T cells.

As discussed above, IL-4 appears to be a key cytokine in the early stages of allergen activation including by the priming of Th2 cells and their initial differentiation [93,94]. The cellular source of the early IL-4 is unclear but basophils, mast cells, esoniophils, NKT cells and T cells are candidates [95-99].

1.1.3.2 Innate immune system in asthma

Unlike adaptive immune responses, innate immune responses are immediate and efficient but have no ability for memory. The respiratory tract is accessible to environmental elements and the non-specific barrier epithelium is the first line of defence. The innate system also has to some extent specific responses, facilitated by a more recently recognised receptor network of pattern-recognition receptors (PRRs) [100]. Furthermore, the innate immune system also has an important role in shaping the adaptive response.

To sense immediate danger or injury, virtually all cells express PRRs which bind to pathogen associated molecular patterns (PAMPs), highly preserved motifs on

pathogens, but also endogenous damage associated molecular patterns (DAMPs) [100]. PRRs consist of membrane and cystolic located forms. The Nobel Prize winning discovery of the membrane associated PRR Toll-like receptor (TLRs) by Hoffmann and Beutler, have been identified in many species [101]. There are numerous TLRs (1-13) and once activated, lead to a MyD88-dependent or TRIF-dependent pathway and downstream gene expression [100,101]. Defects in many TLRs are correlated to disease including asthma [102].

In Th2 development from naïve T cells discussed in section 1.1.3.1.4 above, the T cell requires: priming into an effector antigen-specific T cell population and; differentiation, determining the nature of the T cell responses which is dependent on the surrounding conditions and cytokine environment. TLRs may influence these processes. PAMPs induce TLR signalling on immature DCs providing signals for priming and promoting Th2 differentiation by secretion of Th2-stimulating or non-secretion of Th1 stimulating factors [100,103]. TLRs can have dual contrary roles exemplified by the TLR2 agonist, Pam3CSK4: when this is given during sensitisation, it worsened murine AAI; and when given to established airway inflammation Pam3CSK4 attenuated the AAI [104]. When high dose lipopolysaccharide (LPS) a TLR4 agonist is administered intranasally to mice with established AAI, it is associated with downregulated Th2 responses [105]. However, at low LPS doses, Th2 responses actually predominate [106] and moreover, LPS is required for Th2 priming [103]. Hence the activation of Th1 or Th2 development is likely to depend on timing, dose and nature of TLR agonist as well as genetic background [103].

A further mechanism for TLR influence on the adaptive immune response is via regulation of co-stimulatory molecules on APCs. Along with signals on MHC and co-receptor, T cell and DC engagement is enhanced by a 3rd signal from the Notch family of receptors on T cells. Notch ligands on the APC are upregulated by different stimuli and interaction of the ligand Jagged with Notch induces IL-4 production in T cells [107]. This engagement of Notch at the surface of the T cell with the Jagged on the DC is up regulated by PAMPs[106].

1.1.3.2.1 Macrophages and dendritic cells

As the most abundant inflammatory cell in the lung, the role of macrophages in the pathogenesis of asthma must be considered [108]. Along with their predominant role in maintaining homeostasis and a sterile airway by phagocytosing and removing pathogens and particles, they also direct immune response by cytokine release and can present antigen. Pulmonary macrophages are divided into subpopulations by location: as intravascular, interstitial or alveolar. More recently a distinction by activation is described: classically activated (or M1) macrophages and alternatively activated (or M2) macrophages (AAM). M1 macrophages are activated by TLR ligands and by IFN-γ, express pro-inflammatory cytokines and produce inducible nitric oxide synthase (iNOS). M2 macrophages are stimulated by IL-4 or IL-13 and produce arginase instead of iNOS, and express the scavenger mannose receptor CD206 and IL-4Rα on their surface [109]. In mice, the role of macrophages in asthma models is not clear. Some studies have shown depletion reduces AI and AHR [110] whilst others have shown depletion exacerbates AI [111] or that M2 are responsible for persisting inflammation [112]. Recent developments suggest there is plasticity in macrophage phenotypes depending on the prevailing inflammatory conditions which could reconcile these contradictory results [113].

Dendritic cells are the professional APC and transport antigen to the draining lymph node[114]. Increased numbers of DCs are seen in the airway of atopic asthmatics [115]. The murine lung DC population consists of a variety of DC subsets. Myeloid DCs promote T cell priming [114] whereas plasmacytoid DCs promote regulatory T cells and tolerance [116]. The nature of the DC that determine their role in Th1 or Th2 differentiation include the type of DC[117], the types of cytokines produced by the DCs such as IL-12 and IL-6[118], the maturity of the DC and surrounding environment [119] as well as costimulation provided by the DCs to T cells [114]. A further role for DCs is in the effector stage of airway inflammation, by sustaining AI; this is demonstrated by abrogated airway eosinophilia and AHR in mice lacking DCs during antigen challenge [120].

1.1.3.2.2 Eosinophils

Airway eosinophilia is a hallmark feature of the allergen-induced late-phase response in asthma although the pathogenic role of eosinophils is debated. A number of early post mortem studies described the presence of these cells in airways of patients who died of asthma [121]. Subsequently, eosinophil numbers have been found to be elevated in sputum, BAL, biopsies and peripheral blood in 50-80% of asthmatics, compared with normal controls [8,122,123]. Furthermore, sputum eosinophilia correlates with asthma severity and treatment directed at normalisation of the sputum eosinophil count reduces asthma exacerbations and admissions, without the need for additional anti-inflammatory treatment [8].

IL-5 is critical for eosinophil recruitment, differentiation, proliferation and maturation and its role in asthma has been discussed in section 1.1.3.1.2 [60,124]. IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and eotaxin along with a number of adhesion molecules are also important in the development, adhesion and survival of eosinophils [60,124]. The question of whether eosinophils are an effective treatment target in asthma cannot currently be fully answered. In clinical trials, anti-IL-5 treatment did not alter AHR following allergen challenge despite significantly reduced blood eosinophilia[68]. However, tissue eosinophil numbers were lowered by 60% leaving a potentially clinically significant proportion of these cells [125].

On activation eosinophils can: contribute to the 'respiratory burst' by rapidly releasing highly reactive oxidative species [126]; and degranulate releasing pre-formed proteins such as major basic protein (MBP) eosinophil cationic protein (ECP), or enzymes such as eosinophil peroxidase (EP) and lipid eicosanoids [127]. They can also produce Th2 cytokines and have potential to regulate Th2 cells in an antigen dependent manner [128].

In asthma, the presence of eosinophilic inflammation and associated inflammatory products correlate with AHR and disease severity [129]. Experimentally, there is conflicting evidence about an association with AHR that is predominantly explained by differences in model or mouse strain used. Dissociation of eosinophils and AHR is seen in △dbl-GATA mice, deficient of eosinophils, where similar mucus and AHR levels were measured compared to

wildtype (WT) in an AAI model [130]. Alternatively, others have seen an association of eosinophils with AHR in PHIL mice, another eosinophil deficient mouse [131].

In addition to the effector roles of eosinophils in asthma, their repertoire of potential pathological functions is increasing. Shi *et al* showed that eosinophils can act as antigen processing cells, by processing allergen in the airway and travelling to regional lymph nodes in mice [132]. Furthermore, these allergen containing eosinophils activate naïve T cells [133]. In particular, a role in remodelling is now described. Mice given a prolonged antigen challenge have sustained tissue eosinophilia and features of remodelling that persist without further challenge [134]. Furthermore, mice genetically deficient of eosinophils were protected from remodelling in a prolonged airway challenge model [130]. In clinical asthma, patients with severe asthma who had mucosal eosinophilia, exhibited deposition of collagen in the lamina reticularis [135] and in milder asthmatics, in comparison to non-eosinophilic groups or healthy controls, the eosinophilic group had increased thickness of the subepithelial collagen layer [123]. In support of this association of eosinophils and remodelling, patients given anti-IL-5 treatment had reduced extracellular matrix (ECM) deposition [125]. The mechanism for this appears to be due to eosinophil production of transforming growth factor(TGF)-β. TGFβ mRNA levels are raised in bronchial mucosa and are associated with eosinophilia and fibrosis in asthma patients [136] and experimentally, were reduced in IL-$5^{-/-}$ mice [137].

IgE production and the presence of eosinophils are associated with allergy and asthma. Anti-IgE therapy reduces blood eosinophil count indicating eosinophilia may be a potential biomarker of successful response to this treatment [138]. However, systemic measurements may not always reflect the local environment. Balzar *et al* demonstrated a correlation between local lung eosinophil count and higher local IgE concentrations with more severe exacerbations. This was despite similar serum IgE concentrations in patients with and without eosinophilia [139].

1.1.3.2.3 Mast cells and basophils

Mast cells (MCs) have long been associated with asthma and allergy. As stated previously, activation via FCεR bound IgE, cross-linked by specific allergen triggers degranulation and the release of a number of proteases, histamine and leukotrines [140]. The presence of the released mediators results in bronchoconstriction, vasodilation, increased vascular permeability and increased mucus production. Increased numbers of MCs are seen in airways of asthmatics and whilst their role in immediate hypersensitivity is accepted there is a poor clinical response to mast cell stabiliser treatment[141]. This suggests that de novo eicosanoid synthesis and release has a more important role in chronic asthma and blockade of this is the basis for leukotrine receptor antagonist therapy, e.g. montelukast. In recent years interest has focused again on MCs and their role in later phase responses and chronic inflammation.

In humans, bronchial biopsies in asthmatics had significantly increased numbers of MCs within ASM bundles when compared those with eosinophilic bronchitis, a condition with eosinophilic AI but no AHR. The number of these MCs correlated with the degree of AHR suggesting the relationship between MCs and AHR is critical in asthma placing them centrally in asthma pathogenesis [141]. Supportive data for MCs in AHR comes from mast cell knockout animal models where AHR was dependent on MCs along with eosinophilia, possibly through tumour necrosis factor (TNF)α production, but only in model protocols where no allergen adjuvant was used [142].

MCs can influence Th2 priming and can contribute to Th2 inflammation through promoting an influx of inflammatory cells, up-regulation of adhesion molecules, and production of cytokines and chemokines in the late phase response in experimental AAI [40,143]. In chronic inflammation, MCs located in the ASM potentially influence remodelling [93], by mechanisms including via angiogenesis in response to MC vascular endothelial growth factor (VEGF) production [144]. A role for MCs in non-atopic individuals has also been described in which the mechanism of mast cell activation is non-IgE-dependent [145].

Like MCs, basophils become activated through IgE-mediated antigen stimulation with resultant mediator release [77]. Basophil numbers are increased in the

airways of asthmatics and basophil histamine release decreases during omalizumab therapy [146]. More recently, a deeper appreciation of their role in the initiation and amplification of asthma has emerged. This involves a newly described APC role of basophils [147]; a role in IL-4 dependent Th differentiation [148]; enhanced production of thymic stromal lymphoprotein (TSLP) following protease allergen activation [95]; and a role in IgE mediated chronic inflammation [149].

1.1.3.2.4 Neutrophils

Neutrophils are polymorphonuclear leukocytes that act as a first line of defence in bacterial and fungal infections. Neutrophilic AI is associated with particular phenotypes of asthma including severe [150] and corticosteroid resistant [9] as well as in non-infectious asthma exacerbations [151] and sudden death associated with asthma [152]. Furthermore, the concentration of IL-8, a neutrophil chemo-attractant, is elevated in asthmatic sputum and production is unresponsive to steroid treatment [150]. Animal models support this clinical observation; with neither neutrophilic accumulation nor AHR suppressed by corticosteroid treatment in a model of acute exacerbations [153]. Neutrophilic asthma has been associated with TNFα and the Th17 population. In human studies, inhalation of TNFα results in AHR and neutrophilic inflammation [154] and increased levels of TNFα are seen in the airways of asthmatic patients [155]. IL-17 induces maturation of neutrophilic progenitors and in the sputum of asthmatic patients, expression levels of IL-17 mRNA correlates with neutrophil numbers [156]. Neutrophils release mediators that contribute to asthma pathophysiology. In particular IL-8, TGF-β, matrix metalloproteinases (MMPs) and proteases which together can contribute to inflammation and remodelling depending on the clinical phenotype.

1.1.3.2.5 Innate lymphoid cells

Since the conclusion of this work, an important population of multi-potent innate cells derived from murine lymphoid tissue and capable of expressing type 2 cytokines have been described in three landmark independent studies [157-159]. Collectively termed type-2 innate lymphoid cells (ILC2) [160] these cells appear to have important role in Th2 cell-dependent immunity or inflammation and thus their role in asthma pathology should be explored. The phenotype had some years previously been described as non-B/non-T cells producing IL-5 and IL-13 in

response to IL-25 [161,162]. Although differences in phenotyping and markers are reported, all cell types appear to be characterised as lineage negative along with a lymphoid morphology, $CD45^+$, and expressing stem cell antigen-1 ($Sca\text{-}1^+$) [157,158] and stem cell factor receptor receptor tyrosine kinase ($c\text{-}kit^{int}$) [157], inducible T-cell costimulator (ICOS) [158] and importantly ST2 [157,158]. The importance of these cells *in vivo* was demonstrated as they appeared to be the main source of type-2 cytokines in parasite models [157-159]. A comparable cell type have been reported in humans and make up a small proportion of $CD45^+$ cells; 0.2-0.3% in fetal and 0.02-0.08% in the adult lung [163]. Their role in asthma will be discussed in chapter 7 in relation to the data presented here.

1.1.3.3 Chronic inflammation

Inflammation is the response of the immune system to injury and is normally beneficial to the host. In asthma, an aberrant immune response to non-pathogenic stimuli leads to a chronic inflammatory response characterised not only by the presence of large numbers of innate and adaptive immune cells but also by airway remodelling. Remodelling describes a substantial change in the extracellular matrix with alterations in the number, phenotype and function of structural cells in the affected tissues, altering their functional properties. Once established the repetitive cycle of tissue damage and inflammatory-cell recruitment and repair becomes chronic even in the absence of sustained allergen contact [164]. Furthermore, there is evidence for individual components of the inflammatory response contributing to airway remodelling as well as an imbalance with pro-resolution mediators. Lipoxin AX4 (LXA4), a polyunsaturated fatty acid derivative, is one such factor produced to promote resolution of inflammation and patients with severe asthma have been shown to have lower circulating levels of LXA4 [165]. Levels of IL-8 correlate with LXA4 in sputum from mild asthmatics however in severe patients, an imbalance is seen with deficient production of the anti-inflammatory LXA4 [166]. With remodelling changes reported before symptoms of asthma develop [167], it is not entirely clear if remodelling is a sequential or parallel process to chronic inflammation, or co-dependent which is the most probable. The structural cells themselves recruit, activate and promote survival of multiple inflammatory cells as well as orchestrate remodelling.

1.1.3.4 Structural cells in asthma

Asthmatics display exaggerated rates of lung function decline which is primarily related to remodelling of the airway wall and loss of airway calibre [168,169]. Most if not all elements of the airway wall can become dysfunctional. The major features of remodelling include epithelial damage and hypertrophy, smooth muscle cell hyperplasia and hypertrophy, goblet cell hyperplasia, subepithelial fibrosis and angiogenesis [169].

AHR is associated with airway remodelling but not with sustained inflammatory cell recruitment in asthmatic patients [170]. A better understanding of these mechanisms is required as current therapies are primarily aimed at reducing inflammation typically by using CS, however these have limited effects on remodelling [171]. Future studies aim to address whether omalizumab is capable of reversing or attenuating aspects of airway remodelling. Furthermore, remodelling itself can affect treatment efficacy with loss of bronchodilator response to β2 receptor agonist associated with increased remodelling reported [172].

Many mediators participate in airway remodelling including TGF-β, MMP9 and ADAM-33. ADAM-33 is a metalloprotease and the identification of a genetic polymorphism in ADAM-33 is associated with accelerated decline in lung function over time [173]. Although its biological function is not clear, its expression in epithelium, myo/fibrobalasts and the ASM make it a target for further investigation and highlights there may be many genetic factors that contribute to enhance remodelling in a subset of patients. Osteopontin is a glycoprotein that can act as an ECM molecule and as a cytokine and elevated levels have been shown in the sputum from severe but not mild asthmatics. It was strongly associated with remodelling factors, in particular TGF-β1 levels [174].

1.1.3.4.1 Epithelial cells

The airway is covered by a continuous layer of epithelial cells (EC) which is of considerable size, approximately 100m^2 [175]. It is the direct interface with inhaled air and forms the initial defence in the lungs comprising barrier function and a mucocilary escalator particle clearance. Primary disordered epithelium is itself associated with asthma [175]. Defects in antioxidant and interferon production increase susceptibility to air pollution and viral infection respectively [176,177]. Airway epithelial cells (AEC) can be secretory, ciliated or basal; distally the alveolar epithelium can be subdivided into the majority type 1 AEC population which perform gas exchange and type 2 AECs, which release surfactant.

Beyond their physical barrier role, bronchial ECs are now recognised as important components of both innate and adaptive immunity and also contribute to lung remodelling. The concept of the epithelial-mesenchymal trophic unit (EMTU) has been proposed to play a role in remodelling in asthma (Figure 1.1). Epithelial-mesenchymal transition is a process of cellular reprogramming or de-differentiation whereby epithelial cells can contribute to the fibroblast population and this process has been described in a number of diseases. A number of types of lung and airway epithelial cell undergo EMT *in vitro*. ECs lose cellular polarity and markers of polarised cells and acquire mesenchymal cell markers [178]. In turn, this expanded fibroblast population contribute to ECM deposition and thus remodelling [179]. Human epithelial cells exposed to TGF-β can undergo EMT [180]. TGF-β released from eosinophils and from the EMTU (myofibroblasts) itself, in combination is thought to be a key signal in this phenotypic transition [181]. TGF-β stimulates fibroblasts to produce the increased amounts of ECM proteins. Mice deficient in Smad-3, which mediates TGF-β signalling, have reduced number of peribronchial myofibroblasts [182] and TGF-β expression correlates with subepithelial fibrosis in human asthmatic bronchial biopsies, suggesting a role for TGF-β *in vivo* [183]. Another member of the TGF-β superfamily, activin-A, also signals through the Smad-3 pathway and drives AI. Mice administered follistatin, an endogenously produced protein which inhibits activin-A, had attenuated remodelling in a chronic inflammation model suggesting a further potential target in the TGF-β family [184].

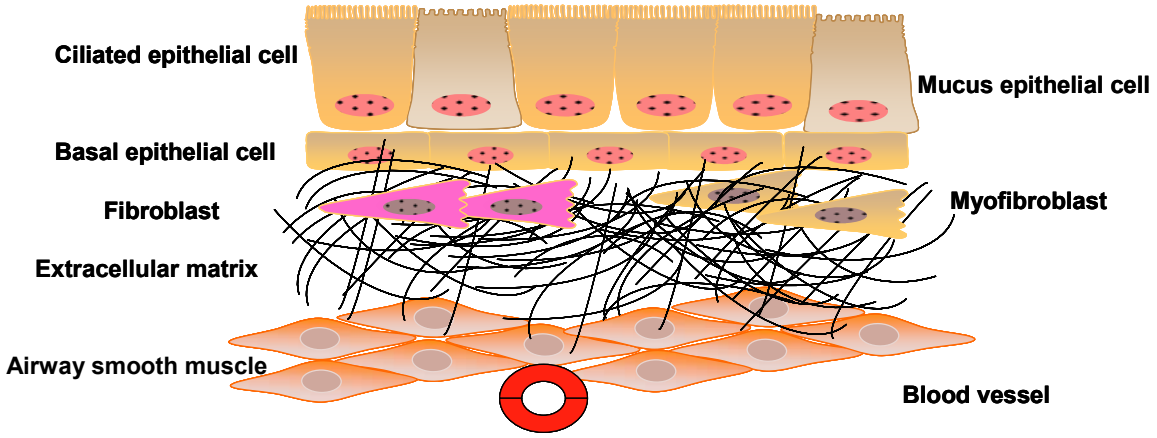


Figure 1.1: The epithelial-mesenchymal trophic unit (EMTU)
Repetitive epithelial injury due to chronic inflammation plus exposure to further insult (virus, bacteria, smoke, pollution, oxidative stress) and repair process results in the formation of an epithelial-mesenchymal trophic unit. Along with sustaining and promoting Th2 inflammation (not shown) this unit regulates remodelling. Myofibroblast activation, airway wall thickening, deposition of extracellular matrix proteins, goblet cell hyperplasia and increased vasculature. Myofibroblasts are predominantly responsible for repair after injury. In asthma subepithelial myofibroblasts are increased in number; migrating fibrocytes, smooth muscle cells, fibroblasts, loss of epithelial cell characteristics to develop a fibroblast/myofibroblast phenotype are responsible.

Following activation of surface PRRs, epithelial cells produce antimicrobial molecules as well as cytokines and chemokines leading to cell recruitment and inflammation [185]. By differential production of cytokines, the EC influences and regulates local DC and subsequent adaptive response to allergen in favour of either Th1, Th2 or Treg responses indicating a further important role for EC at sensitisation [185]. Experiments with restricted TLR expression in bone marrow-chimeric mice, have shown that TLR expression on structural cells is necessary for activation of DC in a house dust mite (HDM) or in a low-dose OVA-LPS AAI model which favours Th2 sensitisation [106,186]. The mice restricted of haematopoietic cell TLR4 were unable to generate a Th1 response to high dose OVA-LPS and instead were able to generate a Th2 response via structural cell TLR4 activation [106]. As well as activation, EC can induce DC migration into epithelium via the production of C-C motif ligand (CCL)-20 (MIP-3α) chemokine [187].

Epithelial cells are an important source of cytokines which contribute to the Th2 inflammation including TSLP, GM-CSF, IL-1, and IL-25 [185,186]. Chemoattractants for Th2 cells including CCL1, CCL17 and CCL22 are produced by EC and this is further induced by the presence of Th2 cytokines [188]. The epithelium is a source of IL-8, contributing to neutrophil chemotaxis. IL-28 and IL-29 are a new family of cytokines also called λ or type III interferons and are important in innate

immunity. IL-28 produced by APC in response to TLR activation, acts on epithelial cell IL-28 receptor IL-28RA suppressing Th2 inflammation [189]. On the other hand, IL-29 is secreted by type II AEC in response to viral infection and may have a role in asthma exacerbation [190].

Mucous cell hyperplasia is a fundamental pathological change in asthma. EC responding to IL-13 in a STAT6-dependent fashion appear to be a critical in mucous overproduction [53]. Furthermore, IL-13 induces apoptosis in EC and upregulation of pro-fibrotic genes in fibroblasts [191] and in agreement, using an antibody to block IL-13 reduced airway remodelling in a mouse model [54]. Notch, an evolutionarily conserved signalling factor has been shown to affect the balance of EC differentiation to ciliated or secretory cells in the developing lung. *In vivo*, Notch inactivation results in an imbalance of EC differentiation resulting in the absence of goblet cells, thus preventing mucous metaplasia [192]. I*n vitro,* Notch-mediated down regulation of MUC5AC, a major component of airway mucus has also been demonstrated however the role of Notch in asthma is as yet unclear [193].

1.1.3.4.2 Smooth muscle cells

The ASM is dysfunctional in asthma and undergoes hyperplasia and hypertrophy [194]. AHR is said to be dependent on ASM remodelling *in vitro* and *in vivo* and contributes to the severity of asthma [195].

ASM may play a more active role in modulating airway remodelling as a source of inflammatory mediators, proteins and expression of cell adhesion molecules suggesting some autocrine responses and interactions with inflammatory cells [194]. ASM cells secrete extracellular proteins including collagen I, IV, V, fibronectin, laminin and hyaluranon and contribute to subepithelial fibrosis [194]. The localisation of MCs into the ASM has been described [141]. MCs are thought to directly modulate the ASM causing proliferation and contraction via the release of inflammatory mediators [40]. There is evidence that MCs can mediate chronic inflammation by cytokine induction of chemokine release from the ASM [196]. ASM is a source of the proangiogenic factor VEGF, also released from ASM in response to Th2 cytokine activation [197]. ASM cells from subjects with asthma promoted

angiogenesis *in vitro* suggesting their fundamental contribution to the neovascularisation that is observed in asthmatic airways [198].

Taken together, the ASM clearly has an important pathophysiological role in asthma and thus an appropriate target of therapeutic strategies. For example, recently, the development of bronchial thermoplasty, the delivery of controlled radiofrequency energy to the airway wall to ablate smooth muscle, was shown to be effective in a subgroup of asthmatics. A reduction in asthma exacerbations, hospitalisation as well as symptoms was demonstrated in the clinical trials (AIR, RISA and AIR2) [199]. The mechanism of this clinical improvement is unknown but reports suggest heat denatures protein impairing the ability of the ASM to contract and a reduction in ASM mass is observed [200]. In addition there may well be an effect of inhibiting ASM secretory function.

1.1.3.4.3 Vasculature

Angiogenesis and microvascular remodelling are features of severe and fatal asthma [201,202] but are also observed in milder disease [202,203]. An imbalance of pro- and anti-angiogenic factors leads to abnormal growth of new vessels in asthma airways [204]. A role for the most potent pro-angiogenic factor, VEGF in mediating these changes is proposed. Associative studies in humans have identified increased concentrations of VEGF in sputum of asthmatic patients as well as mRNA expression of VEGF and its receptor in airway mucosa [202,205]. Supportive evidence from animal models indicate a role for VEGF not only in vascular remodelling but also in extravascular remodelling, as well as in promoting allergen sensitisation and Th2 inflammation [206]. The cellular sources of VEGF are epithelial cells, smooth muscle cells, fibroblasts as well as alveolar macrophages, eosinophils and MCs [204,205]. TGF-β can further promote EC VEGF production [207]. Treatment with the anti-VEGF monoclonal antibody Bevacizumab, already licensed in humans for anti-cancer treatment, caused reduction in remodelling parameters in mice suggesting this may have therapeutic potential in severe asthma [208].

1.1.3.5 Cytokines and chemokines in asthma

Cytokines play a critical role in orchestrating, perpetuating and amplifying the inflammatory response in asthma and one of major targets of CS treatment is to reverse the abnormal expression of cytokines. The movement or trafficking of the immune cells discussed above is primarily performed by cytokines and chemokines. The cytokines IL-4, IL-5, IL-13 and IL-9 have been considered under classical Th2 inflammation (section 1.1.3.1.2). Selected other cytokines with a proposed role in asthma are described here.

TNFα is an inflammatory cytokine involved in the acute response to infection, inducing neutrophil proliferation and also has a role in tumour necrosis. As stated, TNFα is linked to neutrophilic asthma; its concentration is increased in the BAL and it is produced by macrophages, MCs, lymphocytes and eosinophils [155,209,210]. MCs in particular are a rapid source and release pre-formed TNFα on activation and degranulation [93]. The importance of TNFα in asthma pathogenesis is indicated by clinical trials of treatment with Etanercept, a recombinant soluble TNFα receptor, which improved AHR and quality of life in asthmatic patients [211]. The critical issue for anti-TNFα therapies is their narrow therapeutic index with an increased risk of malignancy, thus limiting future prospects for their use[212].

IL-17(IL-17A) is a member of the IL-17 family of six pro-inflammatory cytokines with a role in host defence and autoimmunity. There has been considerable interest in the subset of Th cells identified by their IL-17 producing properties, Th17 cells. Th-17 cells also produce IL-21, IL-17F and IL-22 [213]. Along with their link with neutrophilic asthma, there is an association with Th2-mediated inflammation; levels of IL-17 and IL-5 mRNA in sputum of asthmatics are correlated [156]. Furthermore, both Th17 cells and IL-17 plasma levels correlate with disease severity [214]. Taken together there is a sufficient evidence for a role of IL-17 in asthma and hence a move to clinical trials aimed at blocking IL-17 signalling. However, a recent large randomised double blind placebo control trial of brodalumab, an anti-IL-17 receptor monoclonal antibody, failed to determine any clinical benefit in most subjects with asthma [215]. IL-21 is a pluripotent cytokine that regulates Th17 cells. A recent discovery that IL-21 enhances Th2

cytokine production in a model of AAI through multiple mechanisms, identifies a further target for in the Th17 pathway warranting investigation [216].

IL-6 is a pleiotropic cytokine with pro-inflammatory and anti-inflammatory properties. It is not clear if IL-6 is a marker of ongoing inflammation in asthma or has a specific functional role, as the evidence is conflicting. The concentration of IL-6 in sputum correlates inversely with lung function [217]. IL-6 is reported to be necessary for mucus secretion by epithelial cells[218] and IL-6 blockade ameliorates AHR suggesting a pathological role for IL-6 [219]. However, most *in vivo* evidence suggests an anti-inflammatory role [220]. There also appears to be a regulatory role for IL-6 in effector CD4 T cell fate, promoting IL-4 production during Th2 differentiaton as well as inhibiting Th1 differentiation [221].

The airway cytology characterises the late-phase asthma response, therefore the ability of cells to infiltrate the lungs is important and this is predominantly controlled by chemotactic cytokines or chemokines. The expression of a wide range of chemokines has been described in asthma. The interaction between chemokines and their receptors is complex; one chemokine can bind to multiple receptors and one receptor can recognise and bind multiple chemokines. This complexity provides control of cell movement however dissecting the essential nature of each chemokine is challenging (Table 1.2). The receptor C-C motif receptor(CCR)-8 is found exclusively on Th2 cells and using knockout mice, a reduction in Th2 cytokines and airway eosinophils was observed upon allergen challenge by some [222] but not by others in these mice [223]. The chemokine CCL1 (TCA3), a ligand for CCR8 is detected at increased levels in asthmatic airways thus making it an attractive therapeutic target [224]. However, it appears these experimental findings have not yet been translated into an essential role for CCR8 as its inhibition was ineffective in a pre-clinical primate model of asthma [225]. Other potential receptor and chemokine pair relevant for asthma include, for Th2 cells and eosinophil: CCR3 with ligands CCL11 (eotaxin), CCL24 (eotaxin-2) and CCL5 (regulated on activation, normal T cell expressed and secreted (RANTES)); and CCR4 with ligand CCL17 (thymus and activation regulated chemokine(TARC)); on T cells: CCR5 with ligands CCL11 (eotaxin), CCL5(RANTES), CCL3 (MIP-1α) and CCL4 (MIP-1β); on endothelial cells: CXCR3A and ligand CXCL10 (interferon gamma-induced protein 10(IP-10)) are also of

interest. The main eosinophil-specific chemotactic pathway CCL11/CCR3 is a potential attractive target for asthma therapy. The concentration of CCL11/eotaxin is: up-regulated in mice following allergen challenge [226]; along with CCR3 mRNA highly expressed in atopic asthmatic bronchial biopsies [227]; elevated in plasma during acute exacerbations of asthma [228]; and correlates with levels of forced expiratory volume (FEV1), a marker of AFO in patients with asthma [229]. The sources of eotaxins are predominantly ECs, macrophages and fibroblasts [227,230]. Blocking eotaxin by neutralising antibodies, partially reduces eosinophil recruitment in an ova challenge mouse AAI model [231]. In a similar model using double eotaxin (CCL11/CCL24) knockout but not single knockout mice, eosinophilic inflammation is significantly attenuated [232]. In $CCR3^{-/-}$ mice, eosinophil recruitment following antigen challenge was severely impaired but the affect of CCR3 on AHR is conflicting [232-234]. Attenuated AHR is described by some [233] but in contrast worsening of AHR, attributed to altered mast cell location, reported by others[234]. One explanation for this may be different modes of sensitisation used in these studies but as CCR3 has effects on basophils, MCs, Th2 cells and platelets along with eosinophils dissecting these interacting roles is complex. As chemokine eosinophil regulation through non-CCR3 pathways are also described, the complexity of chemokine interactions are increased [235].

CCR4 gene-deficient mice develop less airway eosinophilia and AHR in a chronic lung allergy model (*Aspergillus Fumigatus)* [236] but not in an allergic allergen model in guinea pigs possibly reflecting different animal model requirements [237]. Additional evidence to support the contribution of chemokines to asthma include the concentration/expression of both CCL5/RANTES and CCL17/TARC elevated in asthmatic airway samples[238,239]. CCL5/RANTES concentration is also elevated in exhaled breath condensate of asthmatic patients and in particular correlates with to patients with the most unstable symptoms and is a potential biomarker [239]. Serum concentrations of CXCL10/IP-10 were increased in patients with viral induced compared to non-viral acute asthma exacerbations [240] but serum levels were reduced in another study in stable asthmatics compared to controls [241].

Receptor	Chemokine	Receptor expression
CCR3	CCL7/MCP3 CCL5/RANTES CCL15 CCL16 CCL11/Eotaxin CCL24/Eotaxin-2 CCL26/Eotaxin-3	Eosinophils, Basophils, Th2, Dendritic cells
CCR4	CCL17/TARC CCL22/MDC	Dendritic cell, Th2, Natural killer cell, monocyte
CCR8	CCL1/TCA3 CCL4/MIP1β CCL17/TARC	Monocyte, Dendritic cell, Th2, Treg
CCR5	*CCL8/MCP-2* *CCL4/MIP1β* *CCL3/ MIP1α*	*Monocytes, macrophage, Th1, Natural killer cell*
CXCR3	*CXCL10/IP-10* *CXCL9/MIG* *CXCL11*	*Th1, Natural killer cell*

Table 1.2: Selected chemokine receptor and chemokine expression profiles implicated in the pathogenesis of asthma

1.1.3.5.1 Novel cytokines in asthma

IL-25 (IL-17E), a member of the IL-17 family is implicated in Th2 responses [161]. In the mouse it is produced mainly by Th2 cells [161], epithelial cells in response to protease allergen exposure [242], and MCs [243]; and in humans is predominantly produced by eosinophils and basophils [162]. Ballantyne *et al* demonstrated that the administration of anti-IL-25 antibody to mice inhibited eosinophilic inflammation, Th2 cytokine response and reduced AHR suggesting an important role in asthma pathogenesis[244]. IL-25 induces the production of Th2 cytokines from Th2 cells [245]. However IL-25 can also induce the production of Th2 cytokines in T-cell deficient mice suggesting other sources [162]. More recently, non-B, non-T target populations have been identified including the innate lymphoid cell, the nuocyte [246] as well as a type 2 myeloid granulocytic population, the later cells being steroid resistant and present in asthmatic blood samples [247]. IL-25 has the ability to promote Th2 differentiation in an IL-4 dependent manner *in vitro* [242] and *in vivo*, IL-25 blockade at sensitisation prevented AHR and reduced other features of asthma in an AAI model [244]. IL-25 dependent antigen-induced AHR is mediated by NKT cell function [248]. In addition, IL-25 modulates Jagged1, the Th2 polarising ligand for Notch on DCs [249]. However, the role of IL-25 in antigen-specific sensitisation does not appear

essential as this develops normally in IL-25$^{-/-}$ mice but here, the structural cell IL-25 production was found to be essential for the effector phase in an OVA model of AAI [250]. More recently, a role for IL-25 has been described in the remodelling phase, in a HDM model of AAI [251].

Thymic stromal lymphoprotein (TSLP) is generated from epithelial cells following activation of TLRs1-3 [252] and influences Th2 differentiation predominantly via effects on dendritic cells [253,254]. The TSLP-stimulated DC induce naïve T cells to differentiate into IL-4, IL-13 and TNFα producing cells[253]. Furthermore, TSLP induces upregulation of MHC and co-stimulatory molecules on DCs including OX40L the ligand for OX40 on T cells [253,254]. In addition, induction by microbial products of epithelial-derived TSLP can activate MCs contributing to Th2 inflammation in non-atopic disease [252]. Interestingly, the presence of IL-4 enhances ds-DNA and rhinovirus dependent TSLP production, suggesting a mechanism whereby a viral infection can further amplify existing Th2 inflammation in asthma [255]. Clinically, elevated numbers of TSLP mRNA^{+ve} cells have been described in endobronchial biopsies from asthmatic patients [238]. Furthermore, mice lacking the TSLP receptor failed to develop an inflammatory response to antigen and exhibited enhanced Th1 responses [256]. Crosstalk of TSLP with IL-25 further amplifies Th2 inflammation in particular via DC-memory Th2 cell interactions [245]. Taken together, there is evidence for TSLP in the pathogenesis of asthma and accordingly clinical trials with TSLP/OX40L blockage are underway. A proof-of-concept small clinical trial with anti-OX40L antibody did not show any benefit in mild asthmatics[257]. Most recently, a trial of anti-TSLP antibody in mild asthmatics showed attenuated allergen-triggered inflammatory and AHR responses but further studies will be required to ascertain if this results in clinical benefit [258].

A role in asthma for many other cytokines has been described. The reports include including IL-11[259], IL-15 [260], IL-16[261], IL-18[262], IL-19 [263], IL-21[264], IL-27[265], IL-31[266], IL-32[267]and IL-35[268].

In summary, interactions between the innate immune response, structural cells and the adaptive Th2 response, orchestrated by many inflammatory mediators are required to produce the spectrum of pathophysiological and clinical phenotypes associated with asthma. In particular, despite the close association

of eosinophils with asthma, this is not an exclusive feature and eosinophilic asthma can be considered as a distinct phenotype associated pathologically with a thickened basement membrane and pharmacologically with steroid responsiveness. In contrast, neutrophilic asthma is particularly associated with severe or non-atopic asthma and with asthma exacerbations. Of course, these phenotypes are not mutually exclusive and overlap [2].

1.1.4 Animal models of asthma

The need for novel therapies in asthma, particularly for the severe end of the clinical spectrum, requires suitable research and disease models in conjunction with human studies. Mice allow us to perform mechanistic studies, evaluate the importance of specific cell types in asthma pathogenesis, and test potential new treatments.

Mice are the most commonly used species for modelling human immune-mediated disease. The allergic airways inflammation model of asthma in the mouse is well established. The use of mice is advantageous as they can be bred easily with uniformity in inbred genetic strains and a wide variety of appropriate reagents are available. In addition, knowledge of the mouse immune system and genome is now extensive and technology to manipulate the genome to specifically consider genetic influence is available. However, mice do not spontaneously develop AHR or asthma-like disease. Mice have structurally different airways to humans with fewer bronchial divisions and smooth muscle present only in larger airways. They also have a different distribution of eosinophilic inflammation and lack MCs in the ASM [134]. These are limitations that must be considered along with ethical considerations when designing and interpreting experiments.

1.1.4.1 Protocols

The clinical features of eosinophilic inflammation and AHR in asthma are those most often replicated in murine models of AAI [269]. Most commonly, mice are sensitised peripherally to a protein antigen and after a time interval subsequently challenged via the airways with the same antigen [269]. Other models of AAI include: passive cell transfer [22]; employing transgenic mice where

features of asthma develop spontaneously [61]; or models of viral infection which cause airway inflammation [270].

Ovalbumin (OVA), a well characterised chicken egg protein, in a highly purified form is the commonest antigen used to sensitise mice in the AAI model. Mice expressing an ova-specific T cell receptor are available which increases the usefulness of this antigen choice. Other antigens used include those from *Aspergillus*[236], cockroach[271] and HDM[74], which may have a greater clinical relevance. Using the OVA model reproduces features of asthma including elevated IgE antibody, eosinophilic airway inflammation and AHR [269]. One criticism of acute OVA challenge models is that AI and AHR resolve and thus mice don't show features of remodelling [272]. To investigate the more chronic features of asthma such as airway remodelling or persistent AHR, a number of chronic allergic inflammation models have been developed using repeated challenge with OVA [134] or using HDM allergen extracts [74]. In both acute and chronic allergen models, dissociation between inflammation, AHR and remodelling has been described [273,274].

When using OVA to sensitise, the response is often boosted with an immunological adjuvant because administration of OVA alone can lead to tolerance [275]. The use of adjuvants is non-physiological and is a weakness to this approach, however, sensitisation has been shown to develop without adjuvant in some models and these may be more informative of clinical disease [276]. The most widely used adjuvant aluminium hydroxide (alum) strongly induces Th2 responses [277]. Furthermore, there are alternative routes of administration of the sensitising antigen and some protocols exist without an antigen sensitisation phase using HDM, which are used to generate AAI [74,278].

Despite its common use, significant differences exist in OVA AAI model protocols in terms of duration, sensitisation route, number of challenges, route of challenge and mouse strain, therefore it is vital to be aware of the advantages and limitation of an antigen when determining a model of choice [134,274,279,280].

1.1.4.2 Measurement of experimental parameters

Irrespective of the AAI protocol used, the gold standard method for assessment of airway inflammation is quantification of differential cell counts in the BAL. This can be done by the Romanovsky staining method and microscopy however, more detailed flow cytometric analysis and fluorescence-assisted cell sorting (FACS) techniques have been described and are being increasingly employed to analyse the BAL with improved knowledge, speed and accuracy [281]. Cytokine, chemokine and immunoglobulin quantification as well as histological assessment of inflammation in lung tissue are often undertaken. Additional assessment of inflammatory cell profile in the lung can be performed by cell counting or FACS following lung digestion, but this is less commonly performed [282].

Perhaps the most controversial aspect of murine asthma models is the assessment of AHR. Whole animal assessment of AHR in rodents may not distinguish between airway and contractile sources of airway obstruction [283]. Furthermore, the methods used to assess AHR are diverse. The gold standard is an invasive measurement of direct airway resistance in intubated, anaesthetised mice. This technique requires considerable time and expense as well as expertise and therefore non-invasive alternatives are often preferred. The most popular of these is using whole body plethysmography measuring a derived variable named enhanced pause (Penh) [284]. Although there are criticisms of this technique [285], it is none the less widely used and Penh has been shown to correlate with airways obstruction measured by the more invasive techniques [270].

1.1.4.3 Genetic background

The choice of mouse strain used in models must be carefully considered. The Bagg Albino, (BALB)/c, strain is the commonest choice for antigen models because they develop robust Th2 responses but other strains (C57Bl/6 and A/J) have been used successfully [286]. However, differences in all measured parameters can be dramatic when the same model is applied concurrently to different strains to exclude genetic differences [276]. Others have found that one strain may be more susceptible to develop particular disease characteristics; particularly BALB/c mice to AHR and C57Bl/6 to airway eosinophilia [287]. In a chronic allergen exposure model, only A/J mice were shown to develop

sustained inflammation as well as persistent AHR and changes of airway remodelling [279]. Certain mouse strain genomes are easier to target than others, therefore the development of transgenic mice are favoured to these genetic backgrounds [288].

Taken together, the relevance of the use of animal models to investigate experimental airways disease has been questioned and humans remain the best biological model [289]. Studies in humans with biopsies or BAL or sputum can characterise the presence of immune and inflammatory cells, cytokines and expressed mediators, and these can be correlated with clinical parameters. However, these studies are limited in defining cause and effect and this aspect can be usefully explored in experimental models. It is clear that asthma is a complex multifactorial disease, it is thus unlikely that a single animal model is able to replicate all these features. Instead we can use animal models to study specific features to characterise detailed cellular information. Choosing the most appropriate model with the relevant phenotypic features of interest rather than necessarily the most common model used is imperative. In addition, improved understanding of chronic models, better techniques to measure lung function, developing better models to reflect exacerbations or non-atopic disease will be useful. As long as the caveats associated with the models are borne in mind when planning and interpreting experimental data, they remain a useful experimental tool.

1.2 ST2

St2 is a gene first described when induced in fibroblast cell lines during the G_0/G_1 transitional cell cycle phase, by two independent research groups. Referred by one group as T1 [290] and by Tominaga *et al* as *St2* [291] it was induced by mitogen or serum stimulation. This gene encoded a protein with similarities to the immunoglobulin super family receptor interleukin 1 receptor (IL-1R) but without an apparent cytoplasmic domain [291]. Subsequent studies identified further products of the *St2* gene and one of these with an intracellular domain named ST2L[292]. Ultimately, 16 years later the ligand for this ST2L receptor was described as IL-33 [293].

1.2.1 ST2 gene

The discovered *St2* gene was identified as a late gene expressed in mouse fibroblasts entering the cell cycle, was found only during growth and was not constitutively expressed [291]. The presence of *St2* was described in a human T cell line and was found to have 68% homology with the murine gene [294].

Further cloning studies of the *St2* gene in mice mapped it to chromosome 1, near to the *Il1R1* locus suggesting a link with this IL-1 super family [295]. In humans, the *St2* gene was mapped to chromosome 2 again close to the *Il1R1* locus [296]. The *St2* gene has been shown to be homologous in a number of other species including chicken, fish and rat [297-299] and it is highly conserved from *Drosophila* and consistent with IL-1 receptor family signalling [300].

Four types of ST2 gene products can be produced by alternative splicing. In the original descriptions, the *St2* gene was a 2.7 kilobase (kb) mRNA sequence giving rise to a secreted cell product which was subsequently termed soluble or sST2 [291,301]. The secreted sST2 protein appears to require the peptide sequence to be heavily glycosylated [301]. It comprises 3 immunoglobulin domains and a short tail with similarities to extracellular IL-1R1 and IL-1R2 [291] (Figure 1.2). An additional, less abundant and longer 5 kb mRNA was subsequently identified and this generated an ST2 protein with a similar structure to the entire IL-1R1; with an identical extracellular portion to sST2 but possessing a transmembrane as well as cytoplasmic portion [302]. This membrane anchored product was named ST2L and from herein will be referred to as ST2 or ST2L [302]. A third gene product identified in human cells and termed ST2V is a shortened form of sST2[303]. It has also been identified in chickens as has a fourth variant ST2LV, but the *in vivo* function of both are unclear at present [297].

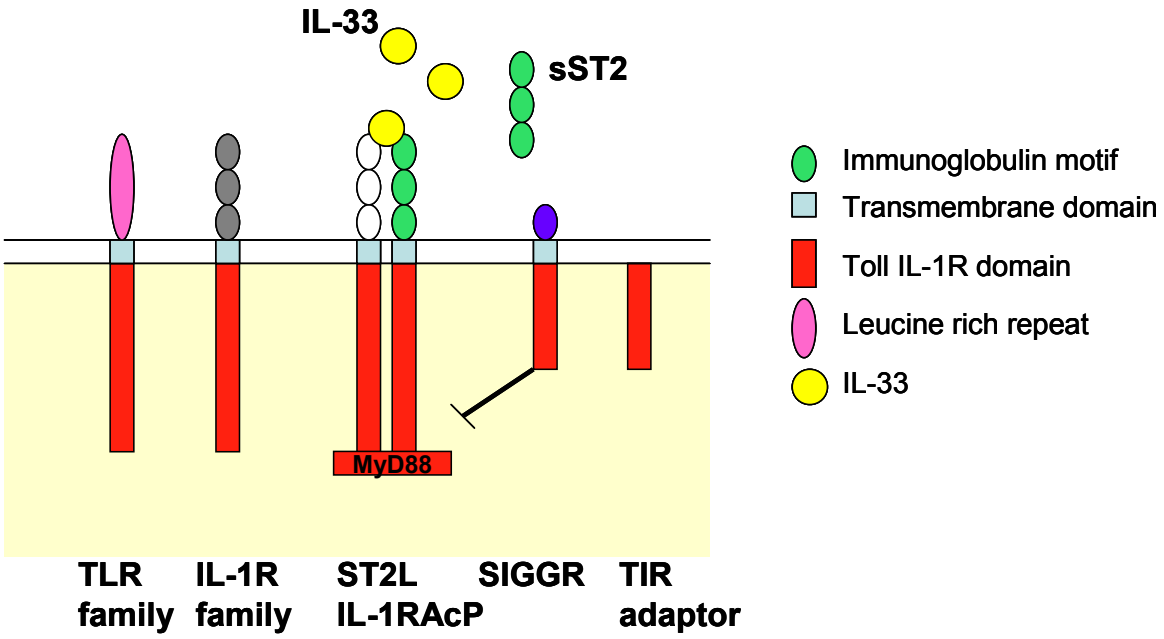


Figure 1.2: Schematic representation of TLR-IL-1R family of receptors

1.2.2 ST2 product expression and regulation

As described above, sST2 synthesis in fibroblasts appears to be induced in growing cells. On the other hand ST2L, is less abundantly expressed in fibroblasts and more highly expressed on haematopoietic cells such as $CD4^{+}$Th2 cells, MCs and macrophages, indicating differential expression [304-306]. In mice the differing expression patterns appears to be regulated by distinct transcriptional promoters with mutually exclusive promoter start sites determined by cell type; a distal promoter in haematopoietic MCs and 10.5kb away a proximal promoter in the fibroblasts. Promoters can result in sST2 or ST2L production by 3' splicing but each promoter is influenced by transcription factors, in particular GATA2, and environmental factors which ultimately determines the dominant protein [307]. The expression of ST2 by cell types is summarised in Table 1.3.

More recently ST2 expression has been identified on other cells that could play a role in asthma or AAI. Basophils show little or no constitutive sST2 and ST2L expression but both are induced by IL-3 stimulation, with sST2 responding rapidly and ST2L after 8 hours [308]. These authors could not detect ST2 expression

on eosinophils but in other studies, these cells did respond to IL-33 suggesting that expression levels for ST2 were below detection limits, and others have identified ST2 mRNA in eosinophils [309,310]. Subsequently, work from our laboratory identified low levels of ST2L expression which was inducible in mice in eosinophils [311] and also neutrophils [312]. ST2 mRNA is expressed in murine lung [313,314] and sST2 and STL mRNA in human lung tissue [315,316].

Cell	*Mouse sST2*	*Mouse ST2L*	*Ref*	*Human sST2*	*Human ST2L*	*Ref*
Fibroblast	RNA and protein	RNA and protein	290,291 305,315,317,318	RNA and protein	RNA and Protein*#	315 314 306,319
Epithelial cell	RNA	RNA	318	RNA* and protein*#	RNA*	306,314 316,320
Endothelial cell	-	-	315	RNA* and protein*#	RNA*# and protein*#	306,315,321 320 319
Smooth muscle cell	-	-		RNA*	RNA*	306,321
Th2 cell	RNA	RNA and protein	304,322 323	RNA	RNA and protein	324,325 326
B cell	-	RNA and Protein	305,327	-	RNA and protein	315 319
NK cell	-	Protein	328	-	RNA	326
Mast cell	Protein	Protein	293,305		RNA and Protein	307,319,329 330
Basophil	-	Protein	328	RNA and protein	RNA and protein	308,310,326
Monocyte	-	-		RNA	RNA and protein	331
Macrophage	RNA	RNA and protein	305,306	RNA	RNA and protein*#	306,319
Dendritic cell	-	RNA and protein	332	-	RNA	326
Eosinophil	-	Protein*#	311	-	RNA and protein	309,310
Neutrophil	-	RNA and Protein#	312,333	-	-	
Innate lymphoid cells		Protein#*	334,335		RNA# and protein*#	163

Table 1.3: Cellular expression of ST2

***pulmonary expression # observation since 2010**

ST2 is a member of the TLR/IL-1R super family of receptors. There are 3 main subtypes according to the structure of the extracellular ligand binding portion, but all share a Toll-IL-1R (TIR) intracellular domain. Type 1 or IL-1R receptors include receptors for IL-1α, Il-1β and IL-1R antagonist (IL-1ra) as well as the IL-18 receptor (IL-18R) [336]. The IL-1R family have 3 immunoglobulin domains, consistent with that described for sST2 and ST2L [291,302], with ST2L having a transmembrane and intracellular component in keeping with the other IL-1R receptors[302]. Part of the regulation of IL-1 is the production of a soluble receptor type 2 or IL-1R2 which is an antagonist to potent IL-1 activity[336]. IL-1R2 acts as a 'decoy' receptor by binding IL-1 in solution and not mediating any biological signals. The similarity with sST2 suggests it may also act as a 'decoy' receptor. The ST2L/IL-33 complex is represented in Figure 1.2.

1.2.3 ST2 function

1.2.3.1 ST2 in Th2 inflammation

The evidence of a role for ST2 in Th2 or type-2 inflammation comes primarily from the discovery of its preferential expression on $CD4^+$Th2 subset of T cells as well as on MCs suggesting a functional role in adaptive and innate immune cells that contribute to this inflammatory phenotype.

A number of studies have conclusively demonstrated that ST2 is expressed on $CD4^+$Th2 cells [304,322,323] and not on naïve T cells [323], Th1 cells which express IL-18R[337] or on Th17 $CD4^+$ cells [324]. Furthermore, ST2 was confirmed to be a stable and selective marker to distinguish Th2 from other helper cell subtypes in cell lines and *ex vivo* cells expressing IL-4, IL-5 or IL-10 but not IFNγ or IL-2 [304,322]. Although $CD4^+$Th2 ST2L expression is not dependent on Th2 cytokine expression [322], their presence increases ST2 expression [323]. In humans ST2 is expressed on activated Th2 cells, which can also produce sST2[324]. These studies demonstrating selective ST2 expression on Th2 cells went onto first show a functional role for ST2 using antibody or a recombinant fusion protein. In these studies, ST2 blockade increased resistance to leishmaniasis, exacerbated collagen-induced arthritis [304] or reduced eosinophilic airway inflammation [322].

The development of $ST2^{-/-}$ mice facilitated further study into the functional role of ST2. These mice appear to have normal development and normal cell populations [338,339], including of Th2 cells supported by *in vitro* studies [338]. However, the antigen mediated Th2 responses appear affected. Using a *Schistosoma mansoni* parasite granuloma formation model, $ST2^{-/-}$ Th2 cell cytokine production was reduced but clinically, the magnitude of effect was influenced by a primary or secondary challenge [339]. *In vivo*, all forms of ST2 will be deficient and so attributing influences cannot be assumed to be loss of ST2 receptor and could be due to loss of sST2.

MCs contribute to innate type-2 inflammation as previously discussed. Mast cells constitutively express high levels of surface ST2 that are the highest among haematopoietic cells [293,305]. Despite this, there is no evidence that lack of ST2 affects mast cell development or function [340].

1.2.3.2 ST2 in murine AAI *in vivo*

Apart from the possible contribution to AAI identified by the presence on Th2 and MCs, the role of ST2 in asthma has been further assessed in context of disease models including using $ST2^{-/-}$ mice, were studies have shown conflicting results. The studies with ST2-IgG fusion protein, sST2 gene transfer or anti-ST2 antibodies indicated a role for ST2 in Th2-mediated disease, in particular AAI [313,322]. A further study using both ST2-Ab and ST2-Ig in a murine AAI model also identified a requirement for ST2 in developing Th2-inflammation and furthermore that this is due to a direct effect of ST2 on $CD4^+$ cells [341].

Elevated concentrations of sST2 in sera of mice following allergen challenge agree with a pro-inflammatory functional role for ST2 [313]. In the same study, sST2 added to OVA-stimulated splenocytes *in vitro* attenuated Th2 cytokine production. This effect was mirrored *in vivo* where following administration of sST2 by gene vector, to mice before sensitisation and challenge, reduced airway eosinophilia and Th2 cytokine production [313]. In other AAI models, ST2 expression on $CD4^+$ cells was increased during antigen challenge [342] and correspondingly in a model generating a Th1 response following over expression of IP-10, ST2 expression on $CD4^+$ cells suppressed [343].

However, some studies investigating the AAI model in the $ST2^{-/-}$ mice conclude there is no critical role for ST2 in murine asthma. Hoshino *et al* found no requirement for ST2 in an OVA-induced AAI model or in a Th2-driven parasitic model [338]. This finding was challenged by Townsend *et al* who found abrogated Th2 responses in a pulmonary granuloma model in $ST2^{-/-}$ mice; however, this group used a short-term parasite model and therefore sensitisation and challenge pathways and role of ST2 may differ [339]. However, Mangan and colleagues contradicted this reporting that loss of ST2, in particular on Th2 cells, demonstrated by adoptive transfer models in transgenic mice, actually exacerbated eosinophilic pulmonary inflammation and AHR [344]. In the OVA-induced model in AAI similar to that used by Hoshino *et al* they similarly showed no change in many parameters including AHR compared to WT mice, although worsened eosinophilia was consistent with the transfer model findings [344]. This phenomenon of ST2 knockout exacerbating Th2 inflammation was also observed in studies within our group; $ST2^{-/-}$ CD4 cells *in vitro* produced increased amounts of Th2 cytokines and exacerbated AI when adoptively transferred into WT mice indicating complexities in loss of ST2 in intact organism versus an individual cell type [345].

1.2.3.3 ST2 in Th1 and innate inflammation

ST2 is a regulator of additional aspects of inflammation and in keeping with other members of the Toll/IL-1R super family; ST2 is involved in innate immunity. $ST2^{-/-}$ mice developed more severe LPS-induced endotoxin shock with increased production of pro-inflammatory cytokines in comparison to WT mice, although mortality was unchanged, suggesting a role for ST2 in tolerance to gram negative microbial infection [346]. The toxic effects of LPS are primarily conferred by macrophages and further analysis *in vitro,* demonstrated that $ST2^{-/-}$ macrophages produced higher amounts of cytokines in response to LPS or IL-1[346]. The effect of ST2 on macrophages is most likely indirect via sequestering MyD88 and Mal which prevent NF-κB activation, the signalling pathway through which most TLR members activate responsive genes. Similarly, blockade of ST2 by monoclonal antibody exacerbated LPS-mediated mortality [347]. On the other hand, blockade of ST2 signalling by pre-treatment of sST2 suppressed *in vitro* and *in vivo* LPS-induced inflammation highlighting distinct roles for ST2L and sST2 [347].

An *in vivo* role for ST2 has also been described in: liver damage whereby sST2 is protective in an ischaemia-reperfusion model [348]; a streptomycin induced pancreatic damage and diabetes model whereby ST2 deletion worsened inflammation [349]; a pulmonary fibrosis model with unexplained upregulation of ST2 identified [314]. ST2 expression was induced in cultured myocytes undergoing biomechanical strain and in an *in vivo* model of myocardial infarction (MI), elevated concentrations of serum sST2 in mice was also detected[350]. Mice treated with bleomycin, a model of acute lung injury, transiently overexspressing sST2 by gene transfer, exhibited suppressed levels of injury [351].

1.2.4 ST2 in disease

ST2 has been associated with a number of diseases. *St2* gene polymorphisms in the distal promoter have been associated with asthma [352] and with atopic dermatitis, functionally correlating with clinical parameters [353]. The role of ST2 in disease and as a biomarker will now be considered further.

1.2.4.1 sST2 as a biomarker

Following the development of an enzyme-linked immunosorbant assay (ELISA) to measure levels of sST2, assessment of its association with various clinical conditions has been performed [354].

In terms of respiratory pathology, the concentration of sST2 is increased in the sera of patients with asthma [354], and this in particular is associated with acute exacerbations [355]. In addition, levels are raised in patients with pulmonary fibrosis [356] and in sera and BAL of patients with eosinophilic bronchitis [357]; returning to normal following CS treatment. In patients admitted as an emergency with acute shortness of breath due to pulmonary diseases, the serum sST2 concentration was an independent predictor for 1 year mortality [358]. In admissions with shortness of breath of all causes, sST2 levels were again a predictor of mortality at 1 year [359,360] or 4 years [361]. In breathless patients with preserved left ventricular ejection fraction (LVEF) sST2 levels were independent of but provided additional prognostic stratification to other biomarkers [362]. In atopic patients, sST2 is elevated in patients with allergic rhinitis [363].

A rise in sST2 has also been associated with Th-1 mediated inflammatory disease. Patients with autoimmune disease including rheumatoid arthritis and SLE have elevated serum levels [364].

Patients in intensive care had high serum sST2 levels compared to healthy controls and amongst these the subgroup of patients with sepsis had with highest levels; sST2 concentrations correlated with serum IL-10 in this cohort [365]. Hoogerwerf *et al* demonstrated that sustained levels of sST2 correlated with severity as well as mortality in the patients with sepsis [366]. Studies of patients with other inflammatory conditions demonstrated elevated sST2 concentrations: in cerebrospinal fluid of patients suffering subarachnoid haemorrhage [367]; and in diabetic patients with critical limb ischaemia, predicting mortality in this group [368]. In stable diabetic patients, the concentration of sST2 in serum was associated with diabetes but not with established vascular risk factors in a cross-sectional study, suggesting that it reflected an independent inflammatory component of diabetes [369].

ST2 is related to cell growth and proliferation and there are a number of associations of ST2 with malignancy which might reflect this [325]. Concentrations of sST2 in malignant pleural effusions were significantly higher than in effusions caused by heart failure or *Mycobacterium Tuberculosis* infection. Transfecting sST2 into a tumour cell line reduced the ability of cells to proliferate when not anchored, suggesting a role for ST2 in tumour survival [370].

The most common area of interest in sST2 as a biomarker and as a predictor of morbidity and mortality, is in the field of cardiovascular disease. Following on from the findings of Weinberg *et al* suggesting that ST2 participates in cardiovascular injury in mice, these authors found elevated concentrations of serum sST2 in patients after MI [350] and this finding has been consistently demonstrated by others since [371]. Following MI, sST2 correlates positively with creatine kinase, inversely with left ventricular ejection fraction (LVEF) [350] and independently predicts 20-30 day mortality as well as the development of subsequent heart failure (HF), suggesting an association of sST2 with extent of injury [371]. Other studies have demonstrated a correlation of sST2 with mortality in stable HF [372] and exacerbations of chronic HF (not in the context of acute MI) with synergistic prognostication with other biomarkers [359,373]. The studies

discussed above of patients with acute presentations of shortness of breath will clearly include some of these HF patients. Further evaluation of the relationship with sST2 and HF following acute MI in our local population has shown an association of sST2 with LVEF, related infarct size and remodelling parameters over time [374]. The findings in MI in patients alluded to so far are in those with ST elevation myocardial infarction (STEMI) and investigators have now extended these findings to Non-STEMI patients where an association with risk of HF post non-STEMI is reported [375]. Some studies have also now shown that sST2 measurement can selectively predict patient responders to drug treatment including with aldosterone antagonists [374] and increased beta-blockade [376], further extending the clinical value of sST2 as a biomarker. Overall there is clear relationship between serum sST2 concentration and worse outcomes in patients with dyspnoea and in MI with or without HF.

As ST2 was acknowledged as having significant homology with IL-1R (also called IL-1R4), the search to identify the ligand for ST2 was focused on the IL-1 family. IL-1α and IL-β itself were excluded because they do not bind or signal through ST2 [377,378]. Other proteins bound ST2 but were found to be non-functional ligands [377,378] until the discovery of an IL-1 family member with binding and functional capacities which subsequently named IL-33 [293].

1.3 IL-33

The cytokine IL-33 is the 11th member of the IL-1 family and was identified by Schimtz and colleagues at the end of 2005. Although a role for ST2 in the pathogenesis of disease has been demonstrated, functional mechanisms were lacking to explain its effect. The identification of the ligand for ST2 has provided insights into the role of the ST2/IL-33 pathway in the pathogenesis of immune-inflammatory diseases.

The majority of current knowledge regarding IL-33 biology was accrued during or following completion of the experimental works in this thesis. In the following sections, a summary of relevant literature forming the basis for the work of this thesis will be presented along with the current understanding of IL-33 biology unrelated to the data shown.

1.3.1 IL-33 gene and product processing

The *IL-33* gene was identified by searching a computational derived database of the IL-1 family members [293]. This gene had been previously described as a gene which was upregulated in canine cerebral vasospasm and called *DVS27* [379]and as a nuclear factor identified in high endothelial venules and called NF-HEV [380] but its function in these locations was unclear. The human and mouse gene sequences of *IL-33* have been mapped to chromosomes 9 (9p24.1) and 19 (19qc1) respectively. The translated protein of human and mouse full-length IL-33 are 270 and 266 amino acids respectively, is a 30 kiloDalton (kDa) pro-domain and like other members of the IL-1 family has a predicted basic structure of 12 β-sheet trefoil fold. There is 55% homology between the amino acid sequences of human and mouse IL-33 [293].

As with IL-1ß and IL-18, the synthesised 30 kDa propeptide IL-33 lacks a clear signal peptide for direct processing in the endoplasmic reticulum and golgi apparatus [293]. Using the examples of IL-1β and IL-18, *in vivo*, caspase-1 cleaves the pro-IL-1β and pro-IL-18 to bioactive forms which is an essential step for their subsequent secretion [381]. A similar mechanism was proposed for IL-33, but supporting evidence is conflicting. At least *in vitro*, human IL-33 can be cleaved by caspase-1 to produce a 20-22 kDa mature form [293,382], and this cleavage can be blocked by a caspase-1 inhibitor [382]. Supporting data demonstrates that triggering of the Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, which mediates caspase-1 activation, by the adjuvant alum, results in the cleavage of full length IL-33 (pro-IL-33) and subsequent secretion [383]. The biological effects of extracellular IL-33 have largely been studied using an 18 kDa recombinant protein thought to represent this active mature cleaved product [293]. However, lysates from cells overexpressing human or murine IL-33 after incubation with caspase-1 predominantly contain the 30 kDa full length IL-33, with only a small and variable amount of the 20-22 kDa protein identified. No difference was seen in the lysate content when mutations in potential caspase-1 binding sites were introduced. Indeed, in a protease free environment, no caspase-1 dependent cleavage 20 kDa product was identified [384]. The described cleaved product, which can be blocked by a caspase-1 inhibitor, actually appears to be a biologically inactive product with the

cleavage site being in the IL-1-like domain [382]. Furthermore, *in vitro* caspase-1 cleavage of full length pro-IL-33 to a 20 kDa product could be replicated by caspase-3 producing an inactive product [385]. Mature IL-33 is found intracellularly independently of the presence of caspase-1, suggesting pro-IL-33 may not be a direct substrate for caspase-1 *in vivo* and other proteases could be involved [384]. In addition, full-length IL-33 cleavage by apoptotic caspase-3 and-7 results in attenuated activity of the cleaved product and indicates a protective mechanism from the release of bioactive and inflammatory IL-33 from apoptotic cells during normal cell turnover [386].

Before its description as a cytokine IL-33, HF-NEV was first described as a nuclear factor in the nucleus [380], and investigators have now established a role for the intracellular IL-33, as a nuclear factor *in vivo* [387-390]. This indicates that IL-33 is a dual-function molecule both as a secreted cytokine and an intracellular nuclear factor. With a lack of caspase-1 binding site, uncertainty regarding a caspase-requiring release of 'mature IL-33' and the full length form of IL-33 shown to be active extracellularly [382], hypotheses for how this pro-IL-33 form might be released to be biologically active by cell damage as an 'alarmin' and how the full-length and other forms function intracellularly as a nuclear factor were developed. Calpain-associated cleavage releases mature IL-33 (20 and 18 kb products) but the full length IL-33 is released in cytotoxic conditions, however the biological activity of these released mature forms were not confirmed [390]. Demonstration of release of pro-IL-33 was also seen from endothelial cells in response to mechanical injury or necrosis resulting from freeze/thaw cycles [382]. Further studies also show murine pro-IL-33 from transfected cell lysates, nuclear extracts or synthesised in vitro, induced ST2 dependent release of IL-6 from MCs [384].

Potential release of a biologically active full length form of IL-33 was likened to the alarmin high motility group box-1 (HMGB-1), a nuclear factor which modulates transcription but when released from a damaged cell, has a potent extracellular action [391]. Like PAMPs, responding to exogenous danger patterns, alarmins can respond to endogenous danger signals with alarmin release also triggered by PAMPs; PAMPs and alarmins are collectively known as DAMPs. HMGB-1 is inactivated during apoptosis [392] and is activated by TLRs as well as enhancing TLR-induced inflammation [393].

Secretion of mature IL-33 has been described in: a monocyte cell line (THP-1 cells), in response to bacterial infection or the adjuvant alum and LPS[383]; culture supernatants of rat cardiac fibroblasts stimulated with phorbol 12-myristate 13-acetate (PMA) [394]; and in mixed murine glial cell cultures supernatants after stimulation by LPS and ATP [395] but the biological activity of cleaved mature IL-33 is unclear. More recently, biological activity of active secreted mature forms of IL-33 released following non-caspase protease cleavage (18-22 kDa), has been demonstrated and demonstrate increased potency [396]. Overall, it is clear that if released from the cell, both pro- and mature-IL-33 have biological function with the cell type and environment likely to influence the 'type' of IL-33 secreted or released in cell damage.

1.3.2 IL-33 expression

Using *in vitro* detection methods and cDNA libraries, IL-33 mRNA is found in virtually every murine tissue but particularly in the central nervous system and in surfaces exposed to the environment such as the stomach, lungs and skin [293]. In the lung, IL-33 mRNA was observed in murine lung following ovalbumin challenge in an AAI model [344,397]. In humans, mRNA is predominantly found in skin and lung tissues [293].

At a cellular level, IL-33 is expressed primarily in smooth muscle, endothelial and epithelial cells [293,389]. Levels of expression vary as constitutive or inducible and nuclear or cytoplasmic, different according to cell type. For example, resting MCs express very low IL-33 mRNA levels but high levels after ionomycin stimulation [398]. Endothelial cells have high levels but these are further inducible and in fibroblasts and epithelial cells mRNA levels are high and unchanged with stimulation [398]. Nuclear localisation predominates in epithelial and endothelial cells [388,389] whilst cytoplasmic location is predominant in MCs [398]. A summary of cellular expression of mRNA and protein is shown in Table 1.4.

Cell	Mouse	Reference	Human	Reference
Endothelial cell (including HEV)	RNA and Protein (rat)	388,398	RNA and protein*	293,387 388,389 382
Epithelial cell	RNA	293 398	RNA *(bronchial and small airway)and Protein	389,390 293
Smooth muscle cell	-	-	RNA* (pulmonary and bronchial) and protein*	293,399
Fibroblast	RNA and protein	400 394,398	RNA* and protein	293,401 402
Myofibroblasts	-	-	Protein	403
Keratinocyte	-	-	RNA	293
Adipocyte	-	-	RNA	404,405
Colonocyte	-	-	RNA and protein	406
Glial cell	RNA and protein	395	-	-
Osteoblast	-	-	RNA	405
Monocyte	-	-	RNA and protein	293,384,386
Dendritic cell	RNA and protein	293 400	RNA	293
Macrophage	RNA and protein	293,400,407 408	RNA	293
Neutrophil	RNA	333	Protein	396
Mast cell	RNA and Protein	409 398	-	
T cell	RNA	293	-	
B cell	RNA	293	-	

Table 1.4: Cellular expression of IL-33

1.3.3 IL-33 receptor binding and signalling

IL-33 binds specifically to the IL-33 receptor, ST2L (Figure 1.2). Recombinant human or murine IL-33 forms a complex with ST2L and does not bind to other IL-1 receptor family members [293]. Consistent with canonical IL-1-like signalling, ST2L recruits a number of signalling molecules via its cytoplasmic TIR domain. The binding of IL-33 to ST2L results in the recruitment of myeloid differentiation primary response protein 88 (MyD88) *in vitro* [409] and *in vivo* [410]. Other signalling components recruited include IL-1R associated kinase 1 (IRAK1), IRAK4 and TNF receptor associated factor-6 (TRAF6) to the receptor complex in cytoplasmic

region of ST2 [293]. Downstream this results in phosphorylation of transcription factor nuclear factor-κB (NF-κB) leading to up-regulation of pro-inflammatory genes. NF-κB upregulation can be inhibited by the administration of anti-ST2 antibody [293]. Other signalling proteins induced include; inhibitor of NF-κB(IkBa), MAP kinase family members extracellular signal-regulated kinase 1 (ERK1), ERK2, p38 and c-Jun N-terminal kinase (JNK), and transcription factor AP-1, leading to the induction of production of inflammatory mediators [293,411].

Adaptor molecule TRAF6-dependent pathways have been identified in fibroblasts [412] and endothelial cells [413] with a TRAF6/NF-κB-independent, MyD88/p38-dependent pathway in basophils [409]. A study using tyrosine kinase inhibitors identified JAK2 as critical in the IL-33-mediated NF-κB pathway, identifying a target molecule for specific cell types [414]. Drube *et al* report that c-Kit activation cross-reacts with ST2L for optimal IL-33 effects on mast cells in a STAT3, ERK1/2 and JNK-1 pathway [415]. Overall this suggests cell-to-cell variation in signalling regulation to fine tune IL-33-mediated effects according to the needs of the cell type.

The signalling mechanism of the other IL-R1 family members requires a co-accessory protein for high affinity binding and this has also been demonstrated to be the case for ST2L. The IL-1 receptor accessory protein (IL-1RAcP), which acts as a shared co-receptor with other members of the IL-1 family is the co-receptor for ST2L-mediated signalling through its TIR domain [416]. In addition, IL-1RAcP increases ST2 affinity for IL-33 by a factor of four [410]. The potent inflammatory effects of the administration of IL-33 to WT mice are completely absent in IL-1RAcP knockout mice [416].

Because of the severe pathophysiological consequences of dysregulated expression, all IL-1 family members are tightly regulated. As mentioned, the soluble form of ST2 has been described as a decoy receptor for IL-33 as it attenuates inflammatory responses suggesting sequestration. *In vitro* direct blockade of IL-33 signalling by sST2 has been shown [397] [394] whilst soluble IL-1RAcP enhances ability of sST2 to bind and inhibit IL-33 [410]. Single Ig IL-1 receptor-related molecule (SIGGR), a further member of the IL-1/TLR superfamily acts as a negative regulator TLR-IL-1R-mediated signalling. SIGGR directly inhibits IL-

33-ST2 mediated signaling *in vitro* which may explain the exaggerated Th2 responses seen in SIGGR deficiency *in vivo* [411].

1.3.4 IL-33 function as a nuclear factor

Pro-IL-33, which was originally described as a nuclear factor, accumulates in the nucleus [387,389]. Here, it associates with heterochromatin and mitotic chromosomes through an evolutionary conserved localisation sequence, homeodomain-like helix-turn-helix (HTH) motif and thus acts with transcriptional repressor properties [387]. In endothelial cell activation, non-proliferating cells express nuclear IL-33 that is strongly down-regulated in early angiogenesis further suggesting regulatory properties [388]. This is supported by the observation that nuclear expression of IL-33 is not uniform but co-localises with chromatin-rich domains containing high local concentrations of DNA [389]. A chromatin binding peptide (H2A-H2B), with similarity to the mechanism of the DNA Kaposi sarcoma tumour herpes virus, has been identified on IL-33, suggesting mimicry of the virus with a biological mechanism for nuclear association and latent infection[417]. Full length pro-IL-33 can translocate to the nucleus and it has been shown to interact directly at its N-terminal region forming an IL-33/NF-κB complex suppressing proinflammatory gene transcription confirming its repressor function [418].

1.3.5 IL-33 function as a cytokine

Despite uncertainty over different forms of IL-33, it now seems that whatever the release mechanism, IL-33 is a biologically active cytokine in its cleaved and mature form. At the outset of this work the description of the biological effect of IL-33 was as a potent inducer of type-2 innate inflammation [293]. Following the discovery of the *IL33* gene, Schmitz and colleagues developed a recombinant (18ka) protein and tested its activity *in vivo* and *in vitro*. In mice, following systemic intraperitoneal (i.p.) administration of IL-33, splenomegaly, blood eosinophilia and severe pathological changes in the gut and lung developed. This was accompanied by a rise in the concentration of type-2 associated cytokines IL-5 and IL-13 in the serum as well as raised IgE and IgA immunoglobulins [293].

The cell populations targeted by IL-33 were poorly characterized but since then considerable work in clarifying the cellular responses have been established with multiple targets and a corresponding wide range of effects. The knowledge that the IL-33 receptor ST2L is expressed on innate MCs as well as adaptive Th2 cells suggests that IL-33 is a cytokine capable of involvement in multiple facets of asthma pathophysiology and may act as bridge between the innate and adaptive immune processes.

1.3.5.1 IL-33 and innate cells

MCs highly express ST2 and are therefore unsurprisingly sensitive to IL-33 stimulation and as such are one the most studied responder cells. IL-33 induces the activation of NF-κB and MAPKs in mouse MCs [293]. In response a range of cytokines are produced including IL-6, IL-13, IL-1β, TNF-α, prostaglandin D2, MCP-1 in BMMCs [419] and IL-13, IL-5, IL-6, IL-10, TNF-α, GM-CSF, CXCL8/IL-8 and CCL1/TCA3 from human MCs [330]. A synergistic effect of IL-33 with IgE crosslinking to enhance cytokine production has been described [330] However degranulation of MCs does not appear to occur [330,419]. IL-33 promotes the development of mast cell from $CD34^{+}$progenitor cells [330] and promotes their survival and adhesion to ECM and endothelial vessels by upregulation of adhesion molecules [329].

Basophils also secrete proinflammatory mediators including IL-4, IL-5, IL-6, GM-CSF and histamine after with IL-33 treatment [310,326]. IL-3 enhances basophil ST2 expression and cytokine release [308,310]. Like in MCs cytokine production is enhanced by IgE crosslinking in basophils [310,328]. Degranulation does not appear to occur, but IL-33 enhances IgE mediated degranulation at least in humans *ex vivo* [310].

As previously discussed in section 1.2.3.3, ST2 is proposed to play a role in endotoxin tolerance by reduced cytokine production by sequestration and blocking MyD88 signalling [346]. However, when macrophages are treated with the ST2 ligand IL-33 *in vitro,* the response to endotoxin was conversely enhanced and LPS desensitisation was not induced [420]. IL-33 may increase MyD88 to enhance this TLR pathway but in the absence of a ligand binding to ST2, ST2 can sequester MyD88 thereby reducing its availability for TLR signalling. Importantly,

other macrophage phenotypes have been described but their response to IL-33 is hitherto unknown and will be further investigated in chapter 4.

IL-33 activates the expression of cell surface molecules: MHC-II and co-stimulatory molecules CD86 [332]; or co-stimulatory molecules CD40, CD80 and OX40L on murine DCs [421]. IL-33 drives production of IL-6, IL-1β, TNFα and CCL17/TARC from DCs [332,421]. In co-culture experiments these IL-33-stimulated DCs drive naïve T cells to a Th2 phenotype producing IL-5 and IL-13, but not IL-4 or IFNγ, suggesting a role for IL-33 in initiation of Th2 inflammation [332]. *In vivo*, in an AAI model, $ST2^{-/-}$ mice had attenuated inflammation which was reinstated by the adoptive transfer of these IL-33 treated DCs [421].

Since the discovery of IL-33 and the recognition that its administration caused profound eosinophilia, some interest has turned to these granulocytes not known previously to express ST2L, as potential IL-33-mediated effector cells. ST2 mRNA expression was detected in human eosinophils but ST2L protein surface expression was only present in activated cells after 24 hours *ex-vivo* and this was enhanced by co-culture with GM-CSF [309]. IL-33 drives modest IL-8 and MCP-1 cytokine production, superoxide production and promotes survival in human eosinophils [309]. In mice, IL-33 enhances eosinophil differentiation, stimulates *in vitro* eosinophils to produce IL-13, CCL17 and TGF-β, and enhances the differentiation of AAM in an airways inflammation model, suggesting an additional role for eosinophils directly in IL-33-mediated inflammation [311]. Given the important roles for MCs, basophils and eosinophils in allergy and asthma, these findings suggest IL-33 is important in allergic responses.

Neutrophil ST2L expression is significantly upregulated by IL-33 and furthermore, IL-33 is a neutrophil chemoattractant in a mouse sepsis model and in patients with RA, suggesting a mechanism for migration into inflamed joints [312,333]. Neutrophils of patients treated with anti-TNFα therapy did not display this IL-33-induced chemotaxis suggesting a possible mode of drug action [333].

1.3.5.2 IL-33 on adaptive cells

Schmitz *et al* demonstrated type-2 inflammation in response to exogenous IL-33 in mice. Th2 cells, known to selectively express ST2, through which a role in Th2/type-2 inflammation has already been described, places them central in a hypothesis of IL-33-driven inflammation [293]. The initial *in vitro* stimulation of Th2 cells by Schmitz revealed IL-33 that enhances Th2 cytokine production from IL-4 stimulated Th2-polarised cells. In support of IL-33 driving a Th2 biased inflammatory response, Th1 cells incubated with IL-33 produced less IFNγ [293,326]. Non-polarised T cells produce IL-4 and IL-13 and polarised antigen-dependent as well as antigen-independent T cells produce IL-5 and IL-13 when treated with IL-33 [326,422,423]. In antigen-dependent stimulation, IL-33 also enhances IFNγ production in blood-derived Th2 cells with loss of intracellular IL-4, although in these experiments there were other T cells sources capable of contributing to IFNγ production in the mixed cell culture [326]. In Th2 cells, IL-33 plus IL-2 induced NF-κB dependent production of IL-13 in an antigen-independent pathway that required STAT5 activation but not nuclear factor of activated T cells (NFAT), a further example of differential cell signalling [423]. Interestingly, IL-33 has also been found to act as a chemotactic factor for Th2 cells in both mice and humans[424].

Administration of IL-33 can induce the production of a type 2-immunoglobulin profile in naïve mice suggesting that IL-33 could interact with B cells directly[293]. Administration of i.p. IL-33 increased the population of B1 cells in an IL-5-dependent manner. Naïve B1 cells expressed low levels of ST2, upregulated by IL-33 treatment producing IL-5, IL-13 and IgM. This direct response is augmented by IL-5 produced from mast cell and T-cells in response to IL-33 [327].

Extending the biological activities of IL-33 beyond Th2 immunity, invariant NKT (iNKT) cells are found in increased numbers in mice treated with systemic IL-33. IL-33 treatment in the presence of TCR stimulation enhanced IL-2, IL-4, IL-5, IL-13 and TNFα production from human iNKT cells *in vitro*. In the absence of TCR stimulation and in the presence of IL-12, the production of IL-4 and to a greater extent IFNγ were enhanced. NK1.1 cells responded similarly indicating the

induction of a pro-type1/Th1 profile as well as type-2 responses [326]. The cytokine responses of NK2 cells to IL-33 cells are not known.

Recent studies show that $CD8^+$ T cells express ST2 and respond to IL-33, and similar to iNKT cells this is augmented by IL-12 and TCR engagement [425]. This has led to the investigation into potential IL-33 driven viral responses. Indeed, a responisve subset of antigen-specific CD8 T cells was found to mediate protective anti-viral T cell responses [426].

1.3.6 IL-33 in disease

Given the effects of ST2 and now demonstrated effects of IL-33 on cell populations relevant to asthma, this overwhelmingly suggests a role for IL-33 in AAI and asthma. Following the description of abnormal lung pathology in mice systemically treated with IL-33, the next description of a role for IL-33 in airways disease was the 2007 description of increased sST2 and IL-33 mRNA expression in an AAI model. The IL-33-driven effects on *ex vivo* splenocytes was blocked by sST2 [397]. In humans, genome-wide association studies have identified single nucleotide polymorphisms or SNPs encoding IL-33 and asthma [427].

Higher levels of serum IL-33 are reported in patients with allergic rhinitis [428]; serum levels of IL-33 were also elevated in patients with atopic dermatitis compared to non-inflamed skin healthy controls or non-atopic psoriasis patients [429]. IL-33 gene polymorphisms are described in rhinitis patients [428] and allergic conjunctivitis patients [430].

IL-33 helps clear the intestinal nematode, *Trichuris muris* primarily via the induction of Th2 responses. Some pathological changes were still seen in SCID mice, deficient in T and B cells, which displayed elevated NK numbers suggesting NK also contribute to the observed pathology [431]. Indeed $ST2^{-/-}$ mice have increased susceptibility to encephalitis secondary to *Toxoplasma gondii* in keeping with a protective effect of IL-33 in parasite responses [432]. An innate source of cytokines induced by IL-33, is now known to be the recently discovered ILC; a population of cells which proliferate during *Neppostrongylus Brasiliensis* infection in mice[158].

A further potentially beneficial effect of IL-33 administration has been shown in a murine model of atherosclerosis [321]. IL-33 treatment attenuated atherosclerotic plaque development by inducing IL-5 stimulated ox-LDL antibody production [321], and reduced macrophage foam cell formation [433]. IL-33 also appears to have a potential beneficial role in heart failure, enhancing fibroblast-cardiomyocyte interaction [394]. As noted previously in 1.2.4.1, sST2 is a biomarker for acute MI and increased serum concentrations were associated with remodelling and HF post acute MI. There are some mechanistic studies to explain anti-inflammatory effects of IL-33 in this setting. IL-33 prevents hypoxia-induced apoptosis of cardiomyocytes as well as improved LV function and survival post MI in WT but not $ST2^{-/-}$ mice [434]. Furthermore, reduced macrophage infiltration and subsequent cytokine production in the myocardium along with suppressed NF-κB and p28MAPK signalling and remodelling was observed with IL-33 treatment in a murine MI model [435].

Adipose tissue is now increasingly being considered as an immune 'organ'. IL-33 mRNA is expressed in human adipocytes and is upregulated by TNFα suggesting a role in obesity where increased levels of TNFα are seen [404]. Treatment of genetically obese mice (*ob/ob*) with IL-33 led to protective metabolic effects and conversely the $ST2^{-/-}$ mice had worsened metabolic parameters [436]. The highest levels of IL-33 were detected in the endothelial cells in human adipose tissue suggesting that the influence of IL-33 on obesity could be via the microvasculature [437]. Interestingly, ILC were first described in adipose tissue [157] and IL-33 responsive ILC subsequently increase eosinophil and AAM accumulation in adipose tissue by IL-5 and IL-13 production [158,438].

IL-33 may play a role in fibrotic injury in the liver. Increased expression of IL-33 mRNA is reported in fibrotic livers of mice and humans and this was upregulated in hepatic stellate cells in response to proinflammatory cytokines [439]. Serum IL-33 levels are elevated in patients with liver failure admitted to intensive care unit or in those with chronic liver disease secondary to hepatitis C infection and correlates with other inflammatory mediators [440,441]. Levels of sST2 in these patients tended to reflect the more acute presentation indicating a possible additional biomarker role [441].

IL-33 protein and mRNA expression is present in normal and diseased synovium in experimental arthritis and in patients with rheumatoid arthritis [387,401,402]. Human RA synovial fibroblasts contain low levels of IL-33 mRNA and protein but the addition of TNFα and IL-1ß to primary fibroblast culture, increases the expression of IL-33 mRNA and protein [401,402]. Several studies have described a role of ST2L and sST2 in regulating inflammatory responses in arthritis[401]. The administration of IL-33 exacerbates the inflammation associated with antigen-induced and in auto-antibody-induced murine arthritis models, by mast cell activation [402,442]. A genetic variant of IL-33 is associated with rheumatoid arthritis [443] and elevated IL-33 serum concentrations correlate with the number of inflamed joints and with disease activity [444].

IL-33 protein expression is increased in skin biopsies of patients with psoriatic arthritis [445] and *in vivo,* IL-33 induces a psoriatic-like pathology in murine skin which is partially mast cell dependent [446].

In patients with Crohn's disease and ulcerative colitis, protein and mRNA expression of IL-33 and ST2 was observed in colonic biopsy specimens and moreover, serum concentrations of IL-33 and sST2 were raised in these patients [387,406]. Administration of exogenous IL-33 exacerbated experimental ulcerative colitis via an IL-4 dependent mechanism [447]. Production of type-2 cytokines by colonic macrophages may in part contribute to the excessive inflammation in experimental colitis; increased numbers of IL-33 positive colonic macrophages were associated with a reduction in macrophage response to TGF-β, which is important in resolution of inflammation [407].

In the nervous system, gene polymorphisms are associated with Alzheimer's disease [448]. *In vitro,* increased mRNA IL-33 expression is noted in activated glial cells [395] and in the brains of mice with parasite-driven mouse encephalitis model [432]. In an autoimmune encephalomyelitis model, the administration of exogenous IL-33 attenuated disease activity [449].

1.4 Objectives

The preceding introduction discusses the current understanding regarding the pathophysiology of asthma. A great deal of research has provided the current paradigm of a Th2-mediated inflammatory disease of the airways with complex involvement of the innate and adaptive immune systems and increasing evidence suggesting that underlying abnormalities in the structural cells and matrix may be at least as important. Much of this information has come from animal models, but it is also apparent that such information can be conflicting due to the variability in models, stains and experimental conditions. As such, many fundamental questions are unanswered; in particular where novel cytokines are placed in the cytokine hierarchy orchestrating this inflammation. One of these cytokines, the recently discovered IL-33, drives type-2 inflammation and its receptor ST2L has been considered a potential modulator of type-2 responses in the airway in particular due to its expression on Th2 cells and MCs.

Thus this thesis aims to address the hypothesis that the IL-33-ST2 axis is critical in the development, maintenance and persistence of AAI and asthma via effects on both the innate and adaptive immune systems.

The objectives of this thesis are therefore to:

- Assess the expression of IL-33 and ST2 in the murine airway *in vivo*
- Assess the presence of and quantify the amount of IL-33 and ST2 in airway fluid and plasma of asthmatic patients
- Characterise the airway inflammation caused by IL-33
- Assess the contribution of T cells to IL-33-induced airway inflammation
- Ascertain the role of structural epithelial cells in IL-33-induced airway inflammation
- Assess the contribution of IL-33 to the adaptive immune response in allergic airways inflammation *in vivo*

- Further investigate the role of ST2 to adaptive immune response in allergic airways inflammation *in vivo*

By performing experiments to address these objectives this thesis aims to demonstrate that the IL-33-ST2 axis plays an important role in the innate and adaptive immune response in allergic mediated lung disease.

2 Methods

2.1 Reagents and buffers

Commonly used buffers and reagents are indicated in Table 2.1. Other chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated.

Reagent	Composition
Phospate buffered saline (PBS)	8g NaCl, 1.16g Na_2PO_4, 0.2g KCL, 0.2g KH_2PO_4 in 1 litre distilled water, pH 7.4
Complete medium (CM)	RPMI (Invitrogen), 10% heat inactivated fetal calf serum (FCS), 5ml penicillin/streptomycin (pen/strep)(100IU), 5 ml L-glutamine (2mM), 50 µm B-mercaptoethanol
Wash medium	RPMI, 5 ml pen/strep, 5 ml L-glutamine (2mM)
FACS buffer	2% FCS in PBS, ±5mM ethylenediaminetetraacedic acid (EDTA)
ELISA wash Buffer	0.05% Tween-20 in PBS pH 7.4
BD ELISA coating buffer	0.1M $NaHCO_3$ pH8.4
R&D ELISA coating buffer	8.4g $NaHCO_3$, 3.56g $Na2CO_3$ in 1 litre distilled water, pH 9.5
OptEIA coating buffer	8.4g $NaHCO_3$, 3.56g $Na2CO_3$ in 1 litre distilled water, pH 9.5
Biosource coating buffer	PBS
Biosource ELISA assay buffer	0.5% bovine serum albumin (BSA), 0.01% Tween in PBS
BD ELISA assay buffer	10% FCS in PBS
R&D assay buffer	1% BSA in PBS 5% sucrose, 0.05%NaN_3 in PBS
70% ethanol	7 parts 100% ethanol, 3 parts distilled water
Tail lysis buffer	10mM Tris pH 8.0, 50mM EDTA, 100mM NaCl, 0.5% Sodium Dodecy Sulphate. 500 µg/ml proteinase K (Qiagen, stock 20 mg/ml) added fresh before use
Tris-acetate-EDTA (TAE)buffer (x50)	242g Tris base, 57.1ml glacial acetic acid and 100 ml 0.5M EDTA (pH 8) in 1 litre distilled water.
Citrate buffer 0.01M	2.1g citric acid in 1 litre distilled water, pH 6
Tris Buffered saline (TBST) buffer	6.05g Tris and 8.76g NaCl in 1 litre distilled water, pH 7.5 In 0.05 Tween
Avertin	1:1 weight volume solution of 2,2,2-tribromoethanol in tert amyl alcohol

Table 2.1: Commonly used buffers and reagents

IL-33 used was from three sources. Human and murine IL-33 produced and purified within the laboratory by Dr Damo Xu was used for *in vivo* experiments. IL-33 complementary deoxyribonucleic acid (cDNA) was cloned from IL-1 stimulated human or murine fibroblasts by real time-polymerase chain reaction (RT-PCR) using specific primer pairs and this sequence was transformed into host *Escherichia coli*. IL-33 protein was induced by IPTG and purified by Ni-NTA affinity chromatography (Qiagen). Endotoxin was removed by a polymyxin B column purification. Endotoxin levels in the generated protein were less than 0.01 EU/µg of as measured by *Limulus* amebocyte lysate QCL-1000 pyrogen testing (Cambrex). Purity of IL-33 was more than 95%. Detailed methods for IL-33

production have been published[424]. Bioactivity and specificity of recombinant IL-33 were confirmed by IL-5 induction in lymph node cells (Figure 2.1). Human IL-33 was injected into mice in the first description of IL-33 as a bioactive cytokine and there is a 55% homology between human and murine IL-33 [293]. Others in our laboratory had determined the bioactivity of murine IL-33 produced was approximately half of human IL-33 in murine cells in an *in vitro* setting (Dr M Kurowska-Stolarska and Dr P Kewin) and thus the dose adjusted accordingly.

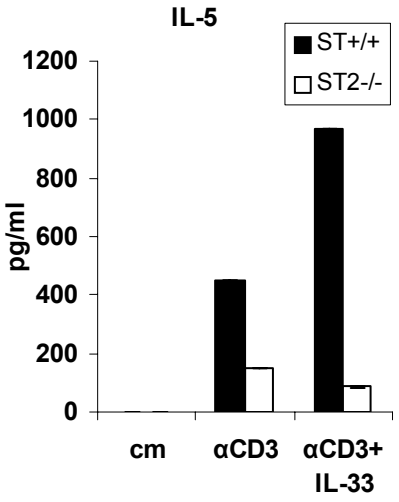


Figure 2.1: Example bioactivity of in-house murine recombinant IL-33
Following processing, WT ($ST2^{+/+}$) and $ST2^{-/-}$ peripheral lymph node cells at a concentration of 1 $x10^6$/ml were cultured in 24 well plate with a combination of complete medium (cm), plate bound anti-CD3(αCD3) (2 µg/ml) or IL-33 (10 ng/ml). Cells were cultured for 72 hours following which supernatants were removed and IL-5 concentration measured by ELISA. Data is mean of experimental triplicates. Example shown is 'Batch 6' 115 µg/l IL-33 tested to confirm bioactivity.

For *in vitro* experiments, recombinant human IL-33 (PeproTech, UK or Axxora, ALEXIS Corporation, Switzerland) were used. Comparable bioactivity of these reagents is shown in Figure 2.2 in the human mast cell line, HMC-1 cells.

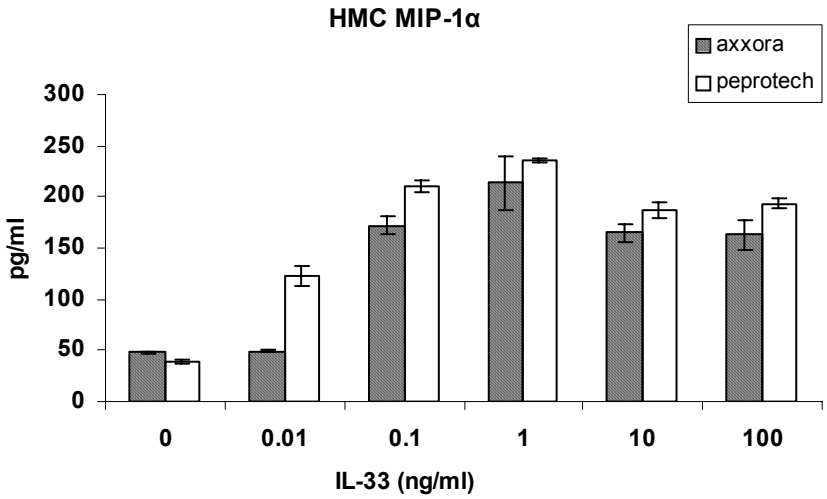


Figure 2.2: Bioactivity of commercial human IL-33
HMC-1 cells were grown in 12 well (2x 10^5 cells/well) overnight and then IL-33 at 0-100 ng/ml added to culture media and supernatants removed 24 hours later and stored at -20°C until analysis. Human MIP-1α ELISA performed as per manufacturer's instructions.

2.2 Mouse strains

All animals were housed in pathogen-free conditions in facilities managed by Biological Services staff, University of Glasgow in strict accordance with United Kingdom Home Office regulations under the Animals (Scientific Procedures) Act 1986.

2.2.1 Commercially available mice

BALB/c and C57BL/6 mice were obtained from Harlan Olac (Bicester, Oxon, UK).

2.2.2 Knockout mice

$ST2^{-/-}$ mice were originally a gift from Dr A Mackenzie (University of Cambridge) and $IL\text{-}4^{-/-}$ mice from Prof J Alexander (University of Strathclyde) with colonies subsequently bred in-house by Biological Services, University of Glasgow. $Rag^{-/-}$ mice were donated by Dr A Michie (University of Glasgow). BALB/c-transgenic D011.10 mice were bred and housed at Biological Services, University of Glasgow.

2.2.3 Breeding IL-4$^{-/-}$/St2$^{-/-}$knockout mice

Female ST2$^{-/-}$ mice were mated with male IL-4$^{-/-}$ mice to produce heterozygous F1 mice and this is discussed in more detail in section 6.3.1. F1 mice were re-crossed to generate double homozygotes F2 (see Figure 6.17). Genotype was identified by PCR of DNA extracted from tail tips. The tail-tip was cut by Biological Services staff under isofluorane anaesthesia with tail cauterisation

2.2.3.1 Screening for IL-4 and ST2 expression by PCR

All techniques were performed using gloves and guaranteed DNase/RNase free plastic ware.

Tail tips were digested overnight at 55°C in 0.5 ml tail lysis buffer. Phenol/chloroform/isoamyl alcohol (0.5 ml) was added and placed in a vortex mixer. Following 5 minutes rest at room temperature, the digested material was centrifuged for 5 minutes. The DNA-containing top layer was decanted into a fresh eppendorf taking care not to disturb the protein layer under. This was mixed with 0.5 ml chloroform and centrifuged for 5 minutes. The top DNA-containing layer was further decanted again and 0.8 ml of ice cold 100% ethanol was added and following mixing left at -20°C for 20 minutes allowing the DNA to precipitate. This was centrifuged for 15 minutes, the supernatant was poured off, and 0.5 ml of ice cold 70% ethanol added to the DNA pellet. This was centrifuged again for 15 minutes, and the supernatant poured off. All centrifugations were at 14000 rpm at 4°C. The DNA pellet was air-dried for 25 minutes at 55°C in a vacuum-dryer, and resuspended in 50µl pure water for injection. The DNA was stored at 4°C until PCR was performed for amplification.

ST2 $^{-/-}$ mice have a targeted vector deletion within the *St2* gene which involves the majority of exons 4 and 5 [339]. ST2 primer sequences were based on his published data and obtained from Sigma-Genosys. IL-4 $^{-/-}$ mice generated by targeted vector insertion transfection into embryonic cell lines [450]. Constructed IL-4 primers were based on published data but modified using BLAST® *mus musculoris* genome search (with assistance from Dr Derek Gilchrist) and synthesised by VHBio [450,451]. All primers were reconstituted in Diethyl Pyrocarbonate (DEPC) treated water (Ambion).

	5' ⟶ 3' sequence
Primer 1	TTG GCT TCT TTT AAT AGG CCC
Primer 2	CTA TCA GGA CAT AGC GTT GGC TAC C
Primer 3	TGT TGA AGC CAA GAG CTT ACC

Table 2.2: ST2 oligonucleotide sequence primers for PCR
Primers were obtained from Sigma-Genosys and diluted to 10OD with pure water and stored at -20°C until use.

	5' ⟶ 3' sequence
Primer 1	GTT GAG CAG ATG ACA TTG GGG C
Primer 2	CTT CAA GCA TGG AGT TTT CCC

Table 2.3: IL-4 oligonucleotide sequence primers for PCR
Primers were obtained from VHBio and reconstituted with pure water as per volume instructed to a 100 µM concentration. A working stock solution of 10 µM was prepared and stored at -20°C until use.

Reaction buffer and sample DNA were mixed in PCR tubes. Reaction buffers for ST2 and IL-4 PCR are as shown in Table 2.4.

Per well	**ST2**	**IL-4**
10x PCR buffer	2µl	5µl
$MgCl_2$ (50mM)	1µl	2.5µl
dNTPs (10mM)	0.5µl	1µl
Taq DNA polymerase	0.5µl	0.5µl
Pure H_2O	13µl	36µl
Primer 1	0.5µl (10 OD)	1.5µl (10 µM)
Primer 2	0.5µl(10 OD)	1.5µl(10 µM)
Primer 3	1µl(10 OD)	
Sample DNA	1µl	2µl

Table 2.4: PCR reaction mix for ST2 and IL-4 polymerase chain reaction

DNA samples from ST2 $^{+/+}$, ST2 $^{+/-}$, ST2 $^{-/-}$, IL-4 $^{+/+}$, IL-4 $^{+/-}$ and IL-4 $^{-/-}$ mice were used as positive controls and pure water as a negative control. PCR was amplified in a thermal cycler (Eppendorf) using the following protocols in Table 2.5.

ST2		IL-4	
94°C 3 minutes	x1	94°C 2 minutes	x1
94°C 45 seconds 60°C 30 seconds 72°C 90 seconds	x30	94°C 30 seconds 58°C 45seconds 68°C 2.5 minutes	x5
72°C 10 minutes	x1	94°C 30 seconds 68°C 30 seconds 68°C 30 seconds	x35
		68°C 2.5 minutes	x1
Store at 4°C until run on DNA detection gel		Store at 4°C until run on DNA detection gel	

Table 2.5: PCR protocol for ST2 and IL-4

The PCR product was mixed with 5µl loading buffer and run on a 2% agarose weight/volume gel with 0.005% ethidium bromide for 30 minutes at 100 mV in TAE buffer, then viewed under ultraviolet (UV) light (Gel logic 200 imaging system) and Kodak software. A DNA sample ladder was used to estimate the size of the bands detected, the ladders were 50 base pair (bp) and 1 kbp (Invitrogen). For ST2, primers 1 and 3 amplified the 500 bp WT band with primers 2 and 3 amplifying the 200 bp disrupted ST2 band. For IL-4, primer 1 and 2 gave the WT band (~120 bp) and targeted band (~1320 bp). Example gels are shown in Chapter 6.

2.3 *In vivo* methods

Procedures were carried under Project Licence Number 60/3119, Procedure 9, or Project Licence Number 60/3791, Procedure 5 in strict accordance with United Kingdom Home Office regulations under the Animals (Scientific Procedures) Act 1986. $RAG^{-/-}$ mice were kept in filter top cages with sterilised food and water.

2.3.1 Intranasal (i.n.) dosing

Mice were anaesthetised by the intraperitoneal (i.p.) injection of 250 µl of avertin solution (1:40 dilution of avertin stock (Table 1) in PBS). Intranasal (i.n.) administration was performed with the animal in the upright position. Using a 200 µl pipette, 30 µl of reagent in sterile PBS was instilled into the nostrils. The mice were placed in the recovery position in a warming chamber until they recovered consciousness, with supplemental oxygen given if required. The number of challenges is described in each experimental protocol.

2.3.2 Intravenous (i.v.) dosing

Mice were placed in a warming chamber at 38°C for 20 minutes prior to injection to induce vasodilatation. Following placement in a restrainer, intravenous (i.v.) access of a tail vein was obtained using a 1 ml insulin syringe and up to 200 µl volume of cells injected. Direct pressure was applied to the puncture site and mice released.

2.3.3 Ovalbumin allergic airways inflammation model

2.3.3.1 Rapid 12 day OVA allergic airways inflammation

An optimised model of experimental asthma based on a published model was already in use within the laboratory[452]. AAI was induced in female BALB/c mice aged 6-8 weeks with senstised by i.p. injection of 100 µg chicken OVA (Grade V) adsorbed to 2 mg alum (Brenntag Biosector, Frederlkssund, Denmark) in sterile 100 µl of PBS on day 1. On days 9-11, were challenged with 10 µg OVA in 30 µl by i.n. administration. Mice were culled on day 12 by i.p. injection of avertin 500 µl before exsanguination. Samples were collected and processed as detailed below. Dosing modifications and addition of IL-33 for some experiments are detailed where relevant in chapter 6.

2.3.3.2 Long traditional 28 day OVA allergic airways inflammation model

For some experiments, a well studied longer 28 day model of AAI was used[104]. Mice had a further identical sensitisation dose on day 14 with i.n. challenges on days 25, 26 and 27 mice and culled on day 28 as above.

2.3.4 Adoptive transfer model

CD4 T cells from WT, IL-$4^{-/-}$ and WT DO.11.10 mice were grown as described below. The cells were transferred i.v. as described above. Mice were challenged i.n. with daily consecutive dose of OVA or OVA peptide (ovapp) as indicated. Mice were culled 24 hours following final airway challenge and specimens collected as detailed below.

2.3.5 IL-33-induced airway inflammation model

Recombinant IL-33 or PBS was instilled into the airway of WT, ST2$^{-/-}$, IL-4$^{-/-}$ and RAG$^{-/-}$ mice as detailed above for 1-7 consecutive days. Murine (2 µg) or human (4 µg) IL-33 in 30 µl PBS was used as detailed in experimental protocols. Mice were culled at 24-172 hours and specimens collected as detailed below.

2.3.6 Airway hyperresponsiveness measurement

Non-invasive plethysmography was used to measure enhanced pause (Penh) as an indicator of airways responsiveness to broncho-provocation with methacholine[284]. Penh is a dimensionless parameter which is derived from the relationship between inspiratory and expiratory pressure as well as the pause which occurs at the end of expiration. Briefly, animals are placed in sealed individual chambers in a 4 chamber unit (EMMS, UK) where pressure changes are measured by a transducer. Following a 30 minute acclimatisation period, baseline measurements were recorded and mice were given nebulised increasing concentrations of methacholine or saline in 2 minute intervals and Penh continually recorded. Methacholine was prepared freshly and delivered at doses of 12.5 mg/ml-100 mg/ml. An average reading for the challenge period was calculated using EMMS EDAQ® software.

2.3.7 Sample collection and processing

2.3.7.1 Serum collection

Mice were given an i.p. terminal dose of 500 µl of avertin. Following the onset of full anaesthesia, the mice were killed by exsanguination. The heart was exposed and blood withdrawn using a 1 ml syringe attached to a 23G needle. The blood was stored in 1.5 ml centrifuge tubes for 2-3 hours at room temperature to allow clotting, before serum was separated by centrifugation at 14000 rpm at 4°C for 30 minutes (Joan BR4i centrifuge). Serum was then collected and stored at -20°C until analysed.

2.3.7.2 Bronchoalveolar lavage (BAL) collection

The trachea was exposed by dissection of the superficial neck structures and following a small incision, it was cannulated using a 1 ml syringe with a 23 gauge needle sheathed in plastic tubing (0.58 mm ID, 0.78 mm OD; VWR International). The lungs were carefully inflated with 800 µl PBS whilst holding the needle in place at the insertion site by blunt forceps to provide a seal. The fluid was aspirated after 10 seconds and BAL fluid collected into a 1.5 ml eppendorf tube and kept on ice until further processing. The process was repeated with a fresh 800 µl aliquot of PBS and the two aliquots pooled. Live cell counts were performed in a Neubauer haemocytometer (Weber Scientific International Ltd, Teddington, UK) using a 1:2 dilution with 0.4% trypan blue solution. After removal of cells for differential cell counting (see below) the remaining BAL fluid was centrifuged at 1400 rpm for 5 minutes at 4°C and the supernatant frozen at -20°C until further analysis.

2.3.7.3 Cytospin preparation

In order to determine the frequency of different cell types in the BAL, 1 x10^5 cells were spun onto glass slides using a Shandon Cytospin3 (ThermoShandon, Runcorn, UK) at 350 rpm for 6 minutes. Slides were air dried before fixing in methanol at room temperature for 10 minutes. Fixed slides were stained by the Romanovsky method using Rapi-Diff II stain kit (Raymond A Lamb, Eastbourne, UK) and coverslips secured in place with Di-n-butylPhthalate in xylene (DPX) adhesive (BDH Laboratory Supplies, Poole, UK).

Differential cell counts were counted at x100 magnification under oil immersion with macrophages, eosinophils, neutrophils and lymphocytes identified using standard morphological criteria. The relative frequency of each was determined by counting 400 consecutive cells. The number of cells/ml was calculated from the total cell count. Squamous epithelial cells were not included in the analysis. The slides were blinded and randomised before counting with selected experiments counted by a second observer to ensure no inter-observer error. Figure 2.3 shows an example of these BAL cytospin preparations at x40 magnification.

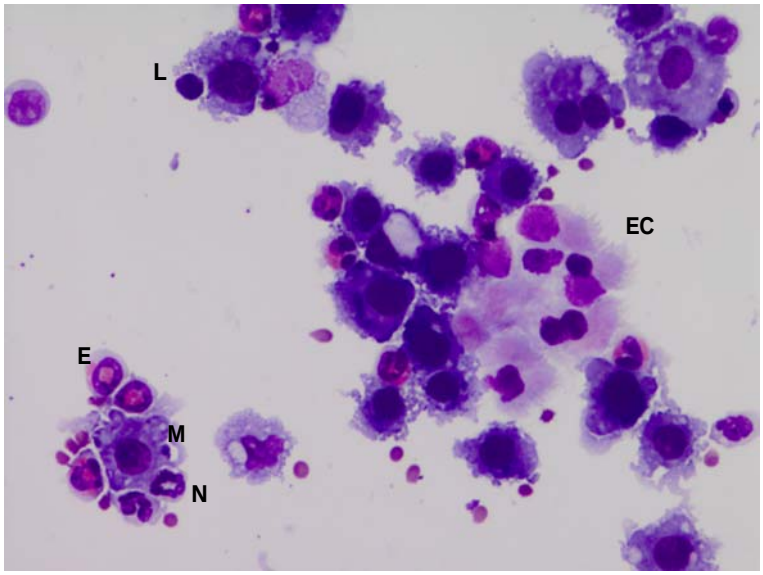


Figure 2.3: Leukocytes in bronchoalveolar lavage fluid cytospin preparation
Example cytospin preparation of BAL indicating eosinophils (E), macrophages (M), neutrophils (N), lymphocytes (L) and ciliated epithelial cell (EC). Magnification x40

2.3.7.4 Mediastinal draining lymph node (DLN) collection

The heart and lungs were removed en bloc from the chest cavity and the mediastinal lymph nodes draining the lung (DLN) from individual mice were removed and placed into cold wash medium. These were placed on ice until further processing. A single cell suspension was created by crushing the lymph nodes with a syringe plunger in wash medium gently through Nitex, a 100 µm nylon monofilament gauze (Cadisch, UK), and filtering again through Nitex into 25 ml centrifuge tubes to remove debris. Cells were washed twice with centrifuging at 1400 rpm for 5 minutes. The cells were resuspended in 5 ml of CM, counted and kept on ice until required for cell culture or FACS analysis.

2.3.7.5 Lung collection

After removal of the heart and lung en bloc the lungs were inflated with 10% neutral buffered formalin (NBF) via the trachea using a 1 ml syringe and 23G needle sheathed with polythene tubing, as used for BAL. The trachea was then tied off with thread and the lungs immersed in 10% NBF for 48 hours then transferred to 70% alcohol. The lungs were subsequently mounted in paraffin blocks and stored at 4˚C.

Where the lungs were required to be frozen, the lungs were similarly re-inflated with optimum cutting temperature (OCT) medium (Raymond A Lamb, UK) and immediately snap-frozen in liquid nitrogen and then stored at -80°C. When lungs were used to perform lung digests, the lung was dissected from the main bronchi and placed in media on ice for further preparation.

When tracheas were taken for epithelial cell culture, the trachea and large airways were carefully dissected from the surrounding heart, lungs, oesophagus and other tissues and then placed in media on ice until further culture.

2.3.7.6 Lung digestion

Lungs were removed and perfused with PBS. The lungs were cut with scissors and then mechanically dissociated with a tissue chopper in wash medium into a 5 ml centrifuge tube. Four millilitres of sterile digest medium containing an enzyme cocktail of 200 µl of 28 Wünsch units/ml liberase Blendzyme 2 (Roche) and DNase1 250 µg/ml (Roche) in wash medium was added and tubes placed in a shaking waterbath at 37°c for 1 hour. The digested material was passed through a 70 µM filter and CM added to stop the digestion reaction. The cells were centrifuged at 1900rpm for 10 minutes and the cell resuspended in 1.5 ml or red-cell lysis buffer for 1 minute. The cells were flooded with CM and centrifuged again. The single cell suspension was resuspended in 5 mls of CM and counted. The cells were incubated in FACS buffer with EDTA and stained as per FACS protocol.

2.3.7.7 Tracheal digestion

Murine tracheal digestion was based on published methods [453]. Following tracheal removal, the specimen was carefully processed to remove all other surrounding tissues, then cut longitudinally, transferred to PBS for 5 minutes and placed in collection media (1:1 Dulbecco's Modified Eagle Medium (DMEM): Ham's F-12, 1% pen/strep). The trachea was incubated in dissociation media [DMEM: Ham's F-12, 1% pen/strep, 1.4 mg/ml pronase (Roche) and 0.1 mg/ml DNase (Roche)] for 60 minutes at 37°C on a rotator. FCS (10%) was added to stop the digestion and tube agitated to dissociate cells. Cell suspensions were transferred into a 50 ml centrifuge tube and digested tracheas rinsed twice more with 15 ml

of media and pooled together into the centrifuge tube. Cell suspension was centrifuged at 300 g for 10 minutes. The supernatant was removed and cells resuspended in culture medium (1:1 DMEM: Ham's F-12, 1% pen strep/5% FCS, 120 IU/ml Insulin) (~5 ml for large Petri dish) and transfer to culture dish for 2 hrs at 37°C to remove rapidly adherent cells. Non-adherent cells were collected spun again, resuspended in FACS buffer to allow cell counting.

2.3.8 Histology

Lungs in paraffin blocks were cut using a microtome into 5 µm sections onto histology slides or superfrost antigen charged slides (VWR) for immunohistochemistry and allowed to dry at 55°C for 30 minutes before being stored at 4°C until used for staining.

Lungs frozen in OCT medium were removed from -80°C and kept on ice then cut into 6 µm sections onto superfrost slides (VWR), allowed to dry and stored at 4°C until used for staining.

2.3.8.1 Lung histology-haematoxylin and eosin staining (H&E)

Paraffin sections were stained with haematoxylin and eosin (H&E). Some of the H&E staining was performed by Mr. Roderick Ferrier, Department of Pathology, Western Infirmary, Glasgow or Veterinary Diagnostic Services, University of Glasgow. Otherwise, the slides were deparaffinised and rehydrated with graded alcohol solutions (Xylene, 100% alcohol, 70% alcohol and distilled water). Following this, the slides were stained in Harris haematoxylin for 2 minutes and excess removed by gently washing with running water. The slides were counterstained with 1% eosin for approximately 2 minutes and excess washed off with running water. The sections were then dehydrated and covered in DPX and a coverslide.

2.3.8.2 H&E scoring method

Sections were examined blinded at x 20-100 magnification and peribronchial and perivascular inflammation assessed. Some experiments were also assessed by a

second assessor. A semi-quantitative scoring system was used to assess the degree of eosinophilic infiltration: 0= no eosinophils; 1= eosinophils comprise less than 10% of total infiltrate or total infiltrate is <20 cells; 2= eosinophils comprise 10%-50% total infiltrate and; 3= eosinophils comprise more than 50% total infiltrate. A total of 10 fields were scored for each mouse and each field contained an airway and vessel in close proximity. Score were averaged for each mouse. Slides were photographed using an Olympus Camera.

2.3.8.3 Periodic Acid-Schiff (PAS) staining

PAS staining on selected experiments was performed by Veterinary Diagnositics, University of Glasgow.

2.3.8.4 Lung immunohistochemistry: ST2 and IL-33

Frozen histological sections as described above were removed from the freezer and slowly dried, then fixed in ice cold acetone/ethanol (75%/25%) for 10 minutes at room temperature, washed in PBS, then dried for 30 minutes.

Endogenous peroxidase activity was blocked using 0.5% H_20_2/Methanol for 30 minutes. Sections were washed in PBS twice. The sections were blocked with 20% appropriate serum (in which the secondary antibody was raised) in PBS with Avidin D block (4 drops/ml) for 30 minutes. Following a PBS wash, primary antibody or control was added and sections incubated overnight at 4°C. The following day following 2 PBS washes, the sections were incubated with the appropriate secondary biotinylated antibody for 30 minutes followed by 2 PBS washes. Avidin-biotin complex (ABC) vectastain (Vector Laboratories) was applied for 30 minutes followed by 2 PBS washes. Immunoreactive sites were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories) substrate which was applied for 1-5 minutes and washed off with PBS. The sections were then washed in running water and counterstained with Harris Haemotoxylin for 10 seconds followed by a running water wash. Finally, sections were dehydrated from alcohol to xylene and a coverslide mounted with DPX.

For paraffin sections, histological sections on electrostatically charged slides were heated in an oven for 35 minutes at 65°C and deparaffinised as before. Peroxidase activity was blocked as described above. Antigen retrieval was performed by pressure cooker method: slides were added to boiling 0.01M citrate buffer pH 6.0 and heated under pressure for 8 minutes. Following cooling, slides were washed in distilled water, then PBS and transferred to a staining apparatus. Non-specific binding block, addition of primary antibody and secondary antibody were added as described above followed by the ABC/DAB system for visualisation.

For secondary antibody, biotinylated (pan-specific) anti-goat/mouse/rabbit IgG produced in horse was used at concentration 4 drops/5 ml. Alternatively, biotinylated horse anti-rat (1:200), rabbit anti-rat (1:200) and appropriate secondary species serum were used (all Vector Labs).

For some paraffin sections, the ImmPRESS polymer reagent kit (Vector Laboratories) was used. Following blocking peroxidase activity and antigen retrieval as above the slides were washed in PBS and TBST buffer. Ready to use normal horse blocking serum (2.5%) was used to block non-specific binding and sections were incubated with primary antibody in 2.5% horse serum/2.5% appropriate species serum (in which primary antibody raised) in TBST. Following overnight incubation and a wash, sections were incubated for 30 minutes with species specific ImmPress reagent (of primary antibody) and Impact DAB applied before counterstaining, washing and dehydrating as before.

Antibody	Manufacturer	Source	Concentration	Isotype
IL-33	Nessy-1; Axxora Life Science	Mouse monoclonal anti-mouse	5-10 µg/ml	IgG1κ
IL-33	R&D	Rat monoclonal anti-mouse	0.8 µg/ml (1:600)	IgG2a
IL-33	R&D	Goat polyclonal anti-mouse	2-15 µg/ml	IgG
ST2	MD biosciences (Switzerland) (Clone DJ8)	Rat monoclonal anti-mouse	0.8-5 µg/ml (1:1200-1:500)	IgG1
ST2	Santa cruz Biotechnologies (USA) (C-20)	Goat polyclonal anti-mouse	0.4-4 µg/ml (1:50-1:500)	IgG
ST2	R&D (clone 245707)	Rat monoclonal anti-mouse	5-10 µg/ml	IgG2b
ST2 (IL-1RL1)	Atlas (Sweden)	Rabbit polyclonal anti-human	0.4 µg/ml (1:150)	IgG
F4/80	Serotec (Oxford, UK) (clone CI:A3-1)	Rat polyclonal anti-mouse	1:50	IgG2b
vWF	Dako (Denmark)	Rabbit polyclonal anti-human (cross-reactive with mouse)	1:300	Ig fraction

Table 2.6: Primary antibodies used in Immunhistochemistry

2.3.8.5 Lung immunohistochemisty (vWF) scoring method

For assessment of vessel changes *in vivo*, an angiogenesis index was developed. Paraffin sections were stained as described above with the ImmPRESS kit using von-willebrand's factor (vWF) or isotype control in lungs from IL-33 or PBS i.n. treated mice. Using a high powered field that included one airway and 90% lung tissue, 10 fields were assessed per slide. Blood vessels were identified with a clear structural lining positive for vWF stain. Images were acquired using Olympus Pro-Cell D software (Olympus). Airway and vessel perimeters were drawn by hand close to the parenchymal edge of epithelial or endothelial cell layer. Perimeter, surface area and blood vessel number were recorded. Sections were discounted if total area of airways on a section fell out with 2 standard deviations of the group mean. Measurements were also recorded independently by Dr M Shepherd.

2.3.8.6 Cell immunohistochemistry

Normal Human Bronchial Epithelial (NHBE) cells (see below for culture methods) were grown on Lab-Teck 4 chamber slides (VWR) and then stained with anti-ST2 human (Atlas) using ImmPRESS kit as described above.

2.4 *In vitro* methods

All cell culture was performed under sterile conditions and cultures were incubated in a humidified incubator at 37°C, supplemented with 5% carbon dioxide. Centrifugation was performed at 380g for 5 minutes at 4°C (Jouan CR3i centrifuge), unless otherwise stated. Cell counts were performed in varying dilutions of 0.4% trypan blue solution. Plastics for cell culture were purchased form Corning and Gibco unless otherwise stated.

2.4.1 Cell culture for adoptive transfer

Previously polarised D011.10 Th2 CD4 cells (Dr Damo Xu) ($5x10^6$ cells/ml) stored in liquid nitrogen CM/20% FCS/10% DMSO were used. Antigen presenting cells (APC) and reagents were prepared before CD4 cells were thawed and cultured. For APC preparation, a single cell suspension was created by crushing the spleen with a syringe plunger in wash medium gently through Nitex and filtering again through Nitex into 25 ml centrifuge tubes to remove debris. Cells were washed twice with centrifuging and pellet resuspended in CM. Red cell lysis solution (2 ml) was added and after 1 minute tubes filled with wash medium and spun again, resuspended ready for counting. Filtered mitomycin C (1 mg/ml) was added, 5µl per 10^6 cells, and incubated for 45 minutes. Cells were washed 3 times in CM, resuspended and counted ready for culture with CD4 cells.

The vials removed from liquid nitrogen were held in 37°C bath for 2-3 minutes to thaw, then transferred and rinsed carefully into 10 ml CM. They were spun and washed again and resuspended in 5 ml ready for transfer to $150cm^2$ culture flasks with 5 mls other reagents for final concentration of APC $0.5x10^7$/ml, OVA peptide 1:1000 and IL-4 10 ng/ml for repolarisation. Media was supplemented with IL-2 (10 ng/ml) and OVA peptide (1:1000) every 2-3 days until use. Cells

were washed twice with sterile PBS, resuspended in PBS and counted before preparing required cells in 200 µl aliquots for each mouse to be injected.

$CD4^+IL\text{-}5^+IL\text{-}4^-$ cells used for adoptive transfer were produced by Dr Mariola Kurowska-Stolarska. Briefly, murine $CD4^+$T cells from D0.11.10 IL-$4^{-/-}$mice were purified by negative selection (AutoMACS) and cultured with mitomycin C-treated APC (spleens from $ST2^{-/-}$ mice) with OVA peptide (10 nM), and IL-2 and IL-33 (10 ng/ml) for 3 days then prepared for i.v. transfer. Unstimulated CD4 cells (Th0) were used as a control.

2.4.2 Cell culture for cytokine quantification

Single cell DLN preparation was described above. Cells were placed in 24 well culture plates at a final concentration of $1x10^6$ cells/ml and cultured with CM or sterile OVA at a final concentration of 1 mg/ml. For *in vivo* experiments without OVA antigen, single cell suspensions of DLN were stimulated in 24 well culture places with 2.5 µg/ml of plate-bound αCD3 (BD biosciences) in CM. After 72 hours of incubation, supernatants were aspirated and stored at -20°C until further analysis.

2.4.3 Cell proliferation assay

2.4.3.1 Thymidine proliferation assay

Cells as described in OVA-recall assay were also cultured in 96 round bottom well plates (200 µl per well) in triplicate for 48 hours to assess proliferation. In the final 8 hours of culture, cells were pulsed with 0.037MBq ^{3}H-thymidine in wash medium. Cells were then harvested onto glass fibre filter paper (PerkinElmer, UK) using a 295-0054 Betaplate 96 well harvester (PerkinElmer). Radioactivity was measured using a MicroBeta TriLux scintillation counter (PerkinElmer). All procedures were performed according to local radiation safety guidelines.

For epithelial cells, cells were cultured at a density of $1x\ 10^4$ cells/well with indicated concentrations of IL-33. ^{3}H-thymidine was added for the final 8 hours of culture.

2.4.3.2 MTT proliferation assay

3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric proliferation assay was performed as per manufacturers' instructions (Cell Proliferation Kit 1, Roche). Briefly, 1x 10^4 cells in 200 µl of medium on a 96 well plate are incubated with the yellow MTT solution and the salt crystals solubilized and spectrophotmetrically quantified using an ELISA plate reader.

2.4.4 Epithelial cell culture

NHBE cell line is referred to as 16HBE in the literature and is a transformed bronchial epithelial cell. NHBEs were grown in CM in 75 cm^2 and media was changed every 2-3 days with cells were passaged approximately weekly by Hank's balanced salt solution (HBSS) rinsing and 1x trypsin (Gibco). For culture experiments, cells were seeded onto 6 well ($2x10^5$/well) or 96 well plates as stated. In some experiments CM was used without FCS supplementation and the addition of 1:100 Insulin transferring Selenium A (Gibco).

Primary human bronchial epithelial cells (pHBE) were obtained from Promocell (Hiedelberg, Germany) and resuscitated according to manufacturers' instructions. Cells were subcultured in 75 cm^2 at recommended density and grown in serum-free complete growth medium (GM) consisting of growth media plus 1 vial of supplement mix (Bovine Pituitary Extract, Epidermal Growth Factor, Insulin, Hydrocortisone, Epinephrine, Triiodo-L-thyronine, Transferrin and Reninoic Acid) (all Promocell). Once 80% confluent, adherent cells were dissociated using Detach Kit (Promocell). Some subcultured cells were cryopreseved using Cryo-SFM (Promocell) under manufacturers' instructions and stored at -80 C. pHBE were used between passage 4 – 6 for FACS, cell culture experiments and transwell experiments. For transwell experiments, 1% pen/strep was added to the media.

Human Umbilical Vein Endothelial cells (HUVEC) were maintained in serum and VEGF free endothelial cell growth medium (Promocell, Heidelberg, Germany) and used at passage 4. Endothelial cell Tube Formation Assay (Life Technologies, UK) was performed under manufacturers' instructions by Dr Malcolm Shepherd.

2.4.4.1 On transwell inserts

Methods were adapted from published studies Lauer *et al* and Tammi *et al* [454,455].

Costar™ (Cambridge, USA) clear transwell 24.5mm culture inserts (pore size 0.4 µM) were placed into 6-well culture dish and a thin coating of collagen basement membrane (BM) added. Collagen volume calculated for 5 µl/cm^2 by adding correct weight of type 1 rat tail collagen (BD Bioscience) to acetic acid for a final concentration of 50 µg/ml. This was kept at room temperature overnight in sterile conditions and washed twice in PBS and used immediately or stored at 4°C for up to 1 week before use.

For polarisation, NHBE or pHBE were seeded 1 x 10^5/well on the collagen BM submerged in their appropriate media, NHBE polarisation media was 1:1 DMEM with high glucose: CM and for pHBE serum-free GM with 1% pen/strep and changed every 2 days for 5-7 days until confluence reached. At this point, the apical medium was removed and media was changed on basolateral side only to expose an air-liquid interface (ALI) for further 14 days.

Cells were exposed to recombinant human IL-33 (0.1-100 ng/ml) in the basal media for 24 hours, following which supernatants were collected and stored at -20°C for later analysis.

2.4.4.2 On transwell inserts with native basement membrane

Thincert™ clear transwell 35 mm culture inserts (pore size 8.0 µM) (Greiner) were thin-coated with collagen BM added as above. On top of this Madin-Derby Canine Kidney (MDCK) cells were added (2 x 10^5 per transwell). MDCK cells were grown in DMEM in 75 cm^2 culture flasks and removed when required with EDTA/Trypsin. Following transfer to transwells, media was changed approximately 3 times a week for 18-22 days. To remove the MDCK cells, sterile 10 mM TRIS-HCL/0.1% bovine serum albumin (BSA)/0.1 mM $CaCl_2$ pH 7.5 was added for for 10 minutes followed by washing with 0.5 % Nonidet with a Pasteur pastette. To solubilise the cells, incubation for 5 minutes in sterile 0.2% deoxycholate was performed for 5 minutes at 37°c followed by 2 washes and inserts viewed under phase contrast microscope to ensure cell removal. The BM

was rinsed in HBSS twice and incubated with media or could be stored at 4°C until required. Cells and IL-33 were added as described above.

Transepithelial electrical resistance (TEER) was measured as an indication of polarisation. The apical surface was washed in HBSS and fresh HBSS added and electrical resistance measured by epithelial voltohmmeter (Prof Tom Evans) according to manufacturers' instruction with a baseline assessed in uncoated transwells without cells ≤200 Ω cm^2 .

2.5 Clinical study

2.5.1 Study protocol

The study was a cross-sectional unblinded use of oral dexamethasone to determine if distinct cytokine profiles could provide mechanistic insights into corticosteroid (CS) insensitivity in smoking asthmatics . Study design and recruitment was approved by the Local Ethics Committee and patients gave informed written consent. All patients were recruited by staff at the Asthma Research Unit, University of Glasgow under the supervision of Dr Mark Spears and Prof Neil C Thomson. Patient demographics are described in chapter 3. Full design, inclusion and exclusion criteria and further information available in the published study by Spears *et al* [456]. Briefly, all subjects had to demonstrate reversible airflow obstruction with a Forced Expiratory Volume in 1 second (FEV_1) bronchodilator response of ≥12% (and >200 ml), Peak Expiratory Flow (PEF) or positive methacholine test to be eligible. Patients completed a questionnaire including Juniper Asthma Control Questionnaire (ACQ), and underwent spirometry, serum and sputum collection before and after a 2 week trial of oral dexamethasone.

2.5.2 Sputum and serum sampling

Sputum and serum collection and processing was performed by staff form the Asthma Research Unit and Division of Infection, Immunity and Inflammation, University of Glasgow as published by Spears *et a*l [456]. Briefly, a whole sputum sample method was used with low concentration dithiothereitol (DTT) and

supernatants collected and stored at -80°C until cytokine analysis. Plasma was collected from heparinised blood samples and similarly stored until analysis. Sputum and plasma sST2 and IL-33 levels were assessed by ELISA according to protocol in section 2.6.2 below.

2.6 Assays and analysis

2.6.1 Flow cytometry (FACS)

Washed 1 x10^5 cell pellets in FACS tubes (BD Biosciences, UK) were resuspended in 50 µl FACS buffer with 1 µl of Fc block (anti-CD16/CD32, BD Biosciences) and incubated for 15 minutes at room temperature (RT). Cells were stained by adding the required amount of antibody to the tube followed by vortex mixing and incubation in the dark at 4°C for 30 minutes. Cells were washed with 500 µl FACS buffer and centrifuged before resuspension in buffer ready for analysis. Three µl microlitres of 7-Amino-actinomycin D (7-AAD, Via-Probe, BD Biosciences) was added before acquisition.

For intracellular (IC) staining, 100 µl Cytofix/Cytoperm buffer (BD biosciences) was added to the cell pellet which was incubated for 20 minutes at RT followed by washing in 500 µl Cytoperm wash (BD biosciences) (made from stock solution diluted in distilled water). Cells were resuspended in 50 µl cytoperm wash and the required amount of antibody before being incubated at 4°C for 30 minutes. The cells were washed again and resuspended ready for acquisition.

For DLN IC staining, DLN were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin for 4 h with GolgiStop (BD biosciences) added during the final 3 hours. Cells were then fixed, permeabilized and incubated with antibody as above.

Antibody-Conjugation	Volume added or dilution used	Manufacturer	Isotype
Human ST2-FITC	5 µl	MD biosciences	Mouse IgG1
Murine ST2-FITC	2 µl	MD biosciences	Rat IgG1
Pan Cytokeratin-PE	1 µl	Abcam	Rat IgG1-PE
CD45-PE	2 µl	R&D	IgG2b
CD4-APC	1 µl	BD biosciences	Rat IgG2b
c-kit-APC	2 µl	BD biosciences	Rat IgG2b
Siglec F-PE	1:200	BD biosciences	Rat IgG2a
F4/80	2 µl	eBioscience	Rat IgG2aκ
Gr-1-Percp/Ly5	0.8 µl	BD biosciences	Rat IgG2bκ
CCR3-APC	5 µl	R&D	Rat IgG2a
IL-4-PE	1:200	BD biosciences	Rat IgG1
IL-5-APC	1:200	BD biosciences	Rat IgG1κ
IFNγ-FITC	1:200	BD biosciences	Rat IgG1κ

Table 2.7: FACS antibodies

Samples were analysed using a FACSCalibur flow cytometer (BD Biosciences) and data acquired using CellQuest software (BD Biosciences). Live cells were gated according to forward scatter (FSC) and side scatter (SSC) and exclusion of 7-AAD positive cells using unstained cells. Using sample cells, isotype staining was used to ensure not that less than 2% of total cells were falsely positive on any axis. Using a combination of single stained samples on each flurochrome with other flurochrome isotypes, compensation was carried out for emission overlap. All data analysis was carried out using the FlowJo package (Tree Star Inc., Oregon, USA).

2.6.2 Cytokine immunoassays

Enzyme linked immunosorbant assays (ELISAs) were performed for cytokine and chemokine quantification using paired antibodies or kits as specified in Table 2.8 Buffers used are as detailed in Table 2.1.

Analyte	Manufacturer	Lower limit detection (pg/ml)
IL-4	BD	40
IL-5	BD	30
IFN-γ	BD	100
IL-13	R&D	5
Eotaxin/CCL11	R&D	5
Eotaxin-2/CCL24	R&D	4
TARC/CCL17	R&D	5
RANTES/CCL5	R&D	5
IL-17E	R&D	62.5
OVA-IgE	BD	dilutions (see text)
Total IgE	Bethyl Labs	3900
Human IL-8	R&D	31.2
Human IL-6	Invitrogen	5
Human Eotaxin-2/CCL24	R&D	15.6
Human MIP1α/CCL3	R&D	7.81
Human VEGF	Invitrogen	23.4
Human IL-33	Genway	700
Human ST2	R&D	31.2

Table 2.8: ELISA kits and antibody pairs

ELISA Immulon 4 HBX plates (Thermo Labsystems, Franklin) were coated with capture antibody 50 µl/well in appropriate coating buffer and incubated overnight. Plates were washed three times with ELISA wash buffer then incubated with 200 µl/well of assay diluent buffer for 1 hour at 37°C as a blocking step. Following three washes samples and standards were added. An eleven doubling dilutions of the standard were added to obtain a standard curve and 2 wells were incubated with assay buffer alone as blank wells. After incubation for 2 hours at room temperature plates were washed five times before biotin-conjugated detection antibody in assay buffer was added to each well. After 1 hour at room temperature plates were washed three times and Streptavidin-horseradish peroxidase (HRP) conjugate (Extravidin, Sigma), diluted 1:1000 with assay buffer, was added. After 30 minutes plates were washed three times and 100 µl/well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, USA) added. Plates were incubated in the dark for up to 30 minutes or until sufficient blue colour had developed. Optical density was read immediately at 630 nm on a MRX II microplate reader (Dynex Technologies, Worthing, UK) running Revelation (Dynex Technologies) software or Sunrise

absorbance plate reader machine and sample values calculated from the standard curves generated. Modifications to this protocol are as noted below.

For R&D ELISAs, assay buffer and Streptavidin-HRP conjugate provided by R&D and diluted to 1:200 were used. In addition, the detection antibody was incubated for 2 hours rather than 1. The Eotaxin-2 detection antibody was diluted in the R&D ELISA assay buffer with the addition of 2% heat-inactivated normal goat serum. Following TMB substrate, the reaction stopped with addition of 100 µl stop buffer and read at optical density of 430nm with a 570nm correction filter.

Total IgE was measured using a kit from Bethyl Laboratories (Texas, USA) using: coating boater 0.05 M carbonate-bicarbonate pH 9.6, wash 50 mM Tris, 0.14 M NaCl, 0.05% tween 20, pH 8.0, blocking solution 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0; and sample diluent blocking solution with 0.05% tween 20. For OVA-specific IgE ELISA, plates were coated with OVA 10 µg/ml in OptEIA buffer and incubated overnight. In the absence of any standard for OVA specific immunoglobulins, serial dilution curves were obtained for each sample using assay buffer as a diluent with an initial sample dilution of between 1:10 and 1:40.

For antibodies from Invitrogen the coating buffer was PBS and the sample and detection steps were combined, with 50 µl/well of sample or standard added to each well followed immediately by 25 µl/well of detection antibody in assay buffer followed by 2 hour incubation.

For the human IL-33 ELISA (Genway Biotech, CA, USA) the reagents are the same as for the Bethyl IgE ELISA with standard ELISA wash buffer.

A murine IL-33 ELISA was developed as described in section 3.2.3. Both capture and detection antibodies were titrated at reducing concentrations measuring blank assay diluent wells and assay diluent wells with a concentration of 1 ng/ml of murine IL-33.

2.6.3 Multiplexed immunoassays

Multiplexed immunoassay is a method by which multiple cytokines or other mediators are measured simultaneously in a small volume of fluid using latex microbeads with antibody covalently attached. Biosource Multiplex kits LHC0009 (Cytokine Human 25-Plex), LMC0006 (Cytokine Mouse 20-Plex) and LMC1031 (RANTES Mouse Singleplex) were used according to the manufacturers' instructions. All reagents and buffers were sourced from the kits. Beads were sonicated before addition to sample well to ensure there was no clumping. Fifty microlitres of incubation buffer, assay diluent and sample were added to each sample well. Data was acquired on a Luminex 100TM analyser (Luminex Corp., Texas, USA). Data was analysed using Luminex software (Luminex Corp.).

2.7 Statistics

Data from *in vitro* and *in vivo* experiments were usually normally distributed with equal variance. Therefore mean values were calculated and expressed with standard errors of the mean (SEM). Comparison between groups of data was made with unpaired Students two-sample t-test, one-way analysis of variance (ANOVA) or two-way ANOVA depending on the data. A p value of less than 0.05 was considered significant. Statistical analysis was performed using Excel (Microsoft, Redmond, USA), MINITAB 16 (Minitab Inc, PA, USA) and GraphPad v4 (GraphPad, CA, USA).

For the clinical studies, Student's t-test or Mann-Whitney test when appropriate for comparisons of clinical data between smokers, ex-smokers and never smokers performed by Dr Mark Spears using SAS software (SAS Institute, NC, USA) and MINITAB (Minitab Inc.). ST2 and IL-33 analysis was performed between groups using Student's t-test or Mann-Whitney test and within groups (before/after CS) analysed with paired t-test and Wilcoxon U test and correlations using Pearson's correlation using MINITAB (Minitab Inc.).

3 Expression of ST2 and IL-33 in the lung

3.1 Introduction and Aims

Schmitz and colleagues reported IL-33 mRNA expression, detected by RT-PCR, was present at a high level in murine lung. On a cellular level, human bronchial and pulmonary artery smooth muscle cells along with bronchial epithelial cells express significant IL-33 mRNA levels[293]. These findings were predated by the detection of protein nuclear factor-high endothelial venule HEV (NF-HEV), subsequently characterised as IL-33[380]. Accordingly, nuclear localisation of IL-33 was predominantly detected in endothelial cells[387,389]. Furthermore, in an inflammatory setting, IL-33 mRNA was detected at higher levels in murine lung in allergic airways inflammation (AAI) model[397]. However, the presence of IL-33 protein in the lungs at a cellular level and localisation within the expressing cell is unknown.

The IL-33 receptor, ST2L is predominantly present on Th2, mast cells and macrophages and lung ST2 mRNA levels are increased in a murine allergic airways inflammation (AAI) model of asthma. Serum sST2 is also elevated following allergen challenge in AAI[313]. In humans, sST2 is being increasingly considered a biomarker with elevated levels shown to correlate in particular with acute myocardial infarction and left ventricular dysfunction [350,374]. In asthma, increased levels of sST2 in the serum have been reported and correlate with severity of acute asthma exacerbations in humans, [354,355] but the potential of sST2 as a biomarker in this condition has not been fully explored.

Elevated serum levels of IL-33 have been reported in allergic patients, specifically with a form of allergic rhinitis [428] but its presence is unknown in asthmatic patients. Elevated mediators in the sputum can correlate with disease severity [8] but the presence of sST2 or IL-33 in sputum samples of asthmatic patients is unknown. Furthermore as many asthmatic patients are being concurrently treated with anti-inflammatory corticosteroids (CS), which can alter concentrations of these markers [457], the potential effect of CS on sST2 and IL-33 in the serum and sputum is also unknown.

The presence and distribution of ST2 and IL-33 in murine lung, including whether IL-33 and sST2 are directly released from the cell in mice as well as humans, will

now be considered. At the outset of this work, there were few commercially available reagents for IL-33. The aim of this chapter is to elucidate murine protein expression by developing immunohistochemical and sandwich ELISA techniques. Furthermore using human asthmatic sputum and plasma samples, the presence of sST2 and IL-33 concentrations in humans, and the effect of CS, will be assessed.

3.2 Characterisation of IL-33 and ST2 expression in murine lungs determined by immunohistochemistry

As published data to date has shown potent effects of exogenous recombinant IL-33[293], it was important to establish the presence of endogenous IL-33 protein in murine lung (in addition to endogenous mRNA expression) and characterise the expressing cell type.

3.2.1 Pulmonary murine IL-33 expression

Following on from the finding of IL-33 mRNA in murine lungs in a model of AAI [397], using lungs from WT PBS- and OVA-treated BALB/c mice in a 12 day AAI model, immunohistochemical staining was performed to assess the presence of IL-33. Heart and lung sections were processed *en bloc* in formalin and then paraffin embedded. At the time of performing these analyses, only one commercial antibody reagent for IL-33 was available. This anti-IL-33 [Nessy-1] (Axxora) is a mouse antibody reactive with both mouse and human tissues. A biotinylated secondary antibody and ABC/DAB system was used to visualise the binding product. Omission of primary antibody was used as a control. Despite optimisation strategies, no specific binding was seen using paraffin embedded lungs. Therefore, as an alternative, some lungs were re-inflated with OCT freezing medium, immediately snap frozen in liquid nitrogen and stored at -80°C until analysis (see chapter 2). Figure 3.1 (A-D) shows the results of anti-IL-33 in frozen sections of murine lung. The architecture is not well preserved in frozen specimens but specific IL-33 staining appears to be present in: (1) lung parenchymal cells that lie around the alveolus; (2) the nucleus of cells that morphologically appear to be macrophages (Figure 3.1A and C). As expected, endothelial vessels showed strong IL-33 expression (Figure 3.1B). To further

investigate that the other IL-33 expressing cell was the macrophage, double-colour staining with anti-F4/80 antibody was attempted. In contrast to the IL-33 antibody, anti-F4/80 staining of macrophages was only determined in the paraffin but not frozen section, therefore double staining to confirm macrophage nuclear IL-33 was not possible. However, the results from the paraffin sections appear to show that the corresponding cells morphologically are macrophages (Figure 3.1E).

Later, with the availability of alternative commercial antibodies, IL-33 was identified in the paraffin-embedded tissues using a R&D rat anti-mouse IL-33 antibody with pressurised citrated antigen retrieval (Figure 1 F-I). As well as expression as noted in the Nessy-1 samples, another cell population, consistent with epithelial cells, show lighter IL-33 staining located in the cytoplasm. Interestingly, although IL-33 expression was seen in the OVA-treated mice, similar expression was seen in the PBS mice indicating no obvious induction of IL-33 expression. However, it can be concluded that IL-33 is present and potentially has a role in AAI.

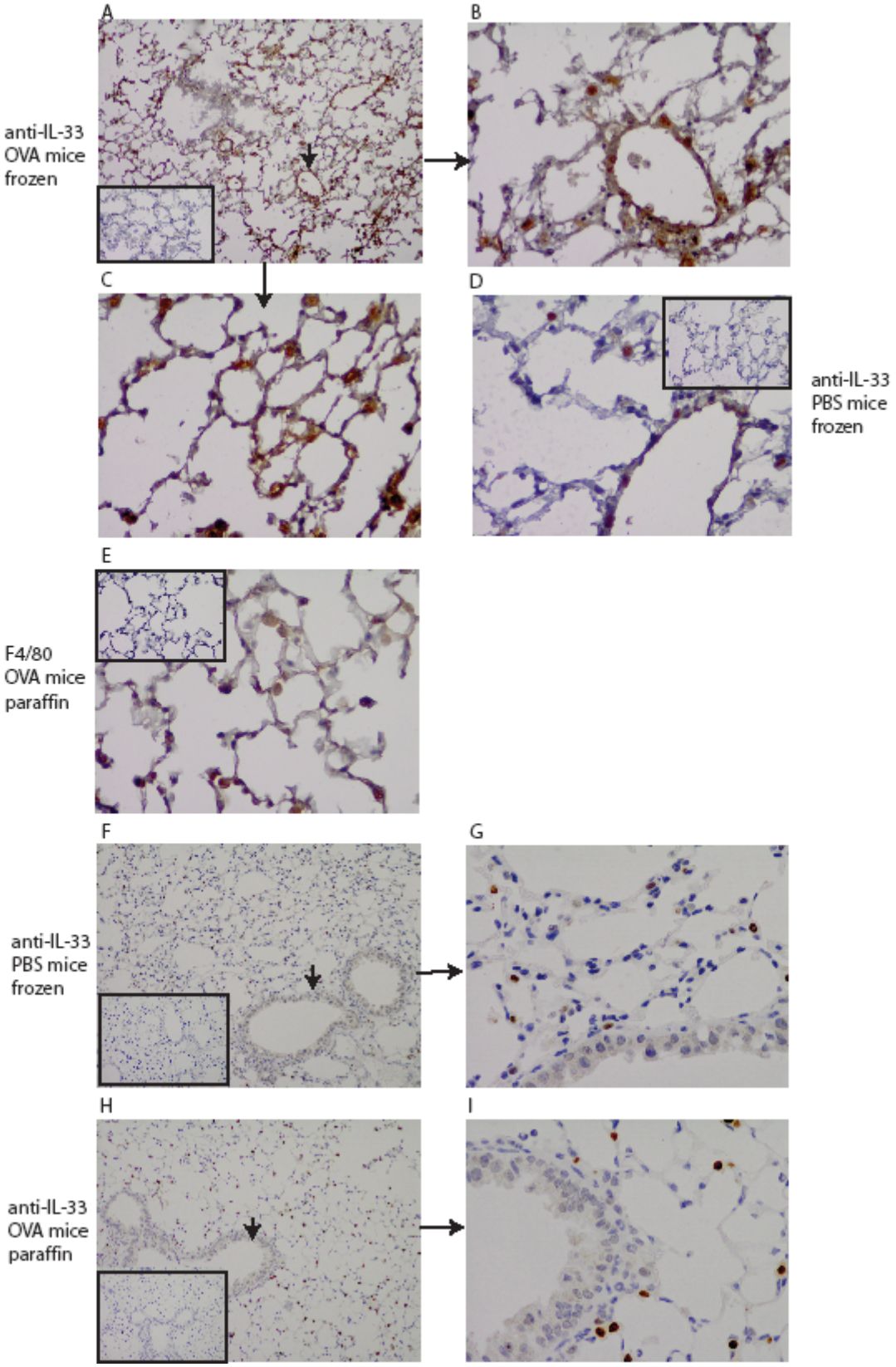


Figure 3.1: Mouse lung IL-33 Immunohistochemistry
See over for legend

Figure 3.1: Mouse lung IL-33 Immunohistochemistry
(A-D) IL-33 expression using primary Nessy-1 IL-33 antibody on frozen sections of lung. (A-C) OVA- and D) PBS- treated mice . A) x10 magnification (insert is isotype control x10). Lung macrophages (B), and vessel with endothelial cells (C). B) & C) are magnified images from A) at x40. D) x40 magnification (insert is isotype control x10). E) Macrophage F4/80 antibody staining x40. Insert is isotype control x40 in OVA-treated mouse. (F-I)IL-33 expression using primary R&D anti-mouse IL-33 antibody in paraffin sections. F) PBS- and H) OVA- treated mice x10 with insert isotype x10. IL-33 expression as F) and H) with magnification x40 of section indicated by arrowheads. G) and I) nuclear macrophage and cytoplasmic epithelial IL-33 staining.(Representative images shown of n=5-10).

3.2.2 Pulmonary murine ST2 expression

In terms of the IL-33 receptor ST2L, similar immunohistochemical methods to IL-33 were used to determine the expression. The primary antibody anti-ST2 however will bind to sST2 and ST2L, so any protein expression identified could represent either isoform. Three ST2 antibodies (goat anti-mouse, Santa Cruz, rat anti-mouse, R&D and rat anti-mouse, MD Biosciences) were tested and titrated with optimal results using a rat anti-mouse anti-ST2 (MD Biosciences). This staining was again optimally performed in the paraffin sections and shown in Figure 3.2. ST2 expression was identified in epithelial cells and scattered parenchymal cell nuclei. In addition, there was some lighter staining in epithelial cell cytoplasm. Results shown are from naïve mice, as no significant difference was noted in OVA-treated mice specimens analysed (data not shown). Specific staining was confirmed with positive staining in cardiac myocytes (with thanks to Dr Ashley Miller for providing the cardiac sections) and no expression was identified in $ST2^{-/-}$ mice confirming specificity.

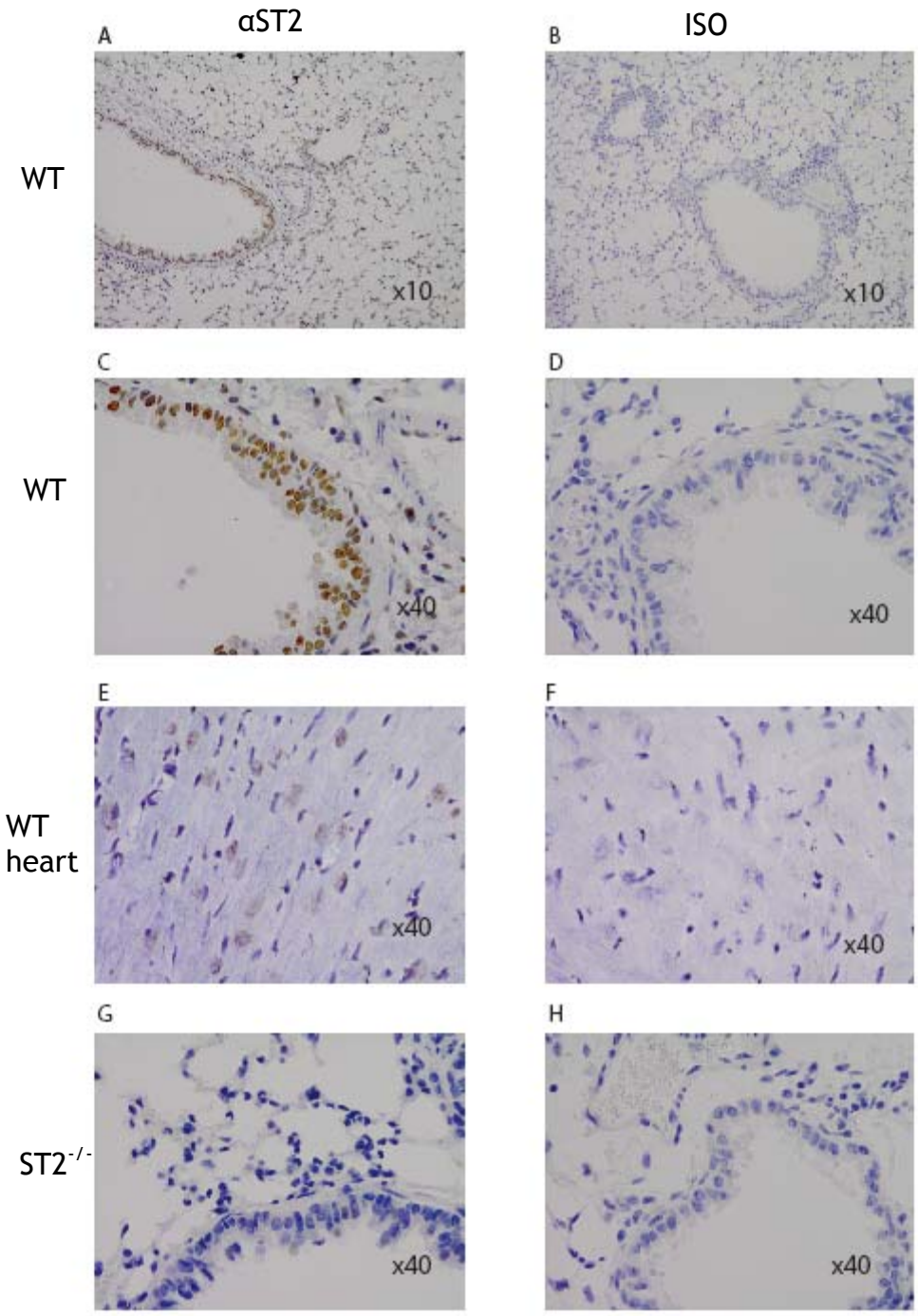


Figure 3.2: Murine lung ST2 immunohistochemistry
ST2 immunohistochemistry on paraffin sections in naïve WT mouse lung stained with rat anti-mouse ST2L in A) x10 magnification and C) x40 demonstrating scattered parenchymal cell and epithelial cell nuclear staining with lighter epithelial cell cytoplasmic staining. B) and D) represent isotype control of A) and C). E) Positive control representing ST2 positive cardiac myocytes in heart muscle and F) isotype control both x40 in WT mice. G) Negative control demonstrating no signal in $ST2^{-/-}$ mouse lungs and isotype control H) both x 40. (Representative images of n=2 for cardiac myocytes otherwise n=5-10).

3.2.3 Levels of IL-33 in mice as determined by ELISA

Following the manufacture of a biotinylated antibody for the Nessy-1 IL-33 antibody (Axxora UK, Nessy-1, biotin conjugated) a working direct enzyme-linked immunosorbent assay (ELISA) for the detection of IL-33 in murine biological samples related to this work was developed; including bronchoalveolar lavage (BAL), digested lung and serum available from a range of animal models. In pilot work (data not shown), a monoclonal anti-IL-33 antibody (R&D systems UK catalogue MAB 3626) was chosen as the capture antibody that was the most sensitive in combination with the secondary biotinylated anti-IL-33 antibody. The optimal diluent identified was 0.1M $NaHCO_3$ coating buffer for the capture antibody. In house as well as newly available commercial recombinant murine IL-33 (R&D 3626-ML-010) were tested as ELISA standards. Figure 3.3 shows optimisation of the antibody pair, choosing the concentration of reagent that minimises use but without losing significant IL-33 detection. The minimum detectable concentration was 200 pg/ml.

A

R&D Capture AB concentration µg/ml
OD

Nessy-1 Detection AB µg/ml
OD

	32	**16**	**8**	**4**	**2**	**1**	**0.5**
16	0.168	0.135	0.097	0.074	0.054	0.047	0.053
8	0.105	0.084	0.073	0.046	0.035	0.029	0.030
4	0.079	0.069	0.052	0.034	0.026	0.020	0.023
2	0.057	0067	0.041	0.024	0.021	0.022	0.018
1	0.062	0.060	0.035	0.020	0.016	0.014	0.016
0.5	0.045	0.042	0.033	0.018	0.016	0.023	0.021

B

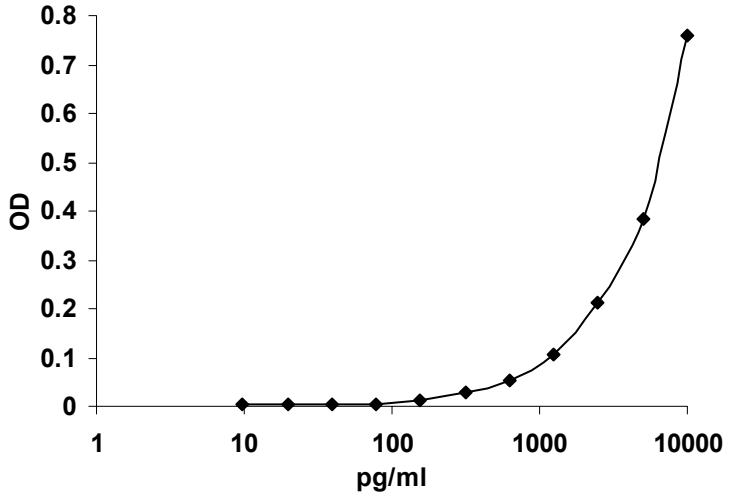


Figure 3.3: IL-33 ELISA optimisation
A) Chequerboard assay to optimise concentrations of capture and detection antibodies. OD=optical density. B) Standard curve using rmIL-33 standard (R&D) using concentrations 16 µg/ml capture antibody and 4 µg/ml for detection antibody.

Following this, the presence of IL-33 in samples generated from a murine AAI model was assessed. Figure 3.4 shows lower levels of IL-33 were detected in the BAL and serum of the OVA-treated group in comparison to PBS-treated controls. Soluble ST2 levels were not measured but would have been useful to determine if high levels of sST2 could sequester IL-33 making it unavailable for detection in the ELISA. These experiments were repeated in $ST2^{-/-}$ mice which lack all isoforms of ST2 (Figure 4). Concentrations of IL-33 were lower in PBS-treated $ST2^{-/-}$ mice compared to the WT PBS-treated group. In response to allergen, $ST2^{-/-}$ mice had elevated serum IL-33 levels compared to the PBS-treated group which could theoretically be explained by lack of sST2 to sequester IL-33 but this

could not be confirmed. No detectable IL-33 was measured in digested tracheas, oesophagus, spleens or BAL (data not shown).

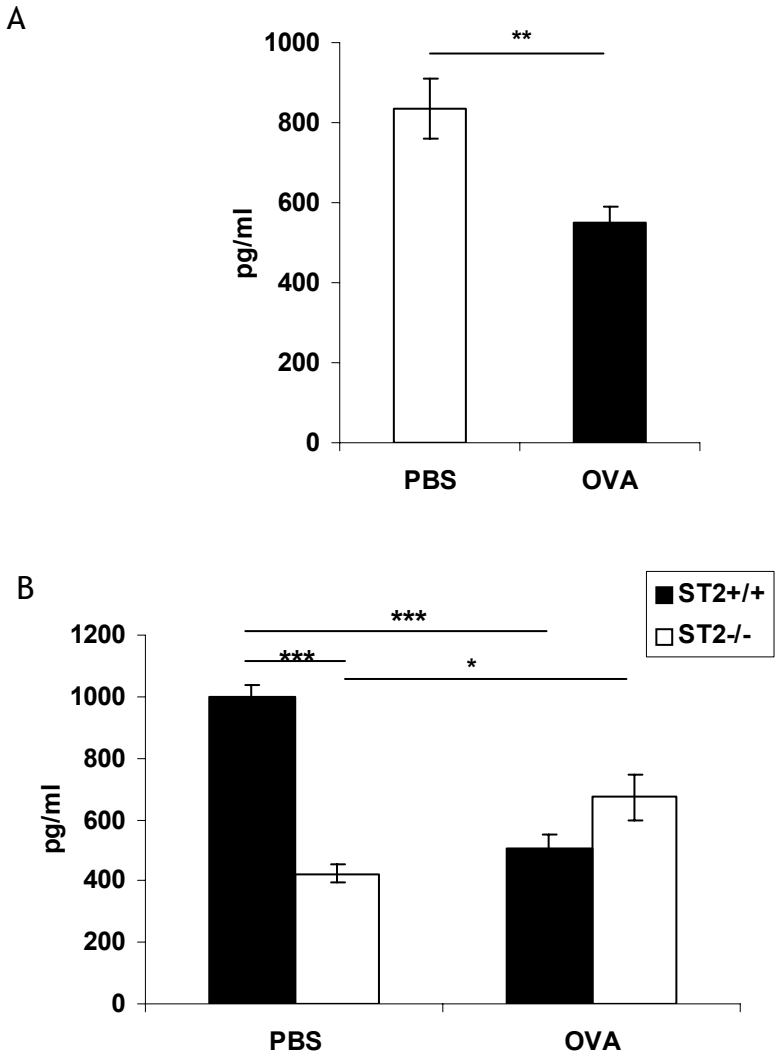


Figure 3.4: Serum levels of IL-33 effects of allergic airways inflammation in mice
IL-33 serum concentrations following a 14 day model of AAI in WT BALB/c mice A) and 28 day model of AAI in BALB/c WT and ST2-/- mice B). n=4-5 per groups. Data shown are mean ± SEM. * $p<0.05$, *** $p<0.01$, *** $p<0.001$.

3.3 Characterisation of sST2 and IL-33 in smoking asthmatics

One of the challenges of modern medicine is having the ability to direct the most suitable treatments towards the most appropriate patients. The measurement of biomarkers, which are proteins in blood or bodily fluids present in disease, can help with prognosis, assessment of disease progress, directing treatment and monitoring treatment effect. Soluble ST2 is one such potential biomarker, predominantly studied in myocardial infarction and heart failure [374].

Two studies measuring serum sST2 in patients with asthma have shown an increase in the levels during acute exacerbations [352,355]. One study performed in adults, demonstrated this increase correlated with the severity of exacerbation by negatively correlating with peak expiratory flow (%PEF) and positively with partial pressure carbon dioxide in the blood (PaC02) [355]. A second study in children however did not demonstrate a correlation with severity [352]. Asthmatics who smoke have an altered cytokine profile and relative CS sensitivity [458]. It is unknown what effect smoking, if any, has on sST2/IL-33. Local research interest and expertise in the area of smoking and asthma led to the development of a sub-study to investigate concentrations of sST2 and IL-33 in this patient population; and assess the effects of smoking and CS on these levels.

3.3.1 Study design

The study design and methods are more fully described in Chapter 2 (section 2.5). Three groups of adult patients with stable asthma were recruited: current smokers, ex-smokers or never smokers. Patients were recruited from hospital outpatient clinics and general practice. Fifty-three patients with asthma agreed to participate. Eligibility for the study required demonstration of reversible airflow obstruction (FEV_1 response to β_2 agonist $\geq$12 %, PEF lability or a positive methacholine challenge test). Patients took two weeks of oral dexamethasone (6 mg/1.74 m^2 equivalent to 40 mg/day prednisolone. Induced sputum and plasma were obtained before and after oral dexamethasone treatment. Medication history including ICS dose and compliance was recorded as well as asthma control questionnaire (ACQ) scores were determined at each visit. Sputum

differential cell counts were performed by a whole sputum sample method and sputum supernatants collected and stored at -80°C until further analysis[459]. Plasma was collected from processed heparinised blood samples stored at -80°C until further analysis.

3.3.2 Patient demographics

Fifty-three patients participated with 22 smokers, 10 ex-smokers and 21 never smokers with asthma. The patients were reasonably well matched although the smoker and ex-smoker groups were prescribed higher daily ICS doses and had higher ACQ scores. Table 3.1 summarises the baseline demographics of the participants.

	Smokers with asthma (n=22)	**Ex-smokers with asthma** (n=10)	**Never Smokers with asthma** (n=21)
Age (yrs)	46.6 (6.7)	49.8 (9.0)	42.5 (10.0)
Sex (F:M)	12:10	5:5	11:10
Asthma Duration (yrs)	22.1 (15.9)	24.6 (15.9)	28.6 (15.0)
Pack years	27.6 (15.7)	28.5 (15.9)	0
Inhaled steroid (mcg/day) beclomethasone equivalent	1046 * (611)	1280 * (551)	679 (419)
ACQ Score (0 to 6)	2.2 * (0.9)	2.3 (0.7)	1.5 (0.8)
Pre BD FEV_1 (% predicted)	73.6 (18.5)	79.7 (24.1)	73.3 (15.3)
Pre BD PEF (% predicted)	81.7 (20.8)	85.4 (24.7)	85.8 (19.1)
Sputum esonophils (%) median (IQR)	0.4 (0,10)	1.0 (0.1,5)	0.3 (0,2)
Sputum neutrophils (%) median (IQR)	34 (24,56)	37 (22,63)	24 (10.5,41)

Table 3.1: Baseline study participant demographics
Data presented as mean (SD). *; p<0.05. p values are compared to non-smokers. Abbreviations: ACQ; asthma control questionnaire, BD; bronchodilator, mcg; microgram, FEV_1; forced expiratory volume in 1 second, IQR; interquartile range, PEF; peak expiratory flow.

3.3.3 Levels of plasma sST2 are similar between groups

Human plasma samples were defrosted and sST2 concentration quantified using a human IL-1 R4/ST2 ELISA (R&D Systems, catalogue DY523). The lower limit of detection was 32 pg/ml [374]. All measurements were performed in parallel in one experimental run with sample duplicates. No significant differences were seen in baseline pre-oral corticosteroid (pre-steroid) sST2 levels between groups (never smokers v ex-smokers p=0.648; never smokers v smokers p=0.120; ex-smokers v smokers p=0.056) (Figure 3.5). Baseline sST2 levels did not correlate with either lung function, FEV_1 or PEF, or symptoms with ACQ score (data not shown).

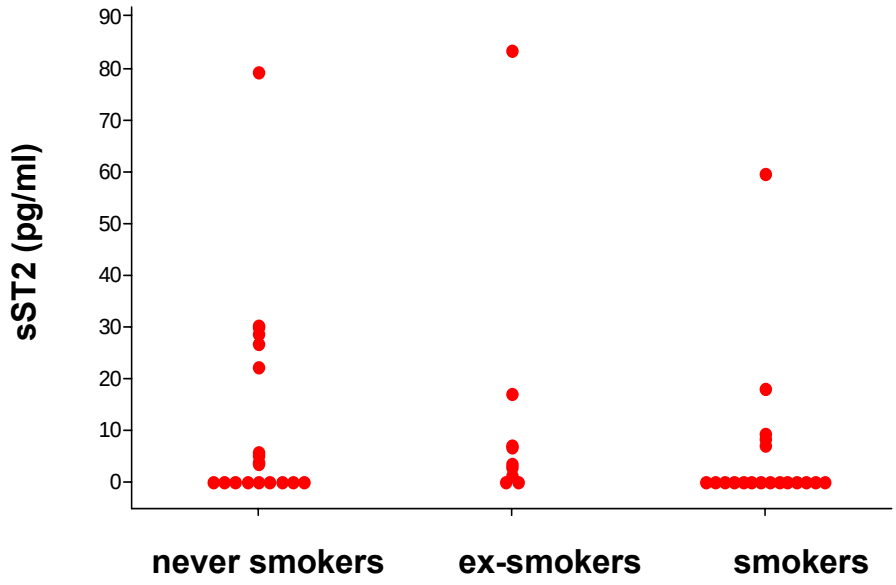


Figure 3.5: Baseline plasma sST2 in never smokers, ex- smokers and smokers with asthma; between group comparisons
Levels of sST2 (pg/ml) were determined in the plasma by ELISA. Data are presented as individual patient measurements. No significant differences between group medians using Mann Whitney *U* test with p<0.05 considered significant.

Normal controls were not included in the primary study design, as the main aim was to compare the effects of smoking on cytokine profiles in asthmatics. Therefore, in a separate pilot study comparison to ascertain if any difference seen could be explained by having asthma, 14 individuals without asthma 'normals' were recruited for baseline sST2 plasma analysis. The only baseline demographic available on the 'normal' subjects is age with a range of 25-48

years. Normals did not have asthma and were never smokers. No differences were seen between median sST2 in normals compared to: never smokers, p=0.96; ex-smokers 0.6318 or; smokers p=0.0929 (data not shown). A trend of the closest association with never smokers with asthma and biggest difference with smokers with asthma was apparent but in context of exploratory data, likely limited by sample size, could not be confirmed.

3.3.4 Plasma sST2 levels are elevated following steroid treatment

Following a 2 week course of oral CS treatment, plasma sST2 was re-assessed. The difference between sST2 pre- and post-steroid within each group of patients was statistically significant (Figure 3.6).

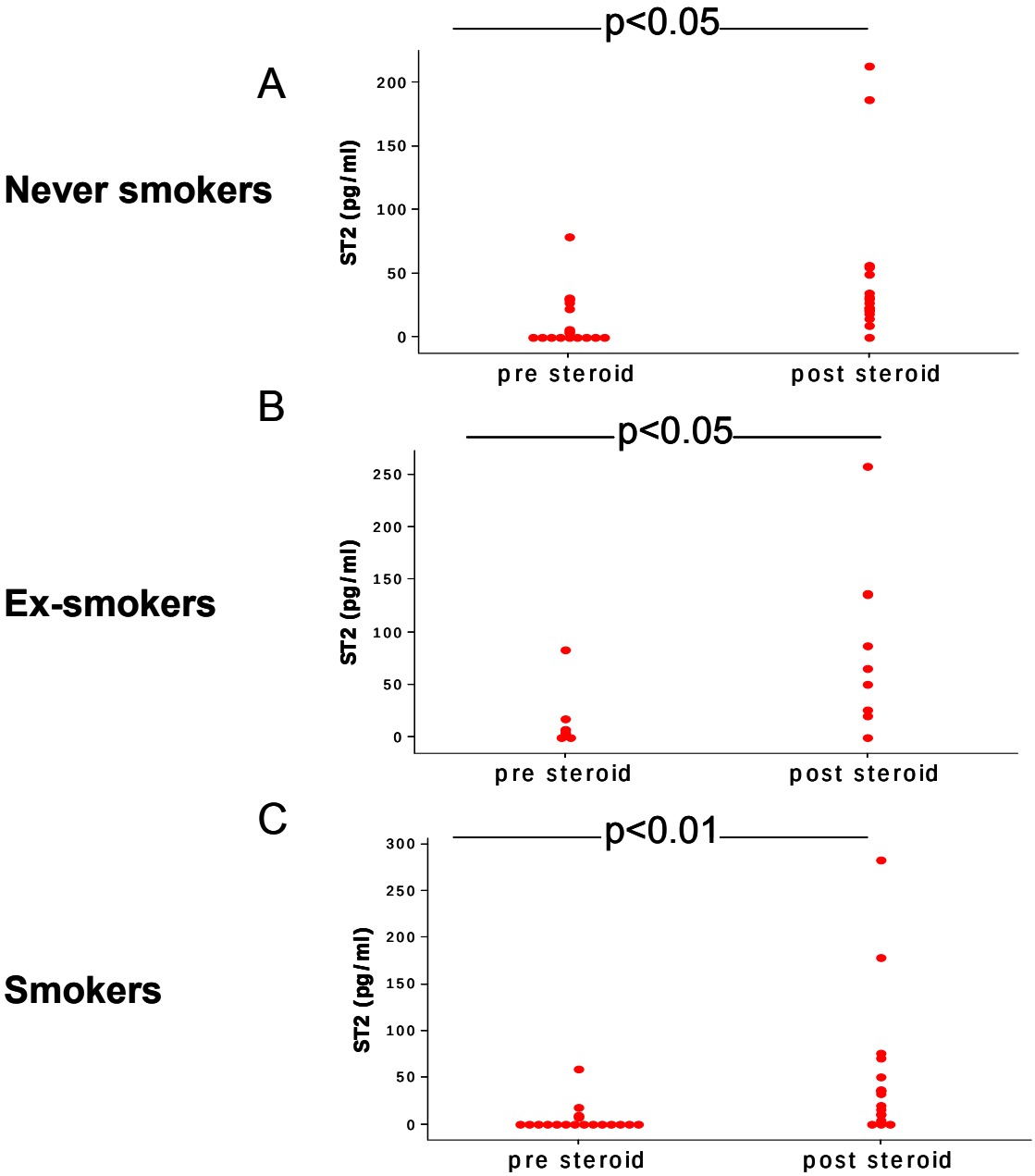


Figure 3.6: sST2 post-dexamethasone treatment; within-group comparison
Levels of sST2 (pg/ml) were determined in the plasma by ELISA. Single data points in each group are displayed. Paired t-test (Wilcoxon signed rank test) was performed between pre-steroid and post-steroid within each group. p<0.05 was considered significant.

There was no significant difference in post-steroid sST2 levels between the groups (t-test of difference; never smoker v ex-smoker p=0.169, never smoker v smoker p=0.597 and ex-smoker v smoker p=0.122). Furthermore, no significant difference was identified in the magnitude of group pre- to post-steroid difference between groups (Mann-Whitney test of differences; never smoker v ex-smoker p=0.2724, never smoker v smoker p=0.6923 and ex-smoker v smoker p=0.0656) (Figure 3.7). Correlations with increased plasma ST2 levels were determined in each group for post-steroid sST2 as well as difference in sST2 pre-

and post-steroid. No correlations between sST2 and FEV_1 change in FEV_1, PEF or ACQ scores was found in any of the groups. The only correlation identified with respect to difference in sST2 was in the never smoker groups with ACQ score (log-transformed sST2, Pearson's correlation r=0.731, p=0.007). Two never smoking patients failed to provide follow-up samples therefore were excluded from post-steroid or difference in sST2 analysis with a new group size n=19.

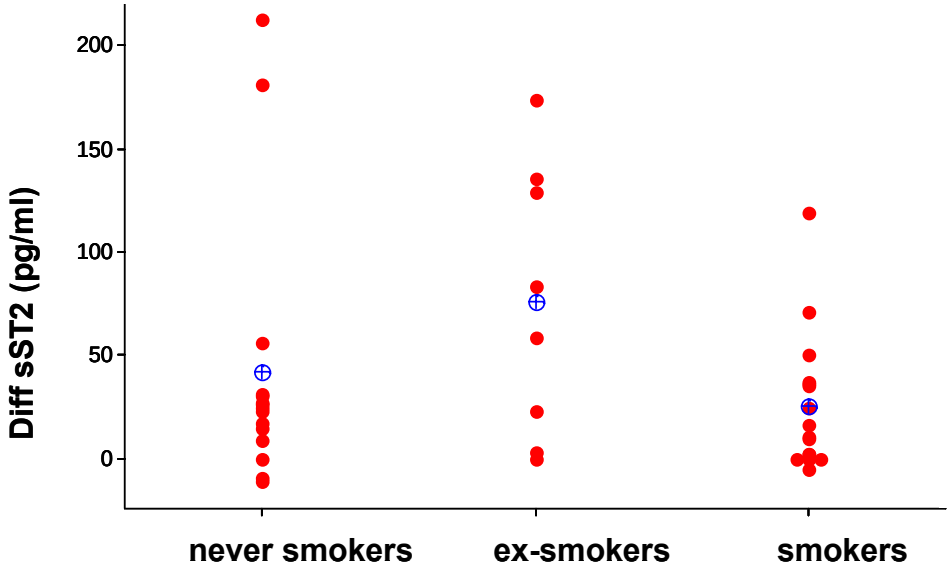


Figure 3.7: Difference between pre- and post-steroid sST2; between group comparisons
Difference in pre- and post-steroid levels of sST2 was calculated for each individual and differences plotted for each group according to smoking status. Mann-Whitney *U* test was performed on the median difference (open cross circle) between groups. No significant differences were shown. Significance was determined if p<0.05.

3.3.5 Plasma IL-33 levels are unaffected by smoking status or steroid treatment

Following the measurement of sST2 levels, the levels of IL-33 in plasma were determined. At the point of experiment design, only limited data existed demonstrating IL-33 levels could be measured in biological samples. Two published reports showed presence of IL-33 in atopic patients with a form of allergic rhinitis [428] and in atopic individuals with anaphylaxis [460] (retracted 2012). The former study used an in-house ELISA constructed from locally manufactured antibodies and the latter using commercial IL-33 ELISA (Centaur or Genway biotech, San Diego, USA catalogue no 40-288-23146) which was therefore chosen for use in this work. The manufacturer minimum detectable IL-33 is 0.7ng/ml.

Recovery was determined by spiking recombinant IL-33 into normal plasma, serum and cell culture media (not shown).

Previously processed specimens stored at -80°C were defrosted as before and Il-33 levels measured in one experimental run. Table 2 summaries the baseline IL-33 levels. There was a wide range of measured IL-33 in all patients but the majority fell below the manufacturer minimal detectable level (0.7 ng/ml). Three, 1 and 2 patients had plasma levels above 0.7 ng/ml in the neversmoking, ex-smoking and smoking groups respectively. Baseline IL-33 was also measured on the group of 14 normals as previously described. No differences were seen between the average values of these individuals and study participants (Table 3.2).

	Median IL-33 (pg/ml)	IQ range (pg/ml)	Maximum IL-33 (pg/ml)
Normal never smoker (n=14)	48	0-108	982
Never-smoker with asthma (n=21)	40	0-257	2635
Ex-smoker with asthma (n=10)	45	5-312	1508
Smoker with asthma (n=22)	28.3	0-316	1116

Table 3.2: Baseline plasma IL-33 concentration in stable asthmatics

Comparison of baseline pre-steroid IL-33 levels did not show any difference between the group medians (Figure 3.7A). Again, following steroid treatment, no difference between median levels in each of the groups was seen (Figure 3.7). Within-group comparison did not attribute any change in IL-33 to steroid treatment (non-smokers p=0.191; ex-smokers p=0.793; smokers p=0.808).

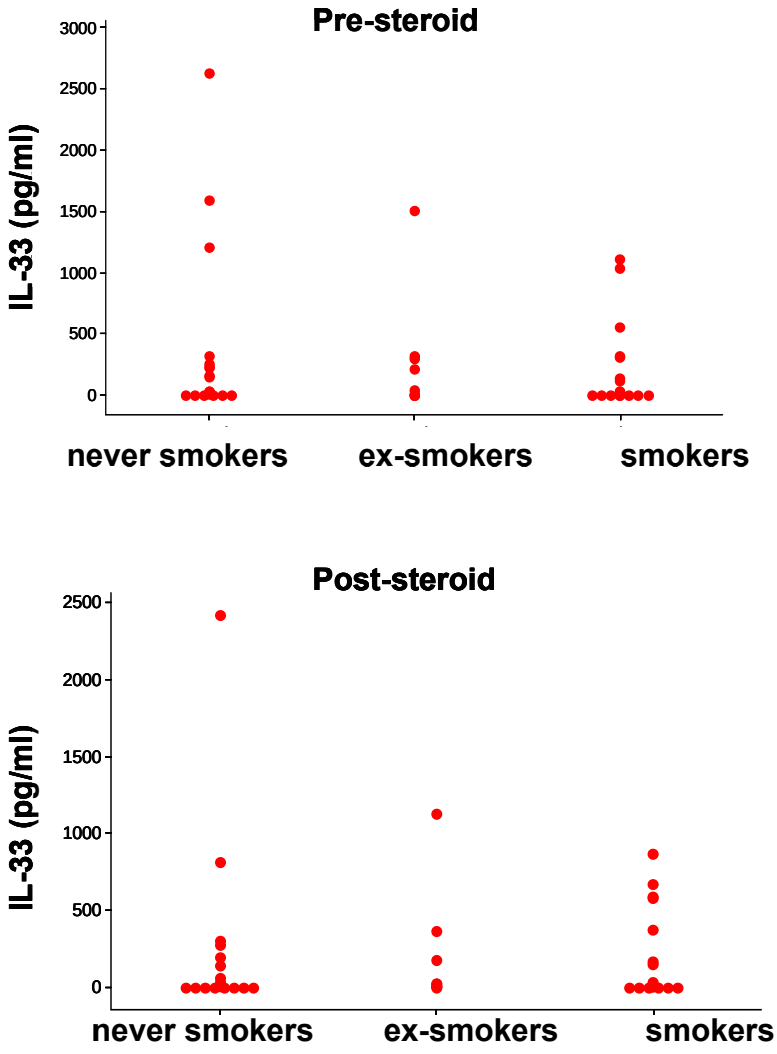


Figure 3.8 Plasma IL-33 pre- and post-steroid in all groups
IL-33 concentrations (pg/ml) as measured by ELISA in non-smokers, ex-smoker and smokers pre- and post- steroid treatment. No significant differences were seen in baseline or post-steroid median group levels assessed by Mann-Whitney *U* test. Within-group comparison using paired *t* test did not find any difference in levels related to steroid treatment. p <0.05 was considered significant.

There was no difference between the groups comparing the ratio of baseline ST2 to IL-33 or post-dexamethasone ST2 to IL-33 levels (not shown).

3.3.6 Levels of sST2 and IL-33 in sputum

The same ELISA kits and protocols as described for plasma samples were used to go on to measure the levels of sST2 and IL-33 in the induced sputum specimens. Levels of sST2 in the sputum of all participants were very low, around 15 pg/ml and no change was identified with steroid treatment (data not shown). Furthermore, there was no detectable IL-33 in the sputum pre- or post- steroid so no further assessments could be performed (data not shown).

3.4 Conclusions

This set of experiments aimed to examine the expression of ST2 and IL-33 in murine and human samples. IL-33 protein was detected in murine lungs by immunohistochemisty. Endothelial cell IL-33 expression in murine lungs is consistent with other reports of vascular expression [387,389]. New findings presented here show the cellular distribution appears to be strongly expressed in nucleus of parenchymal inflammatory cells, most likely macrophages, and in the cytoplasm of epithelial cells. As the antigenic content is generally better preserved in snap frozen sections, the finding that the Nessy-1 IL-33 antibody was not effective in paraffin sections is consistent with current understanding [461]. The R&D antibody subsequently worked well in paraffin sections with enhanced antigen retrieval techniques. The R&D antibody also avoided specificity problems caused by using the Nessy-1 mouse antibody on mouse tissue as this antibody was raised in rat. As the morphology is improved by using this methodology, enhanced definition of IL-33 staining was appreciated. It should be noted that successful use of anti-IL-33 Nessy-1 in paraffin samples has now been described by others in mice [389] and humans [399]. However, using specimens processing techniques in our laboratory, optimal antibody efficiency in frozen and paraffin sections was achieved with Nessy-1 and R&D antibodies respectively.

IL-33 mRNA is expressed predominantly in non-haematopoietic cells and in restricted inflammatory cell populations including macrophages [293]. However, the finding of strong expression of IL-33 in a nuclear location in murine parenchymal macrophages is novel and of interest as it suggests important function in these cells in IL-33 dependent effects in the lung. IL-33 has been now shown to bind NF-κB directly therefore reducing its ability to turn on gene transcription suggesting a predominantly regulatory role in these cells [418]. Recently, very similar results to these presented in this thesis have been published, showing IL-33 expression in inflammatory, epithelial and endothelial cells in OVA treated mice with identical paraffin section methods [462]. Epithelial cell IL-33 expression as determined by immunofluorescence and anti-IL-33 antibody (R&D), has been confirmed in AAI and control mice [463]. Although the form of IL-33 detected in cytoplasm of epithelial cells is unclear, its presence is

in keeping with a translated protein available to be released either by necrosis or secretion. As extracellular release from epithelial cells has been shown, IL-33 is now considered in the category of epithelial derived cytokine akin to IL-25 and TSLP [186,246]. *In vitro*, co-culture of epithelial cells with a gastrointestinal nematode leads to necrosis and release of full-length IL-33 [464]. In the lungs, extracellular IL-33 is detected in BAL following influenza infection in a murine model [465] and 2 hours following flagellin challenge [466]. Both findings would be in keeping with epithelial cell damage; IL-33 was also detected *in vitro* in the supernatant from the influenza infected murine respiratory epithelial cells (MLE-15 cell line) [465]. Secretion of full-length nuclear IL-33 from intact undamaged bronchial epithelial cells (NHBE) is also described, induced by a fungal extract *Alternaria*, and dependent on intracellular calcium concentration by ATP [467]. IL-33 secretion from human AEC is enhanced by IL-1β, TNFα and IFNγ [468]. IL-33 is released from primary cells in tracheal murine cells and primary human NBEC cells in response to HDM allergen [469].

Human tissue was not examined here for IL-33 expression but its presence has now been clearly demonstrated by others. In asthma, lung IL-33 mRNA was elevated in endobronchial biopsies of moderate and severe asthmatics but not in those with mild disease, compared to healthy controls. In terms of cellular expression, the same investigators demonstrated IL-33 in epithelial and endothelial cells in both the healthy and asthmatic samples, similar to murine expression results presented here[470], but they also identified expression in airway smooth muscle (ASM) cells, a feature only apparent in the asthmatic patients [399]. ASM changes in murine models of AAI are often not a reproducible feature of human asthma and hence these changes may not been seen in samples used here[134]. Epithelial cell staining was found in the nucleus along with the cytoplasm and increased staining intensity was measured in subjects with severe asthma compared to healthy controls but not compared to other asthmatics. The human studies did not describe in IL-33 positivity in parenchymal inflammatory cells or macrophages; this could be a finding restricted to murine lung.

This demonstration of IL-33 expression in murine lung is consistent with the finding of IL-33 expression at other comparable mucosal surfaces such as the gut

and skin in humans. In psoriatic skin biopsies, IL-33 immunohistochemistry, also using anti-IL-33 Nessy-1 in frozen sections, was associated with blood vessels and inflammatory cells, and more strongly in affected than unaffected skin sites [445]. IL-33 and ST2 expression is also seen in the gut mucosa. IL-33 was detected in the nucleus and cytoplasm of the intestinal epithelial cell, the lamina propia mononuclear cells of normal controls and in diseased mucosa with varying intensity with the highest expression was in ulcerative colitis biopsies [406]. This study in the gut utilised an anti-human IL-33 IgG with results similar to those described in murine paraffin staining results presented in this chapter. Expression clearly does not assume presence of inflammation as nuclear IL-33 can act as a transcriptional regulator. Further studies show IL-33 expression is present in converged endothelial cells but lost in those which are dividing including tumour cells, suggests control of endothelial cell activation [388]. Whether nuclear epithelial IL-33 plays some role in regulating division in the murine lung is unclear but a further study in the gut identifies IL-33 redistribution in intestinal epithelial cells; from cytoplasmic expression in healthy tissue to strong nuclear expression in diseased tissue suggests this is possible[471].

Results here show pulmonary ST2 protein staining was predominantly identified in unstimulated epithelial cells. Previously, ST2L mRNA expression was first noted to be weakly inducible in a murine mammary epithelial cell line whereas sST2 was expressed constitutively at low levels but strongly inducible [318]. A subsequent study found inducible sST2 in alveolar (A549) and bronchial (NHBE) cells, with no baseline ST2L expression in either cell type identified but it was weakly inducible in the NHBEs [306]. Another study showed sST2 mRNA expression is inducible in the A549 cells by IL-1β plus IL-4; however, it was not released extracellularly as determined by ELISA [314]. Data presented here shows constitutive ST2 expression however the isoform cannot be determined; experiments in Chapter 5 will further examine ST2L expression in epithelial cells. Since the presented experiments were performed, further published studies show sST2 release, with low level sST2 and ST2L expression in epithelial cells during growth phase [320] or sST2 expression in primary alveolar and bronchial epithelial cells with high sST2 production in the alveolar cells [316]. In comparison, ST2 is also present at other mucosal epithelial surfaces. In human colon biopsies,

ST2 is expressed in epithelial cells but in contrast to IL-33 expression it is specifically decreased in patients with ulcerative colitis. The isoform identified was ST2L by mRNA, which could indicate ST2L down regulation in the presence of chronic disease; both circulating IL-33 and sST2 levels were elevated in these patients suggesting this is the case [406]. Another similar study shows similar dysregulated sST2/ST2L expression in the human gut but this not seen consistently in all the examined bowel and in this case was predominantly explained by increased sST2 production [471]. ST2 is also described in parenchymal inflammatory cells, most likely macrophages, in keeping with these known ST2 expressing cells [306].

To confirm these findings presented here, follow up work would include double staining with epithelial cell markers (Cytokeratin) and as well as F4/80 with IL-33 in the paraffin sections. Furthermore, ST2 and IL-33 co-staining would be valuable and this work has already been completed in collaboration with another investigator in our laboratory (data not presented here) [472]. Alternatively, FACS using individual cell expression markers combined with ST2 and IL-33 antibodies, including intracellular staining techniques, can now be undertaken.

Unexpectedly, levels of IL-33 measured in sera of mice with AAI were lower than in the control mice. The possibility that sST2 sequestered IL-33, thus making it undetectable is supported by a rise in IL-33 in sera of OVA-treated $ST2^{-/-}$ mice [397]. However, no corresponding sST2 measurements were performed here to confirm these findings, due to the lack of an available ELISA. Furthermore, as $ST2^{-/-}$ mice can have diminished inflammatory responses in this OVA-model of AAI compared to control WT mice (see chapter 6) this could affect interpretation of these results. However the experiment examined here showed no significant differences in airway inflammation between WT and $ST2^{-/-}$ mice following a long (28 day) model. A further significant limitation of this data is that it was obtained from an ELISA system developed in house and should be repeated with one of the multiple mIL-33 kits now available commercially. Murine IL-33 detection by ELISA has been demonstrated in multiple cell cultures and systems [464,467-469],[473] although the ELISA used is not stated nor absolute values measured in the former and using duoset R&D in the later; the presence in serum was not reported. IL-33 was detected in digested lung tissue 24 hours and 7 days following final AAI challenge in C57BL/6 and BALB/c mice respectively [282]. This

could be explained by the view that IL-33 peaks in the lung at the peak of inflammation in C57BL/6 mice but that in BALB/c mice IL-33 is associated with persisting AHR demonstrated in these mice 1 week following challenge. IL-33 detection was not reported in the serum but these findings suggest IL-33 is elevated in AAI, in contrast to a reduction demonstrated in data presented here. Despite a different compartment analysed and although sST2 was not measured it could be assumed that if sST2 were to be sequestering IL-33, it could also be doing so in these published studies. Observational findings by the thesis author are consistent with the report of higher levels of IL-33 in C57BL/6 mice 24 hours following airway challenge, and suggest the measurement interval for correct peak levels interpretation in BALB/c might need to be extended. A further study in C57BL/6 mice is in agreement with this finding showing increased lung IL-33 levels in OVA treated mice compared to control mice with a corresponding trend to increased sST2 levels. A parallel experiment using a house dust mite (HDM) allergen protocol showed increased IL-33 and sST2 levels in the treated group compared to controls [462].

Corticosteroids are effective in reducing eosinophilic inflammation, a range of inflammatory cytokines and symptoms in asthmatics. Not all patients have these beneficial effects and those who smoke cigarettes are recognised as a group that modulate the response to CS in asthma [474]. Therefore as part of a wider study investigating altered plasma and sputum cytokine profiles in smokers compared to never smokers with asthma, the levels of sST2 and IL-33 in plasma were determined to investigate if any associations exist.

Data presented here from a clinical study is exploratory and therefore limitations apply. No primary endpoints were identified and numbers recruited were not powered to necessarily detect significant differences between groups; the expected difference was unknown. Full age, sex and smoking matched controls were not recruited and the small number of controls used cannot be considered an adequate control group. Plasma and sputum levels of sST2 and IL-33 were measured in this study. Numerous previous studies of sST2 levels have reported measurements predominantly in serum. However, analysis in plasma samples has also been reported and furthermore, long term stability of up to 18 months in plasma samples stored at -80°C was shown validating the results presented here [475]. Personal, unpublished observations based on performing

ELISA in serum and plasma samples from renal dialysis, post-MI and pulmonary hypertension patient cohorts by the author, detect no appreciable difficulties or discrepancies in performing the sST2 assay on plasma or serum samples. For IL-33, plasma detection has been identified by others [476].

sST2 and IL-33 levels were low or undetectable in the induced sputum samples. Cytokines have been detected in induced sputum and correlate with clinical features [38,51,60]. However, there are criticisms of measuring cytokines in sputum supernatant, predominantly due to sputum processing technique, in particular the use of the reducing mucolytic agent dithiothreitol (DTT). Use of DTT results in the reduced detectable concentrations of some cytokines and the potential effect on this on sST2 and IL-33 levels was unknown [477]. Varieties of laborious sputum processing techniques can be employed and as such a lack of consistency in published data exists. New standardised filters and kits could potentially improve consistency if widely employed [478]. The only report of sST2 and IL-33 detectable in sputum was recently published and identifies their presence in induced sputum from children processed with the DTT technique suggesting this processing technique may not impede detection. The ELISA used were the BioPorto Diagnostic, Denmark and GenWay biotech, California, USA respectively. These investigators looked at 37 patients with mild to moderate asthma and found both sST2 and IL-33 in sputum samples were higher in asthmatics than healthy controls [479]. These results are not in keeping with the findings in results presented here which also measured activity in non-severe asthmatics. Despite the lack of normal controls measured in our data, no appreciable levels were seen in the asthmatics assessed. This difference could be explained by technical issues including dilution or that there are indeed no appreciable levels in this adult cohort.

Since the construction of an ELISA to determine human sST2 protein, levels in the sera have been assessed in many conditions (chapter 1.2.4.1) [354]. When considering sST2 in plasma levels in this study, the main finding of the data presented here is that despite small patient numbers, there was a clear association of increased sST2 levels with oral CS treatment. Smoking does not appear to have a significant effect of sST2 at baseline or influence this CS-related increase. Based on this presented data, sST2 levels do not correlate to clinical parameters measured therefore sST2 is unlikely to be useful in reflecting

disease process or use as a biomarker in this cohort of stable moderate patients or reflect altered cytokine profile of smoking status.

It must be noted that the patients taking oral CS in this study are not steroid naïve. All patients were already taking inhaled CS and this was not 100% matched between the groups with the smokers having the highest inhaled CS usage. No statistical correction was employed for this in data interpretation. Smokers have higher symptom scores [480] and data presented here is in agreement with the ACQ scores higher in smokers and ex-smokers compared to non-smokers which explains the higher steroid usage amongst this group. Despite this however, as no inter-group differences were measured and an increased sST2 response to oral CS steroid was seen in all groups, this is unlikely to affect the results presented. Furthermore the smokers with higher inhaled CS had a trend to lowest baseline sST2 compared to other groups and normal controls and so the response to oral CS was inversely related to this association and strongest in the smokers, thus it is unlikely to have been influenced by inhaled CS dose.

It is not clear what the mechanistic cause is for the association of CS with raised sST2 levels. Although many studies have demonstrated sST2 correlating with clinical measures as a biomarker, less is clear about the direct role for sST2 in pathogenesis. Soluble ST2 has been shown to be directly released by cells undergoing biomechanical stress in *in vitro* models [394]. Clinically, diastolic load appears to modulate ST2 production where patients with isolated diastolic heart failure (elevated left ventricular end diastolic pressure (LV EDP)) and normal LV systolic function with raised sST2 compared to controls. However despite this, the source of the sST2 was apparently extra-myocardial [481]. In patients with a form of nephrotic syndrome, sST2 was a marker of recurrence after transplantation but neither *in vitro* nor *in vivo* studies identified an actual role in kidney injury [482]. The mechanism of action of sST2 is postulated to be via ability to bind to IL-33 preventing its binding to ST2L [397]. If in the asthmatics cohort sST2 is truly raised without a rise if IL-33 it could indicate a separate non-IL-33 pathway stimulated by steroids; the cellular source of sST2 production in this circumstance is unclear.

The association of sST2 and oral CS must be taken in account when we consider sST2 as a biomarker, particularly in the context of acute exacerbations of

asthma. Many patients may well have received oral CS in primary care before a measurement is taken in hospital. This could significantly alter the interpretation of sST2 in this context. Previous studies have shown sST2 levels are elevated in acute asthma [352,355]. A study of 56 asthmatics showed a difference in levels of sST2 between moderate asthmatics with persistent symptoms compared to controls. No difference was seen in intermittent, mild or severe persistent cases compared to controls with 33% and 57% of moderate and severe groups respectively taking oral corticosteroids. Thirty of the patients had an exacerbation during the study and a strongly significant increase in sST2 was shown in these patients. It is possible therefore this elevation could reflect a response to an oral steroid 'boost'. However, this would not necessarily explain other clinical correlations of %PEF and PaCO2 reflecting the severity of the exacerbation with sST2, as all patients would be expected to have a similar steroid boost [355]. In the second study performed in children, steroid doses are not recorded but sST2 was measured acutely and in convalescence, most likely after steroid treatment. In these patients levels of sST2 are lower in the convalescence period whilst patients are taking steroids which would be contrary to the findings in presented here [352]. In the children where the presence of atopy was assessed sST2 levels were elevated but it is unclear if atopy or indeed age is relevant to the interpretation of sST2 levels [483]. The data presented here would suggest caution in interpreting sST2 levels in asthma as concomitant steroid, which will be common in these patients, may influence these. Further information, particularly the effect of oral CS in normal controls would be essential as dissecting oral CS from an exacerbation effect will be difficult unless pre-steroid samples at time of exacerbation are assessed for clarification. Despite these considerations, sST2 in unselected group of dyspnoeic patients is accurate in predicting mortality up to 4 years where steroid use and proportion patients with HF or asthma/COPD is unknown [361].

In the study presented, levels of IL-33 in the plasma were generally low with a small number of high IL-33 level individuals present in all groups. There was no association with smoking status or steroid treatment. Several explanations for these findings can be considered. Firstly, the concentrations measured are an accurate reflection of IL-33 in this cohort of patients. Considering these are stable, non-exacerbating and non-severe asthmatics, we could expect that levels

of inflammation would be low and thus as IL-33 is pro-inflammatory, could well be at low or undetectable levels. In comparison to IL-1, a member of the same super family as IL-33, levels of IL-1α are present in high amounts constitutively in epidermal surfaces but not readily measured in plasma or serum [484] and its antagonist IL-1Ra is produced in 10- to 100-fold excess [485]. This suggests tight regulation of this potent cytokine and increased concentrations are likely to be present locally rather than systemically, an explanation that could also apply to IL-33.

If sST2 only acts as a decoy receptor, the detection of levels of sST2 in the serum does suggest that IL-33 could be detectable. Therefore another explanation for the levels detected is that the detection system is not sufficiently sensitive at measuring these lower levels. The ELISA kit used has an apparent high minimally detective IL-33 level of 700 pg/ml. Although it was possible to titrate this downwards in our laboratory to a detection level of 400-500 pg/ml, this is not validated by the manufacturer. In comparison to the other published study available at the time, levels of IL-33 were measured in controls, infectious rhinitis and allergic rhinitis patients by an in-house ELISA with a lower limit of detection 30 pg/ml [428]. The profile of measurements is similar in this published study to that presented here whereby the majority of patients had very low or undetectable levels and those with elevated levels were predominantly under 1000 pg/ml. Interestingly, in this population there was a similar picture of outliers with nanogram levels, the highest 19 ng/ml in a control group individual [428]. A subsequent publication using the same ELISA as in the data presented here, similarly found most patients had a concentration of IL-33 beneath the detection level in keeping findings here [486]. The patients in that study had systemic lupus erythematosus (SLE) and although cannot be considered directly comparable, this cohort had elevated sST2 which correlated with disease activity. In this group, 3/70 patients and 2/28 age and sex matched controls had detectable IL-33 [486].

Following the undertaking of this work, several other manufacturers have developed improved human IL-33 ELISA kits and subsequently reports of these assays in detecting human IL-33 have been published. Using a R&D systems human IL-33 detection assay, studies have shown elevated serum IL-33 in patients following MI [487] and in those with acute phase SLE or RA patients using a

Peprotech kit [488]. The lower level of detection in these assays are 23.4 pg/ml and 32 pg/ml respectively with participant measurements averaged 150-200 pg/ml or 500 pg/ml in the later study. Beltran *et al* used an ELISA with a low limit of detection (5 pg/ml) and measured very low serum levels of IL-33, maximum 15pg/ml (Apotech range 0-500pg/ml) was detected with some differences determined between controls and those with CD and UC [471]. These patients had IL-33 expression demonstrated in biopsies as discussed previously.

Taken together with the results presented here, serum/plasma IL-33 is detected in patients with asthma but there is no correlation with disease. Recommendations for further work would be a controlled study including patients with increased severity with age-matched controls analysed by an assay system with lower detection limits. As IL-33 could be a poor marker of stable disease, exacerbating patients should be included. Also with increased numbers, more information about correlation of the sub-group of very high responders could be obtained. A subsequent small study has shown no change in serum IL-33 between control and a group of asthmatics. However subgroup analysis shows characteristics associated with increased levels including IgE, current treatment and interestingly smoking [489]. However, two just published studies show increased IL-33 in serum of asthmatics with Raeiszadeh *et al* recording mean concentrations of 79.10 ± 20.62 pg/ml IL-33 compared to controls 0.51 ± 0.26 in pg/ml (RayBiotech, Norcross, GA ,catalogue ELH-Il-33-001, sensitivity 2 pg/ml)[490]. Guo *et al* measured higher levels using an alternate R&D assay kit with IL-33 903.62 ± 523.78 pg/ml and controls 158.1 ± 81.74 pg/ml (human IL-33 quantikine ELISA kit R&D systems, sensitivity 1.65pg/ml, assay arrange 6.25-400 pg/ml). Furthermore, this cohort included mild, moderate and severe asthmatics and IL-33 correlated with FEV_1 and severity [491]. Finally, another group of patients who have pulmonary eosinophilia but not asthma show increased IL-33 plasma levels compared to controls but correlation with disease activity was not clearly found [492].

In conclusion, plasma sST2 is increased by oral CS in asthmatic patients. It is unclear if this finding can be extrapolated to other conditions or to control individuals. Although the mechanism for this is unclear, it requires further investigation and suggests important implications into interpreting sST2 in many other inflammatory conditions where use of CS is commonplace. Plasma IL-33

levels were not significantly elevated in this cohort of patients but limitations based on detection system and subsequent studies suggest serum/plasma IL-33 may be related to asthma severity and this suggests value in further studies to determine its validity as a biomarker. Assessment of different forms of IL-33 with asthma phenotypes could enhance clinical correlations.

4 IL-33-induced innate airway inflammation

4.1 Introduction and Aims

Schmitz *et al* administered IL-33 intraperitoneally to C57BL/6 mice, which resulted in eosinophilic peritoneal inflammation and systemic Th2 or type-2 inflammation [293]. Investigations in our laboratory subsequently demonstrated that IL-33 given to BALB/c mice via the i.n. route resulted in a similar potent local Th2 response demonstrated by eosinophilia and cytokine release in the bronchoalveolar lavage fluid (Kewin PK, 2007, PhD thesis, University of Glasgow).

ST2L, the cell bound receptor for IL-33, is present on Th2 cells and mast cells, both of which are influential cells in the pathogenesis of asthma[304 317]. In chapter 3, evidence of additional cellular ST2 and IL-33 expression in the murine lung demonstrates cell types capable of producing and responding to IL-33. Taken together, this demonstrates that the ST2/IL-33 axis is present in the lung and therefore IL-33 can potentially modulate Th2 and innate cells, suggesting a role for this cytokine in asthma.

Experiments in this chapter aim to extend the knowledge of the direct response to IL-33 in the lungs. The hypothesis that IL-33 drives a potent Th2 type response in the lungs via the activation of ST2L, in particular on Th2 cells of the adaptive immune system which are essential for development of this phenotype, will be tested.

Firstly, to further investigate this response of IL-33 in the airway, the cell types involved will be characterised and the dynamics of inflammatory mediators in this response examined in more detail. Secondly, using this information, the detailed contribution of select relevant mediators will be investigated. Thirdly, the role of the adaptive immune system using $RAG^{-/-}$ mice, which completely lack T and B cells, will be elucidated together with the assessment of whether IL-33 in absence of antigen can generate other key features of asthma, in particular AHR.

4.2 The effect of intranasal IL-33 on airways inflammation

In the first instance, the aim was to confirm earlier laboratory findings that direct intranasal application of IL-33 to the murine airway resulted in a Th2 type inflammatory profile. Schmitz *et al* used a dose of 4 µg human IL-33 intraperitoneally but experiments in our laboratory using T cells *in vitro* (M Kurowska-Stolarska, unpublished data) demonstrate an approximately equivalent inflammatory response using 2 µg recombinant murine IL-33, which was generated in our laboratory [424]. Based on the Schmitz data and pilot experiments done by others in our group, an initial dosage schedule of 7 days was chosen. In some subsequent experiments, where recombinant murine IL-33 was not available due to technical problems, recombinant human IL-33 (hIL-33) was used and this is acknowledged in the relevant figure legends.

4.2.1 Intranasal IL-33 initiates cellular Th2 inflammation profile

Recombinant murine IL-33 (2 µg) or phosphate buffered saline (PBS) was administered i.n. for 7 consecutive days to BALB/c mice. Twenty four hours after the final dose, the mice were culled, bronchoalveolar fluid and lungs collected and cellular profile analysed.

Administration of IL-33 to the airways resulted in a massive influx of cells measuring 18.32 x 10^5 cells/ml compared to 1.08 x 10^5 cells/ml in control mice given PBS (Figure 4.1A). The numbers of all cell types increased but eosinophils were the dominant cellular population at 13.48 x 10^5 cells/ml (73.5%) (Figure 4.1B). Macrophages (19.1%) and neutrophils (7.3%) were the other most abundant immune cells identified. Th2 cytokines in the BAL were increased with IL-5 shown in Figure 4.1C.

Similarly, histological examination of the lungs show extensive eosinophilic inflammation, predominantly in the peri-vascular but also peri-bronchial areas in the lungs treated with IL-33 (Figures 4.2A and 4.2B).

These data confirm that IL-33 administered directly to the airways causes a potent Th2 innate inflammatory response.

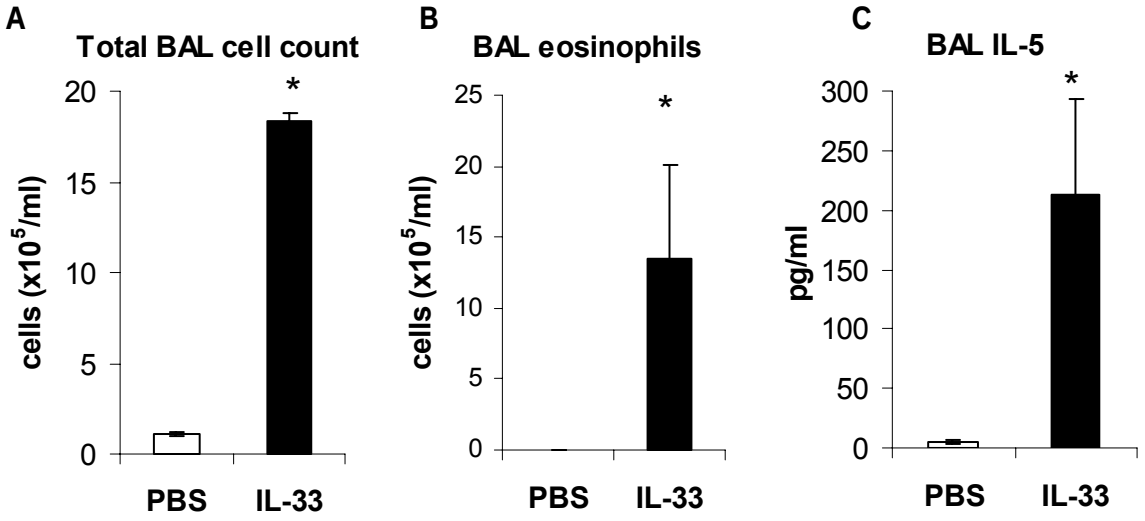


Figure 4.1: Intranasal IL-33 increases BAL cell counts and IL-5
BALB/c wildtype mice were given PBS or 2 µg IL-33 intranasally for 7 days. Mice were culled on day 8 and BAL collected. Total and differential BAL cell counts were performed and cytokine levels measured by ELISA. Data shown as mean +/- SEM, n=5-9 per group. *p<0.05 compared to the PBS group. Data representative of at least 3 experiments.

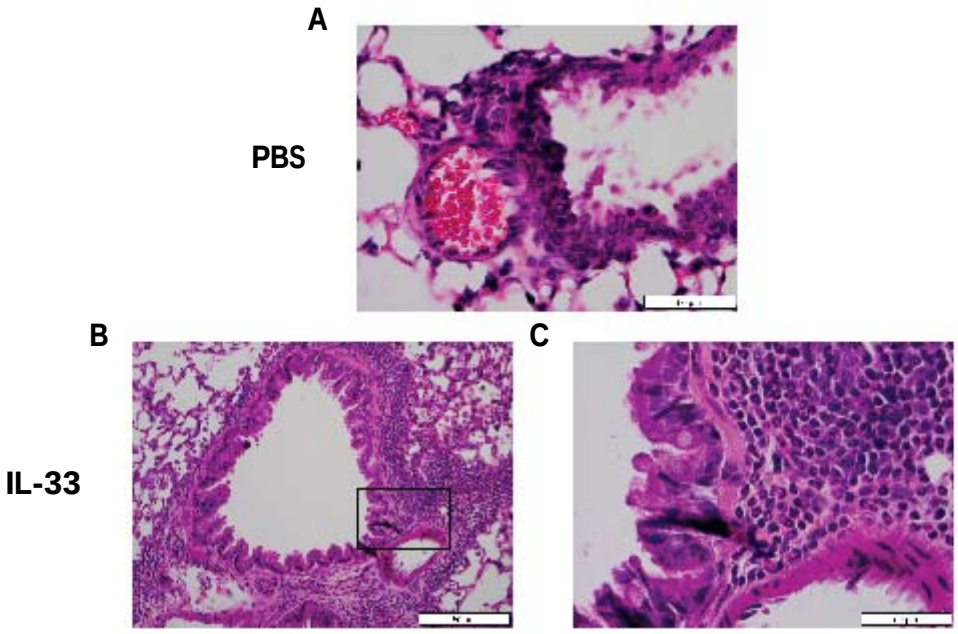


Figure 4.2: Intranasal IL-33 induces eosinophilic lung inflammation
Lungs from experiments in Figure 4.1 were fixed in formalin and stained with H&E. A) PBS treated mice x 40 magnification. (B-C) IL-33 treated mice with severe eosinophil peri-bronchial and peri-vascular inflammation shown x10 magnification B) and x40 C) Representative histological sections shown (n=5-9 per group).

4.2.2 IL-33 increases Th2 cellular inflammation in the lungs

BAL fluid sampling is thought to reflect the degree and type of inflammation within and around the bronchoalveolar lumen and deeper alveolar compartments [80]. Given the now well demonstrated findings of IL-33-driven cellular inflammation in the airway, the cellular contents of the lung were further investigated. Simultaneous evaluation of multiple cell types in the lungs and BAL provides a fuller evaluation of the cellular infiltrate including cells embedded in the tissue, as well as opportunity to assess lung tissue cells. Furthermore, using the technique of lung digestion, a large number of cells are obtained in suspension which is necessary for FACS analysis, overcoming a limitation of low cell numbers of performing FACS analysis on BAL fluid, in particular on control or naïve mice. Lung digestion methods were based on published methods and using pilot studies modified for use in our laboratory [493,494]. These methods are more fully described in chapter 2.

In order to characterise which cells are capable of responding to IL-33, the level of cell surface ST2 expression in whole lung preparations from control mice was ascertained. The specificity of the chosen ST2 antibody (FITC-conjugated anti-T1/ST2, DJ8, MD Biosciences, UK) was tested in pilot studies on high ST2 expressing mast cell lines[293] and has been previously used on mast cells[495].

Following digestion of the lungs, a single cell suspension was stained for ST2 or with isotype control. In the PBS treated lungs, 29.1 % (±1.9 %) cells expressed ST2 whilst in the IL-33 treatment group, the percentage rose significantly to 55.4% (± 2.8%), indicating an influx of these cells or up regulation of ST2 on resident cells (Figure 4.3).

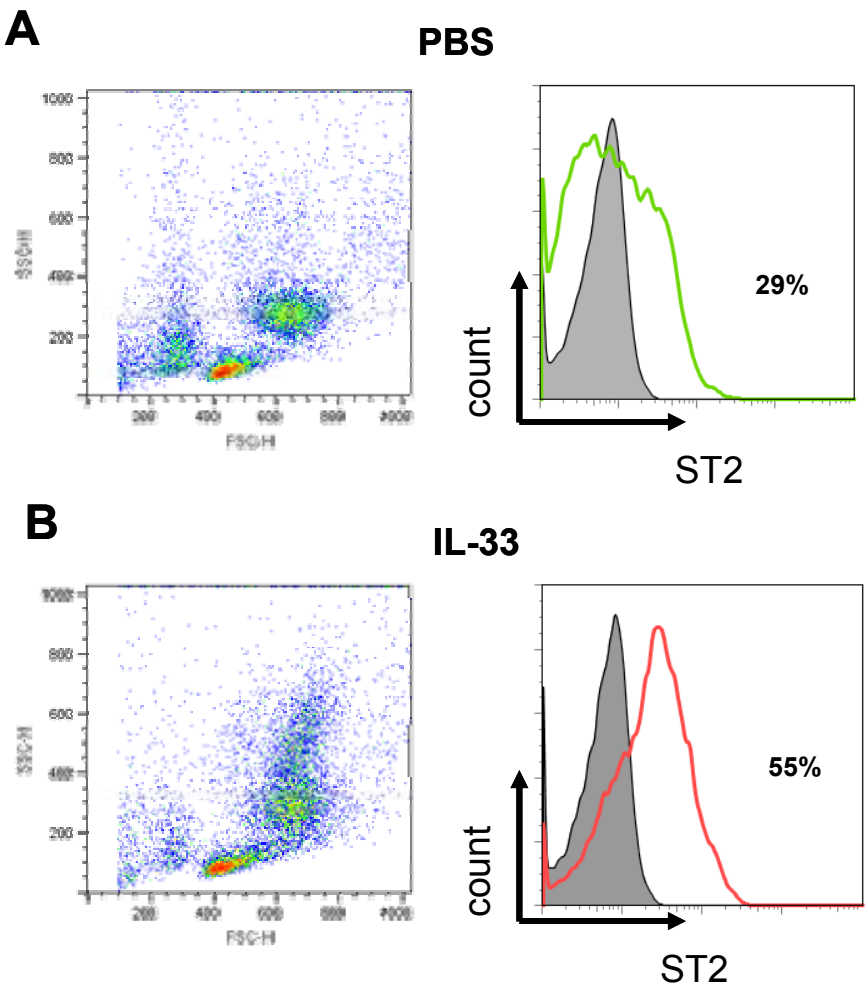


Figure 4.3: Intranasal IL-33 increases total lung cellular ST2 expression
BALB/c WT mice were given PBS or 2 µg IL-33 intranasally for 7 days. Lungs from individual mice were isolated, digested and single cell suspensions obtained. Cells were gated following exclusion of dead cells (7-AAD). Cell surface was stained with FITC-ST2 or FITC-IgG antibodies. Scatter dot plot of viable cells and histogram of ST2 expression in PBS A) and IL-33 B) treated mice. Data shown representative of 3 separate experiments. n=5 per group.

In mice, cellular expression of ST2 was originally described in fibroblasts, subsequently ST2L expression was demonstrated on Th2 lymphocytes [304], mast cells [305] and macrophages [306,346].

In order to further study which cell subpopulations demonstrate ST2L expression in PBS treated lungs seen here and to assess to what extent these populations are influenced by IL-33 administration, cell specific surface antibody staining on the digested lung cell population was performed. Figure 4.4 shows FACS cell surface staining for individual cell types plotted against ST2 expression. F4/80 is a well characterised marker for mature mouse macrophages and using this, 6.32% of the total population were identified as macrophages and 70% (4.43% overall population) of this group co-expressed ST2. In mice treated with IL-33,

overall the macrophage population increased to 23.65%, 54% of which were $ST2^{+ve}$ (12.83% overall population).

ST2 is a stable selective cell marker on $CD4^{+}$Th2 cells but not $CD4^{+}$Th1 cells [304]. Approximately 10% of cells in PBS treated lungs expressed CD4 but unsurprisingly in non-inflamed lung, less than 1% co-expressed ST2. In IL-33-treated lungs, the proportion of $CD4^{+}ST2^{+}$ cells increased to 4.13% indicating IL-33 influences the adaptive immune response directly by driving differentiation or recruitment of Th2 cells $ST2^{+ve}$ cells. Although studies in $ST2^{-/-}$ mice show ST2 is not essential for Th2 differentiation [338,339], *in vitro* analysis in our laboratory shows IL-33 can induce differentiation of $IL\text{-}5^{+}IL\text{-}4^{-}CD4^{+}$ cells from naïve $CD4^{+}$Th cells independently of factors typical for Th2 differentiation, namely IL-4, STAT-6 and GATA-3 [496].

Mast cells highly express ST2, with mature mast cells identified by the presence of SCF receptor (c-kit) [305]. IL-33 treatment increased the population of mast cells in the lung to 2.32% compared to a 0.22% in PBS-treated mice. Ideally, mast cell identity should be confirmed with further co-staining with FCεRIα and the gating strategy used optimised.

Neutrophils were identified as a population of high Gr-1 (Gr^{hi}) expressing cells in Figure 4.4D. There was no change in proportion of neutrophils between PBS and IL-33 treated lungs, at approximately 7-9%. Other granulocytes can express Gr-1, in particular eosinophils which are of interest in these experiments. However, Gr-1 is expressed at a lower level ($Gr\text{-}1^{med}$) in eosinophils [497]. Thus eosinophils probably represent the $Gr\text{-}1^{med}$ population seen here and these cell populations were further discriminated from each other by using scatter parameters (data not shown).

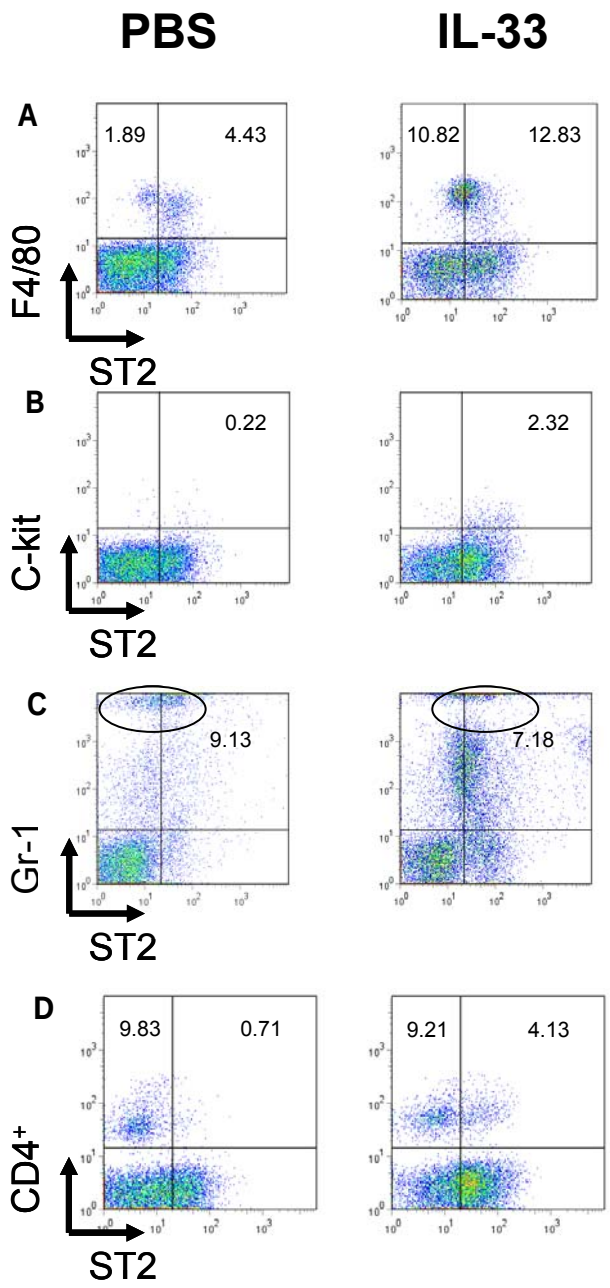


Figure 4.4: Lungs treated with IL-33 demonstrate an increased proportion of ST2^{+ve} cells
The surface expression of A) F/480, B) c-kit, C) Gr-1 and D) CD4^{+} versus ST2 on single cell suspensions of whole lung digests in mice treated with intranasal PBS or IL-33 were analysed. FACS plots were gated on live cells, with 7-AAD positive dead cells excluded from the analysis. Numbers indicate percentage of cells in each quadrant. FACS plots are of individual mice representative of 3 independent experiments.

Eosinophils were identified using the cell surface marker Siglec-F [498] or CCR3 antibody (Figure 4.5). Eosinophils were not seen in the PBS treated lungs. Technical problems with the Siglec F antibody (or unavailability of alternate flurophore from the ST2 antibody) meant difficulties obtaining ST2 and Siglec F co-expression data. A population of Siglec-F^{hi} eosinophils appeared to co-express ST2 at low levels in IL-33 treated mice. Siglec-F is also expressed on alveolar macrophages[499] and so the second population of Siglec-F^{med} cells were eliminated

with negative gating for F4/80 to exclude macrophages, and the remaining cells had morphological characteristics of eosinophils on SSC analysis. Direct microscopy of the digested cell suspension, stained by Romanovsky method, shows eosinophils, macrophages, neutrophils and epithelial cells in the IL-33 treated group confirming the identity of the cells (Figure 4.5).

Small numbers of B cells ($CD19^{+}$) were identified in the lung digests but numbers were similar in both groups (1.8% vs. 2.2%) (data not shown).

These data show a range of immune cells expressing ST2 that are capable of initially responding to IL-33. Immune cells not assessed include DC, NK, iNKT, NKT and recently described ILCs. However, these cells are unlikely to be present in sufficient numbers to account for the total remaining proportion of $ST2^{+ve}$ cells seen in Figure 4.3. The other unidentified cells were larger on FSC and therefore, reasonable to assume are predominantly structural cells, which could include epithelial cells, smooth muscle cells, endothelial cells or fibroblasts. Epithelial cells in particular are well placed to encounter IL-33 initially, and as such efforts to assess epithelial and endothelial cells in the response to IL-33 are further described in chapter 6.

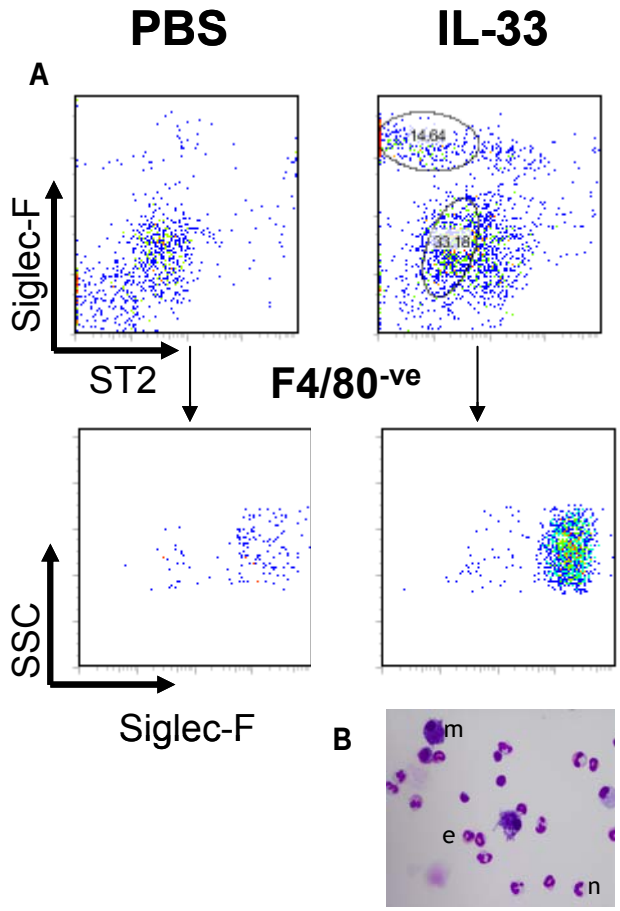


Figure 4.5: Eosinophils population in lung digest of PBS and IL-33 treated mice
A) The surface expression Siglec-F, F/480 and ST2 on single cell suspensions of whole lung digests in mice treated with intranasal PBS or IL-33 was analysed by FACS. FACS plots were gated on live cells, with 7-AAD positive dead cells excluded from the analysis. FACS plots are of individual mice representative n=3. B) Light microscopy of IL-33 treated lung digest showing macrophages (m), neutrophils (n) and eosinophils (e) stained with Romanovsky method x20 magnification.

4.2.3 Dynamics of the response to IN IL-33

Cytokines, chemokines and their networks are implicated in orchestrating the innate and adaptive immune responses that drive asthma[1]. To further define the biological response of the cytokine IL-33 and how it may relate to asthma, a set of experiments to assess the dynamics of cytokine and chemokine profiles using multiple doses of IL-33 were designed. The choice was made to perform these studies on mice on the C57BL/6 background as the subsequent aim is to use $RAG^{-/-}$ mice (C57BL/6 background), to assess the contribution of T cells to the IL-33 response. The 8 day experimental protocol detailed in Figure 4.6 shows mice were given daily dose of IL-33 for between 0 and 7 days, and samples obtained for analysis 24 hours following the final dose. These were analysed with particular interest in cell count, cytokine and chemokine profile.

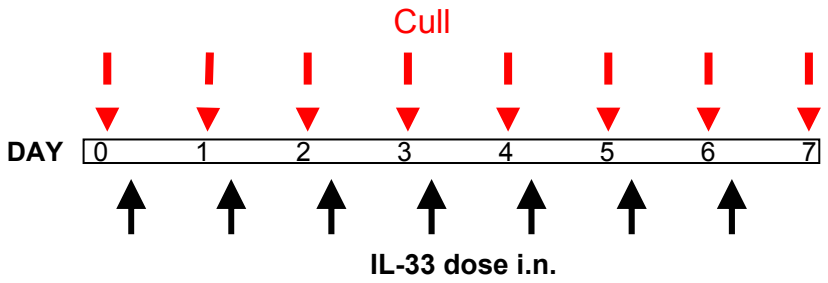
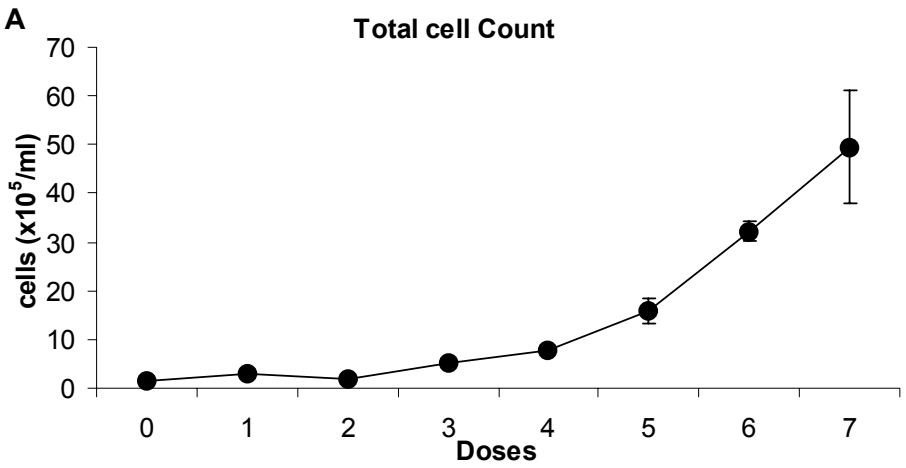


Figure 4.6: Experimental protocol to study dynamics of the response to Intranasal IL-33
C57BL/6 mice received between 0 and up to 7 once daily doses of 2 µg of recombinant murine IL-33 via the i.n. route. Mice were culled 24 hours following each dose and BAL, lung and serum samples obtained.

Total cell counts in the BAL were very low when measured following 0-2 doses. Numbers began to increase following the administration of the 3rd dose (5.16 x 10^5 cells/ml) and continued to increase following each subsequent dose to maximal level of 49.53 x 10^5 cells/ml following administration of the 7th dose (Figure 4.7A). All cell populations increased in number over the time course as shown in Figure 4.7B. Macrophages were the most dominant cell type initially and up to following dose 5. Numbers increased steadily from a mean of 1.4 x 10^5 cells/ml to a mean of 9.48 x 10^5 cells/ml following the 7th dose. Eosinophils, neutrophils and lymphocytes, were initially completely absent from the BAL, appearing in small numbers only following the 3rd dose. Eosinophil numbers then increased exponentially to become the dominant cell type in the BAL by dose 5 at 6.43 x 10^5 cells/ml (40.2%) and following 7 doses measured 31.52 x 10^5 cells/ml (59.3%)(Figure 4.7C).



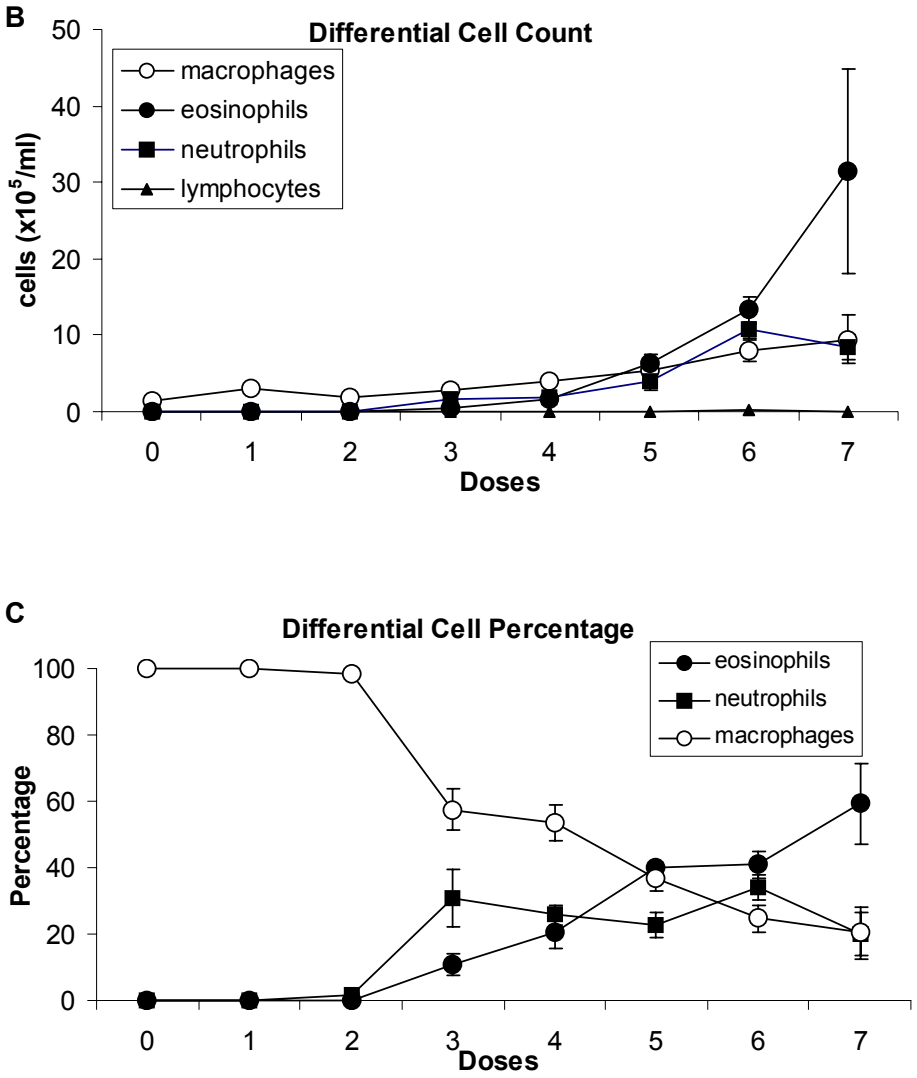


Figure 4.7: Successive doses of IL-33 lead to predominantly increasing eosinophil numbers in BAL

Mice were administered IL-33 as per Figure 4.6. A) Total BAL cell count, B) Differential cell count and C) Differential cell percentage. Results are the mean of each group of mice +/- SEM. n= 4-5. One way ANOVA for the effect of repeated IL-33 dosing on macrophages $p<0.001$, eosinophils $p<0.001$ and lymphocytes $p<0.001$ in B).

In order to ascertain if the histological characteristics of this time course were comparable to airway inflammation, the lungs were collected and cellular infiltration assessed on H&E staining on paraffin embedded sections. Compared to control PBS treated mice, IL-33 treated lungs showed a clear predominantly eosinophilic infiltration in the peri-bronchial and peri-vascular regions. This was first noted by dose 2 where a few eosinophils are seen, preceding the first appearance in the BAL by 1 dose/24 hours. By dose 3, when eosinophils are appearing the BAL, severe histological eosinophilia has occurred with maximal infiltration (by scoring technique) by dose 6. Increasing architecture distortion with epithelial hyperplasia and shedding occurs following dose 3 (Figure 4.8).

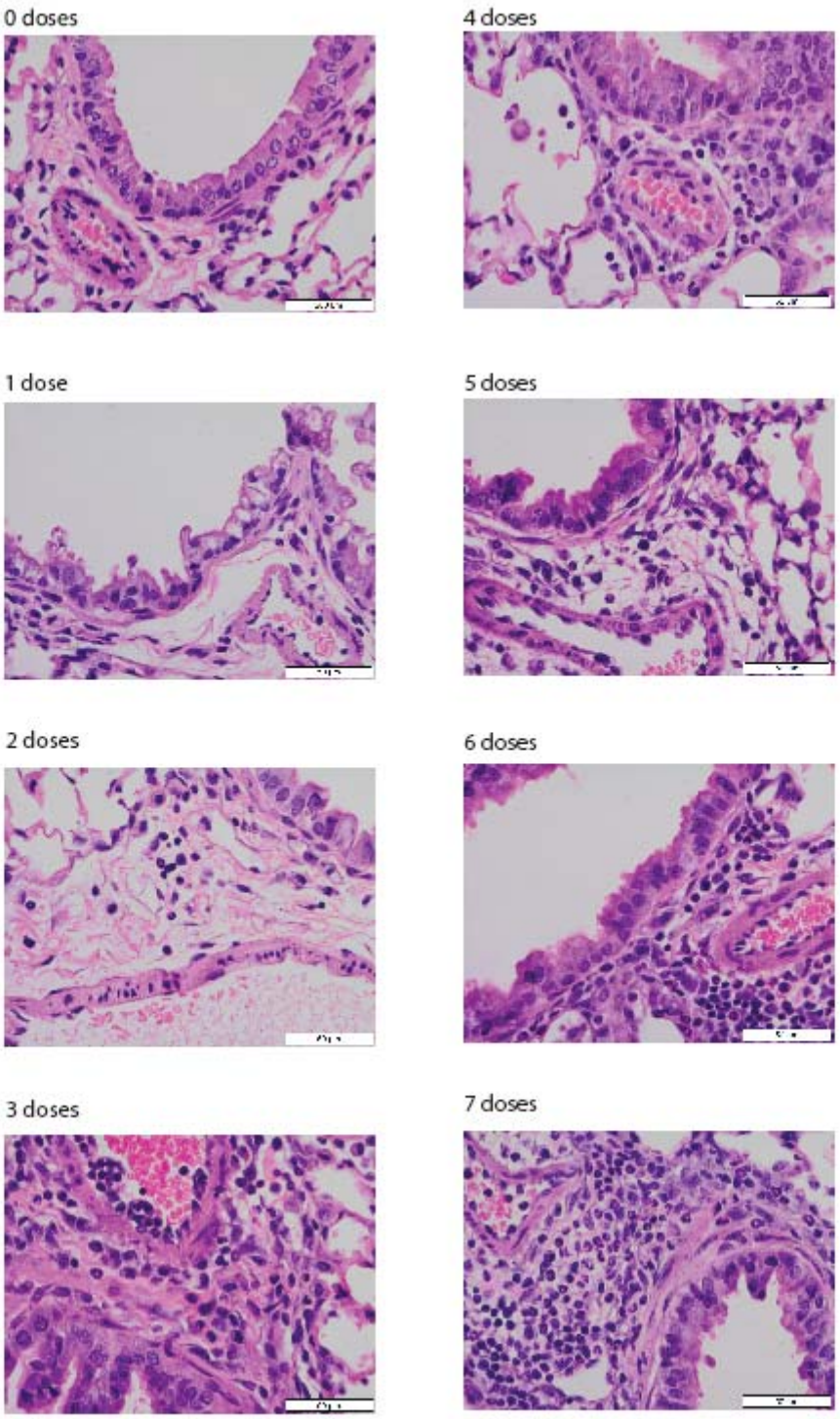
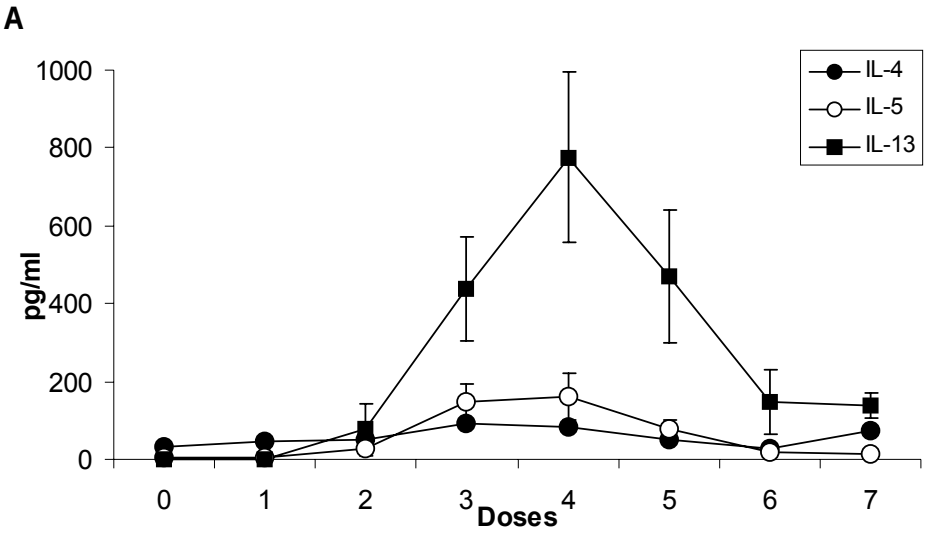


Figure 4.8: Intranasal IL-33 induces eosinophilic inflammation in the lung
Lungs were administered IL-33 as per Figure 4.6. Lungs were fixed in formalin and stained with H&E. Sections were scored according to previously described parameters. Zero dose=score 0, 1 dose=score 0, 2 doses=score 1, 3 doses=score 2, 4 doses=score 2, 5 doses=2, 6 doses=score 3, 7 doses=score 3. Panels shown are representative sections following each dose of IL-33. Scale bars represent 50μm.

A wide range of cytokines and chemokines were detected in the BAL. IL-13 was the cytokine detected at the highest concentration. There were no or very low levels of IL-13 present after doses 0-2. It appeared in significant amounts following 3 doses and peaked following 4 doses at (777 pg/ml); thereafter levels fell with further dosing (Figure 4.9A). Other Th2 cytokines, IL-5 and IL-4 followed a similar pattern with peak levels following dose 4 and dose 3 respectively (Figure 4.9A). Levels of IL-12 and IP-10 also rose to peak levels on day 4 and 3 respectively (Figure 4.9B and C). Concentrations of IL-2, KC and MCP-1 rose a small but significant amount, peaking at dose 3 (Figure 4.9B and C).

Alternative patterns of mediator production in response to IL-33 were also demonstrated. Interleukin-17 levels rose from zero to a peak of 205 pg/ml following 5 doses (Figure 4.9B) and VEGF showed a rapid response by peaking on day 1 with just one dose of IL-33 then levels declined with repeated IL-33 dosing (Figure 4.9C). Eotaxin/CCL11 and eotaxin-2/CCL24 levels rose sharply following 3 doses to a peak concentration of 281 pg/ml at day 4 and 820 pg/ml at dose 3 respectively (Figure 4.9D). Levels however remained raised with repeated dosing suggesting a role in the continued eosinophil accumulation (Figure 4.7B). Concentrations of MIP1α, GMCSF, IL-6, IFNγ showed trends of a pattern similar to IL-13 but levels were low (<50 pg/ml). Levels of FGF, IL-1α, TSLP, IL-10, IL1-β, RANTES (CCL-5) and MIG were zero or low (data not shown).



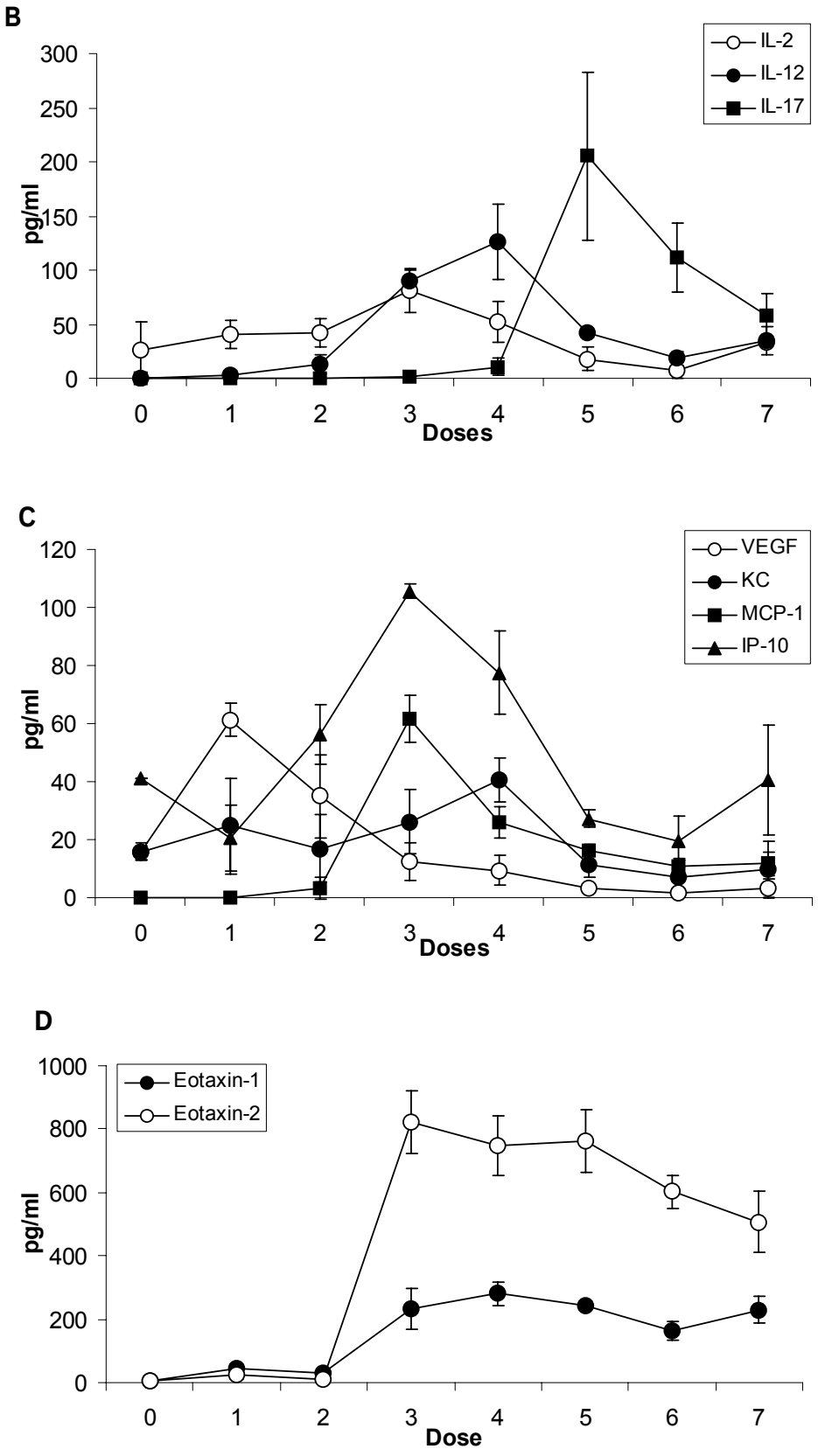


Figure 4.9: BAL cytokine and chemokine profile during repeated intranasal IL-33 administration

IL-33 was administered as per the protocol in Figure 4.6. Analysis was performed by multiplex immunoassay or ELISA. Results shown are group mean, n=4-5. One way ANOVA for the effect of repeated IL-33 dosing on BAL IL-4 $p<0.01$, IL-5 $p<0.01$, IL-13 $p<0.01$, IL-2 $p<0.05$, IL-12 $p<0.001$, IL-17 $p<0.01$, VEGF $p<0.0001$, KC $p<0.01$, MCP-1 $p< 0.01$, IP-10 $p<0.001$, Eotaxin-1 $p<0.0001$ and Eotaxin-2 $p<0.0001$.

Within the serum, the most notable cytokine profile was that of IL-13; the concentration of this began to rise following dose 3, and then steadily increased to a peak following 7 doses at 614 pg/ml (Figure 4.10A). Concentrations of IL-5 in the serum mirrored that of the BAL peaking following the 3rd dose (Figure 4.10B). IL-4 levels were low and did not significantly alter throughout (Figure 4.10B). Other results include: IP-10 which rose following 7 doses; and FGF whose levels were elevated throughout but with no discernable pattern (Figure 4.10C). Levels of IL-2, IL-10, IL-12, IFNγ, IL-1β, IL-6, GMCSF, IL-17, KC, VEGF, IL-1α, MCP-1, MIP1α, RANTES and MIG were below the limit of detection for the assay or zero.

These data show that repeated serial intranasal dosing of IL-33 results in eosinophilic inflammation within the BAL after 3 doses and is associated with release of multiple inflammatory mediators, in particular Th2 cytokines and eotaxins.

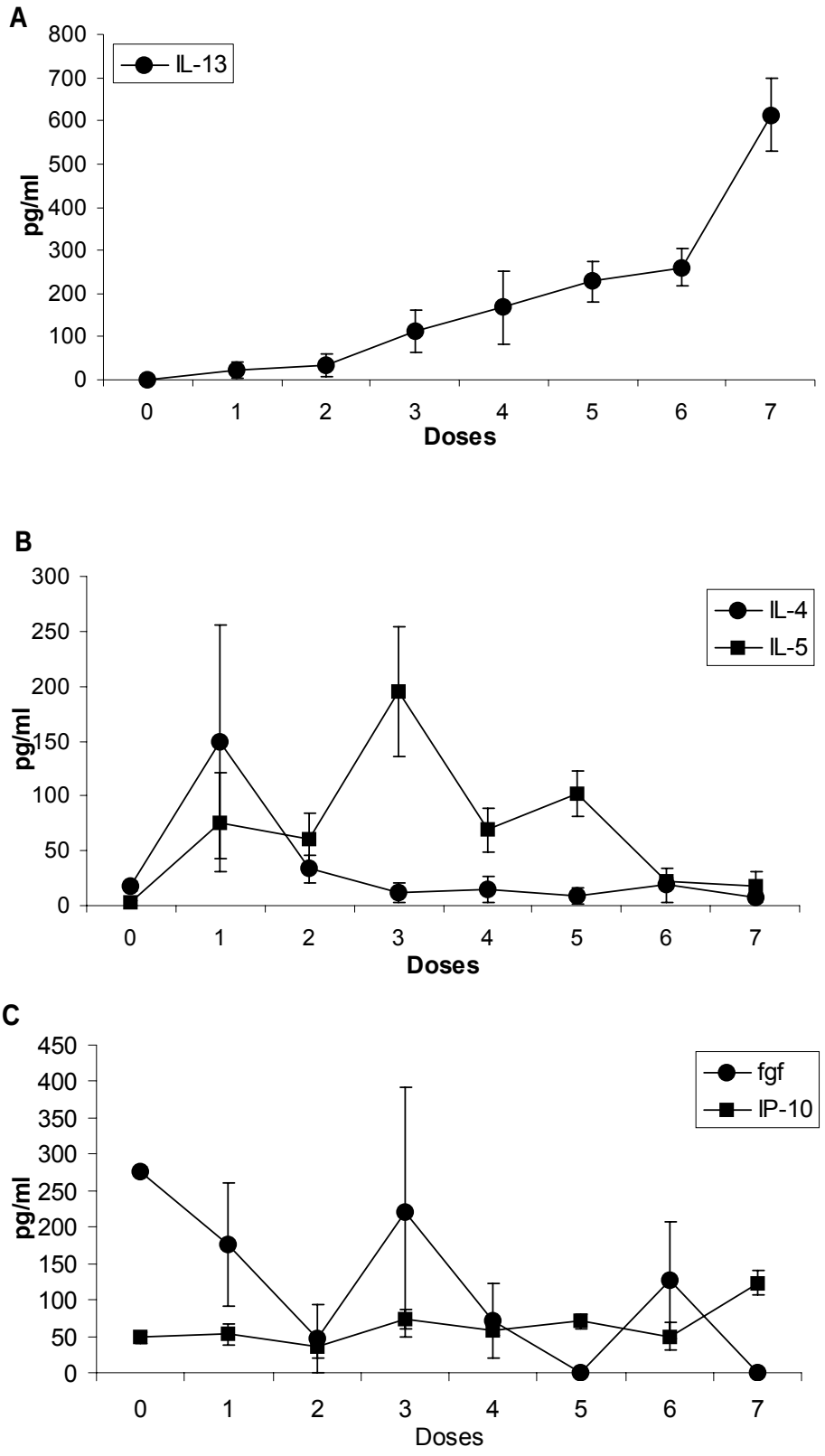


Figure 4.10: Serum cytokine and chemokine profile during repeated intranasal IL-33 administration
IL-33 was administered as per protocol in Figure 4.6. Cytokine and chemokine analysis was performed by multiplex immunoassay or ELISA. A) IL-13. B) IL-4 and IL-5. C) FGF and IP-10. Results shown are group mean, n=4-5. One way ANOVA for the effect of repeated IL-33 dosing on BAL IL-13 $p<0.01$, IL-4 p=ns, IL-5 $p<0.05$, FGF p=ns, IP-10 $p<0.05$.

4.2.4 Dynamics following intranasal IL-33 administration

Having shown inflammation following administration of 7 days of IL-33 in BALB/c and C57BL/6 mice, the aim was to further assess the response to IL-33, in particular the contribution of the adaptive immune response. It was important to refine the experimental protocol whilst minimising animal requirements, excessive inflammation, experimental duration and reagent use.

BAL cellular inflammation continues to escalate with repeated IL-33 dosing and after 4 doses of IL-33; inflammation is established in C57BL/6 mice. In the experimental protocol in Figure 4.6, animal sacrifice and sample retrieval was performed 24 hours following the final dose in each group, which is standard protocol in airway challenge models [500]. It is not clear whether inflammation will continue without ongoing ST2 receptor stimulus over time. Therefore an experiment to study changes in the BAL and serum following cessation of IL-33 application choosing time points over 8 days following the final dose was designed. A total of 4 doses were chosen, reflective of a moderate degree of inflammation comparable to that seen in asthma models(Figure 4.11) [282].

Results are shown in Figure 4.12. Following the administration of 4 daily doses of IL-33, total cell counts in the BAL continued to rise before peaking at 4 days after the final dose at 35.8 $x10^5$ cells/ml before gradually declining over time. Eosinophil counts followed a similar pattern, peaking at day 4 post dose at 24.5 x 10^5 cells/ml. Macrophages numbers peaked earlier at day 2 measuring 7.73 x 10^5 cells/ml. Despite this, the rising eosinophil numbers replaced macrophages as the predominant cell type in the BAL at day 2. Eosinophils remained the dominant cell type until the final recorded sample at day 8 where eosinophils and macrophages each comprised more than 49% of the population. This indicates that even 8 days following a series of IL-33 doses there is still significant eosinophilic inflammation.

Within the BAL, unsurprisingly elevated levels of most cytokines significantly fell away in the days following the final dose. IL-13, IL-5 and IL-4 peaked on day 1 (Figure 4.13A) along with IL-2, IL-12 and KC (data not shown). IL-10, IFN-γ and IL-17 levels peaked on day 2 (Figure 4.13B). Eotaxin/CCL11 and eotaxin-2/CCL24 levels reduced much more slowly over time and remained significantly elevated

for several days (Figure 4.13C). IL-1β, IL-6, GMCSF, TNF-α, FGF, IL-1α, MCP-1, MIP1α, RANTES IP-10 and MIG were low (<50 pg/ml) or zero (data not shown).

IL-13 was the predominant cytokine in the serum. Levels of IL-13 and also IL-5 peaked one day following the last administration of IL-33 before gradually reducing (Figure 4.14A). IL-4 concentrations were low. Concentrations of both chemokines KC and IP-10 appeared to increase up to 8 days post final dose although overall levels of these remained low (Figure 4.14B). IL-6, IL-12 and TNF-α levels were unchanged and those of IL-2, IL-10, IFN-γ, IL-1β, GMCSF, IL-17, VEGF, FGF, IL-1α, MCP-1, MIP1α, RANTES and MIG were low (<50 pg/ml) or zero (data not shown).

These data indicate IL-33 driven inflammation continues to accumulate following removal of receptor stimulation.

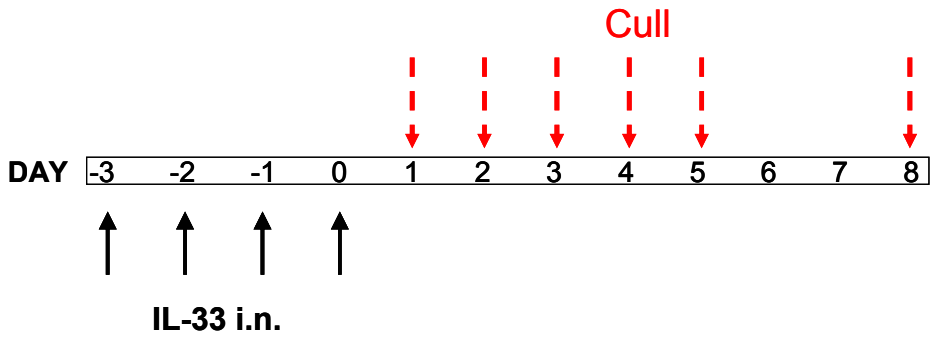


Figure 4.11: Experimental protocol to study dynamics following Intranasal IL-33 administration
C57BL/6 mice received between 4 once daily doses of 2 µg of IL-33 via the intranasal route. Mice were culled between 1 and 8 days following the delivery of the final dose and BAL and serum samples obtained.

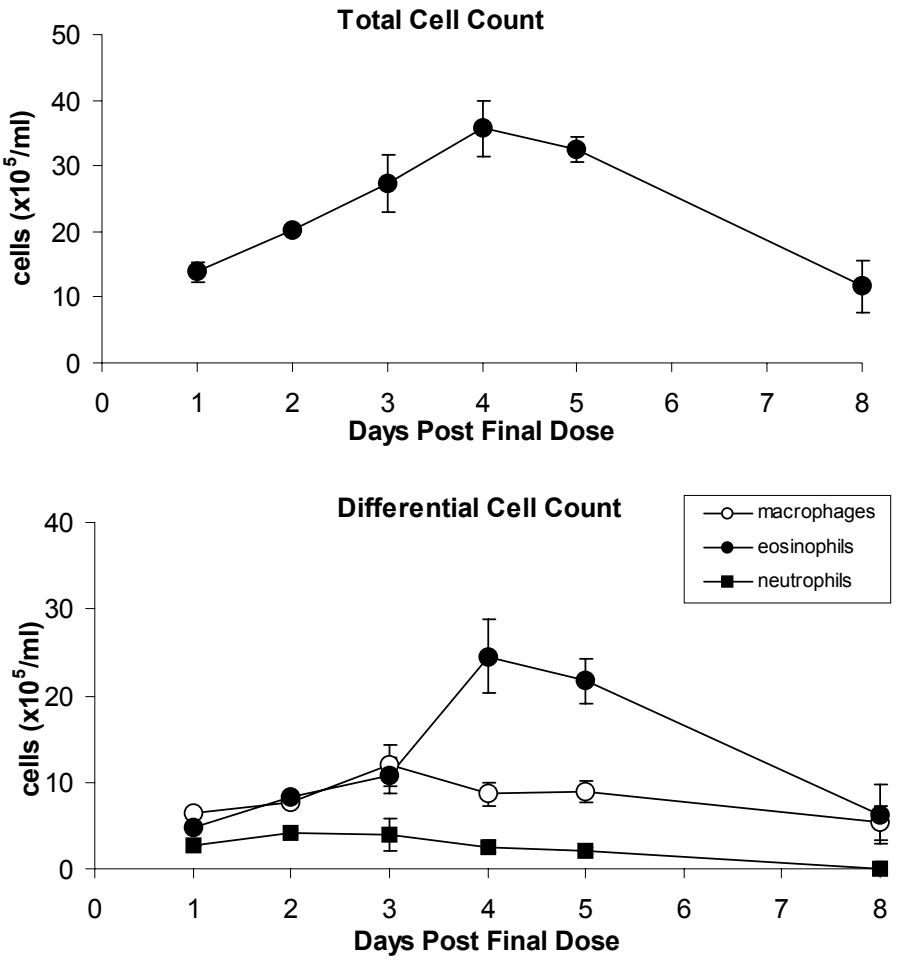


Figure 4.12: BAL eosinophils peak 4 days following 4 daily intranasal IL-33
Mice were administered IL-33 as per Figure 4.11 and BAL cellular composition measured A) Total BAL cell count and B) Differential cell count. Results are the mean of each group of mice ± SEM. n= 4-5. One way ANOVA for the effect of IL-33 on BAL cellular composition over time: total cell count $p<0.0001$, macrophages p=ns, eosinophils $p< 0.0001$ and neutrophils p=ns. ns= not significant.

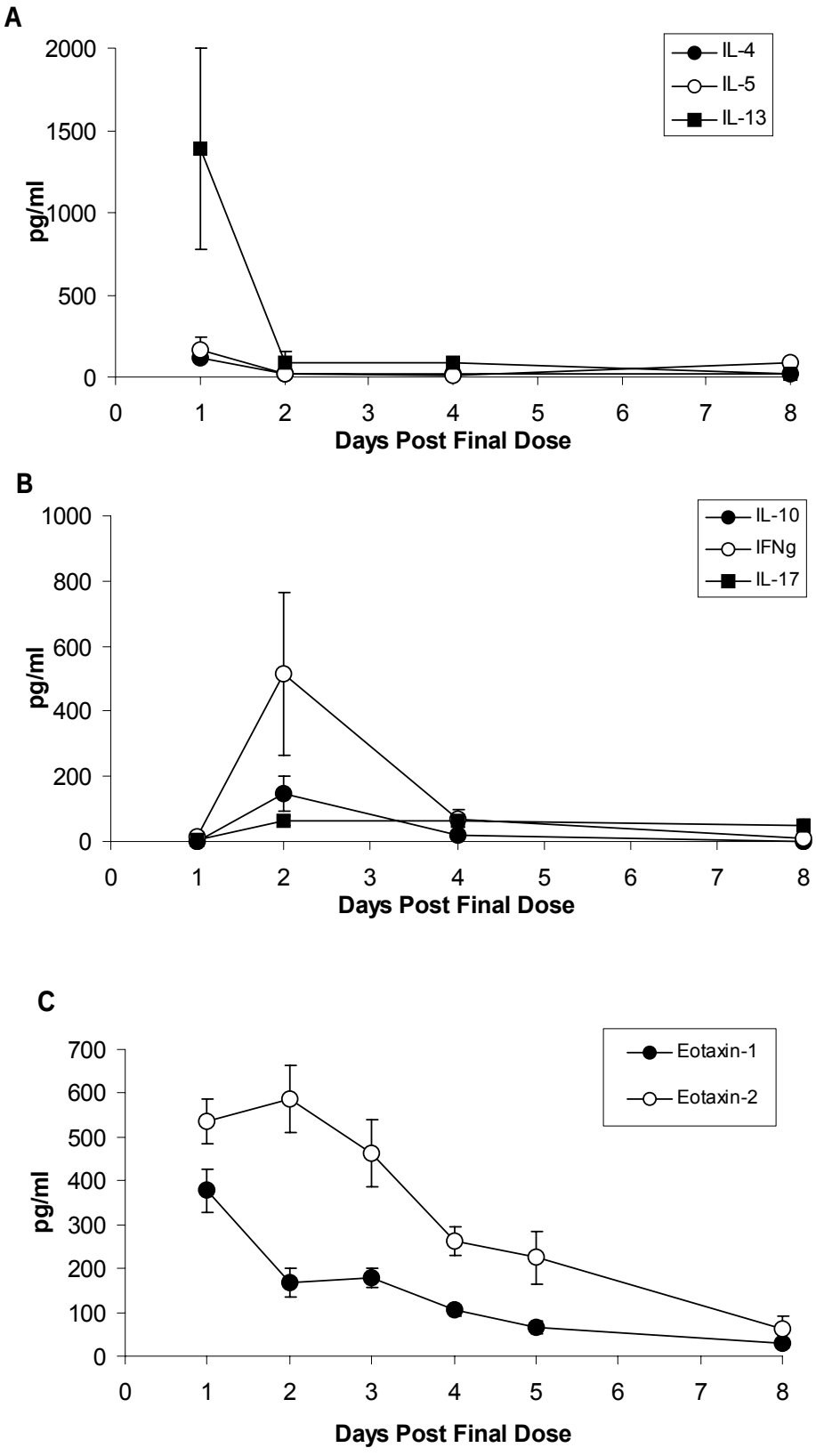


Figure 4.13: BAL cytokine and chemokine profile following intranasal IL-33
IL-33 was administered as per the protocol in Figure 4.11. Analysis was performed by multiplex immunoassay or ELISA. A) BAL IL-4, IL-5 and IL-13. B) BAL IL-10, IFNγ and IL-17. C) BAL eotaxin/CCL11 and eotaxin-2/CCL24. Results shown are group mean, n=4-5. One way ANOVA for the effect following IL-33 dosing on BAL IL-4 $p<0.01$, IL-5 $p=0.01$, IL-13 $p=0.01$, IL-10 $p<0.01$, IFNγ $p=0.05$, IL-17 $p<0.05$, Eotaxin-1 $p<0.0001$ and Eotaxin-2 $p<0.0001$.

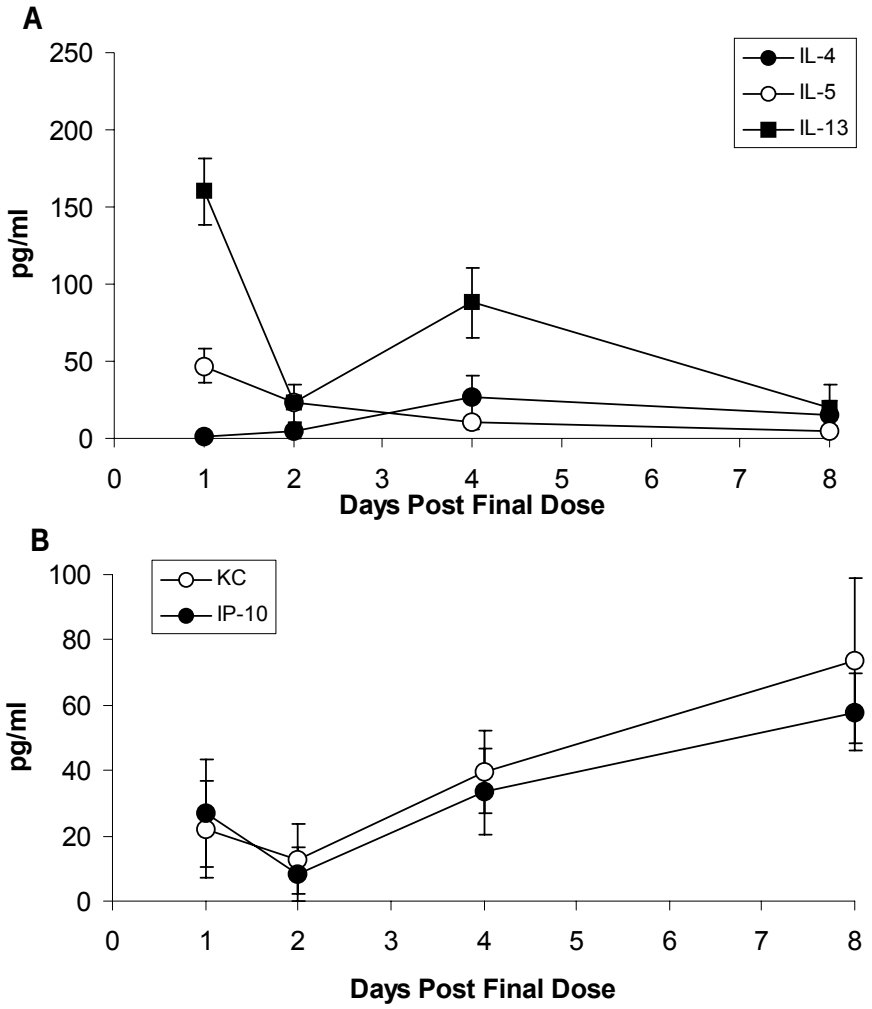


Figure 4.14: Serum cytokine and chemokine profile following intranasal IL-33
IL-33 was administered as per the protocol in Figure 4.11. Analysis was performed by multiplex immunoassay or ELISA. A) Serum IL-4, IL-5 and IL-13. B) Serum KC and IP-10. Results shown are group mean, n=4-5. One way ANOVA for the effect following IL-33 dosing on serum IL-4 p=ns, IL-5 $p<0.01$, IL-13 $p<0.001$, KC $p<0.05$ and IP-10 $p<0.05$.

4.2.5 Protocol for IL-33 induced inflammation in C57BL/6 mice

Using the data detailed in the experiments above, an experimental protocol to use in C57BL/6 mice to accommodate the analytes of interest was chosen. As BAL inflammation continues to increase up to 4 days following IL-33 dosing, a time point of 3 days was chosen to reflect the mid-point in the rise of eosinophilic inflammation; this would allow sufficient inflammation to study the influence of inhibitors, but not at peak levels which may be affected by exacerbating parameters. Furthermore, as 3 days post dose as the eosinophilic inflammation was still high at 27.4×10^5 cells/ml, the number of challenges was

reduced from 4 to 3 aiming for inflammation levels in keeping with current asthma protocols. The protocol is shown is Figure 4.15A. As expected, a predominant moderate eosinophilic inflammation was induced in the BAL as shown in Figure 4.15B.

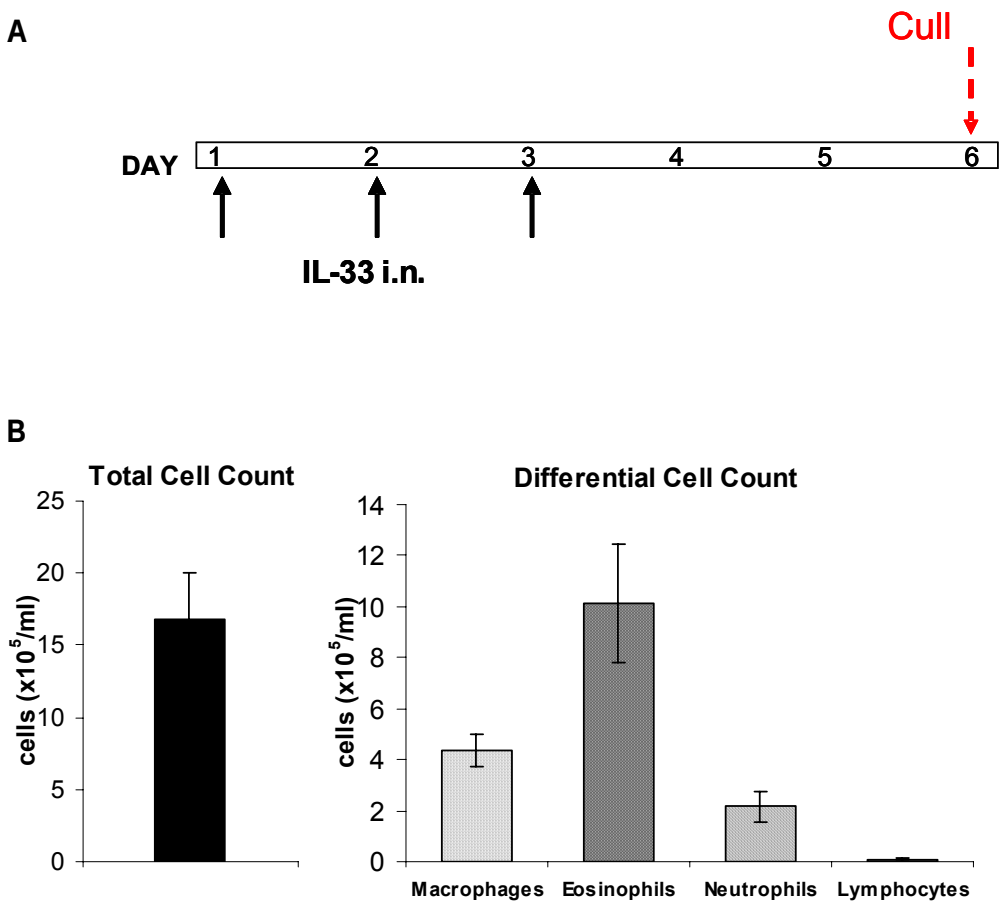


Figure 4.15: Final experimental protocol for intranasal IL-33 in C57BL/6 mice

A) 2 µg of recombinant murine IL-33 was administered daily for 3 days and mice culled following a further 3 days on day 6. B) Total and differential cell counts in the BAL were performed. Results are mean ±SEM. n= 5.

4.2.6 The response to intranasal IL-33 activity is dependent on ST2 expression

IL-33 binds to ST2 and forms a receptor complex with IL-1RAP [293,416]; with the crystal structure of IL-33/ST2 binding recently solved [501]. This complex activates a range of signalling proteins including NF-κB [293]. To confirm the effects of i.n. IL-33 that are described above are dependent on ST2 expression, the experimental protocol in Figure 4.15A was repeated in $ST2^{-/-}$ mice. The

available ST2$^{-/-}$ mice in the laboratory were on a BALB/c mouse strain genetic background so this protocol was also performed in WT BALB/c mice as a control. As expected, IL-33 generated an increase in total cell count in WT but not in ST2$^{-/-}$ mice (Figure 4.16A). In particular, no eosinophils were found in the knockout group (Figure 4.16B). Of interest, the BALB/c mice generated a more modest level of inflammation in the BAL using this protocol compared to the C57BL/6 mice, indicating a strain difference in the responses to IL-33 (Figure 4.16 A and B).

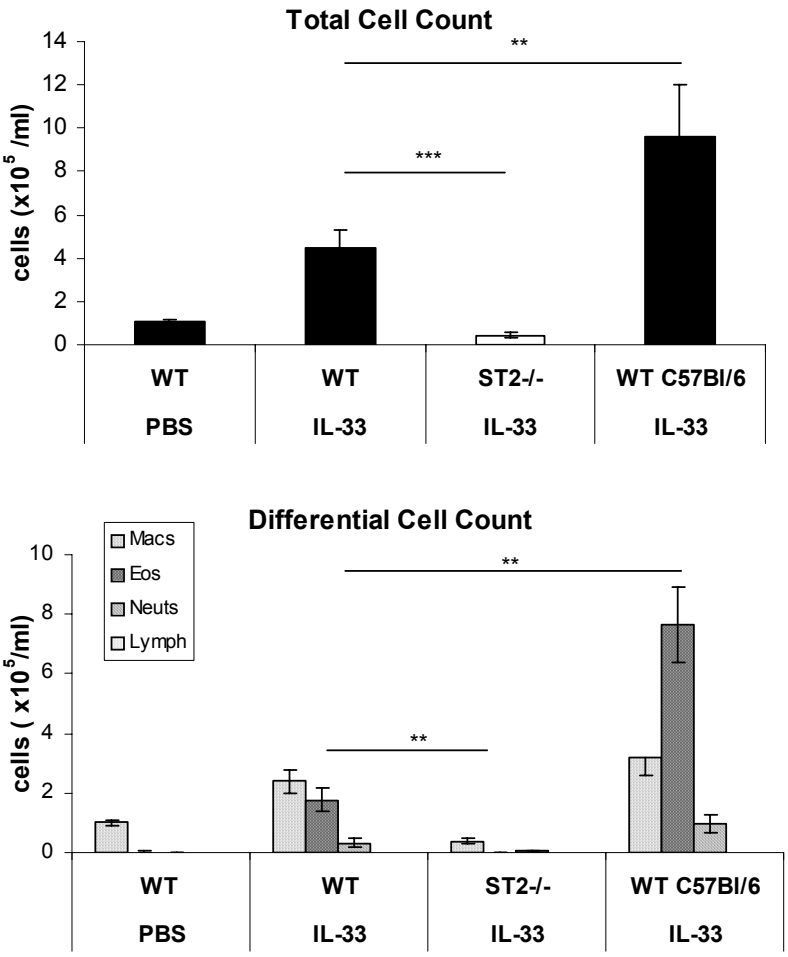


Figure 4.16: IL-33-induced BAL inflammation is ST2 specific

WT (BALB/c) , ST2-/- (BALB/c) or WT (C57BL/6) mice were given 2 µg of recombinant murine IL-33 or PBS as per protocol in Figure 4.15. A) Total and B) differential cell counts were performed in the BAL. Results shown are mean ± SEM. n=5. **p<0.01, ***p<0.001.

4.3 The adaptive immune system is not required for intranasal IL-33 induced inflammation

Asthma in humans and AAI in mice has long been seen as predominantly Th2-mediated inflammation [20-22,24]. In response to i.n. IL-33, a predominantly Th2-type inflammatory profile of eosinophilia and inflammatory mediators is seen in the airway and lungs *in vivo*. Furthermore, an increased population of $CD4^+$ $ST2^+$ Th2 cells in lung has been demonstrated (Figure 4.4). Despite an absence of antigen priming in this particular model, this suggests that Th2 cells could be involved; either being directly activated by IL-33 or indirectly by the cytokine cascade generated in response to IL-33. To further consider the role of Th2 cells, mice deficient in the adaptive immune system can be utilised. Pilot experiments in SCID mice, which lack T and B lymphocytes due to a defect in VDJ recombination of antigen receptors [502], demonstrated a trend of reduced inflammatory profile in comparison to WT BALB/c mice (data not shown). Some SCID mice are considered "leaky" (in particular those on BALB/c and C57BL/6 backgrounds) [503](www.jax.org/strain/001803 or 001913) and therefore not completely deficient in all lymphocyte subsets [504]. Therefore it was decided to perform the attenuated experimental protocol above (Figure 4.15A) in $RAG1^{-/-}$ mice on a C57BL/6 background which are completely deficient in T, B and NKT cells [505,506].

When IL-33 was administered intranasally to C57BL/6 and $RAG^{-/-}$ mice, the complete deficiency of the adaptive immune system did not have any influence on the ability to mount a robust inflammatory response to IL-33. In fact, there was a suggestion that the cellular inflammation was worse in the BAL and lungs of the $RAG^{-/-}$ mice, but this did not reach statistical significance in all experiments. Figure 4.17 shows similar total cell counts in both groups and a trend towards increased proportion of eosinophils in $RAG^{-/-}$. The cytokine response within the BAL was lower in the $RAG^{-/-}$ group when compared to the WT group suggesting that CD4 Th2 cells may contribute to cytokine production (Figure 4.17B). The levels of analytes were generally low in the serum and no differences between the groups were identified (data not shown).

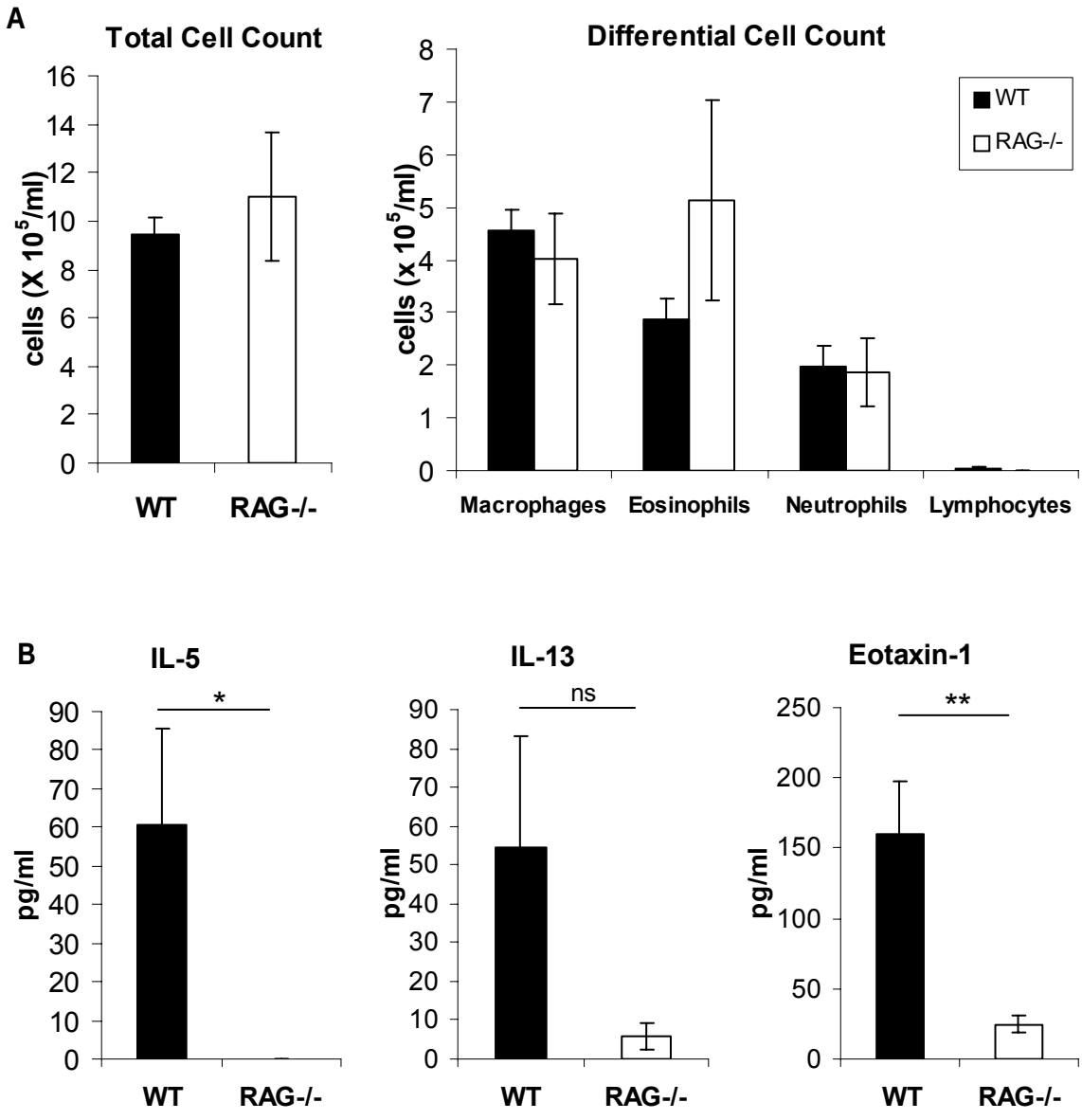


Figure 4.17: The adaptive immune system is not necessary for the generation of airway inflammation in response to intranasal IL-33
Intranasal IL-33 was administered to wildtype C57BL/6 or RAG$^{-/-}$ mice as per Figure 4.15A. A) Total and differential cell counts and B) Cytokine and chemokine analysis was performed on the BAL. Results shown are the mean of each group of mice ± SEM. n=8. Results are representative of several experiments.

Figure 4.18 shows a trend towards worse parenchymal eosinophilic inflammation in the RAG$^{-/-}$ mice.

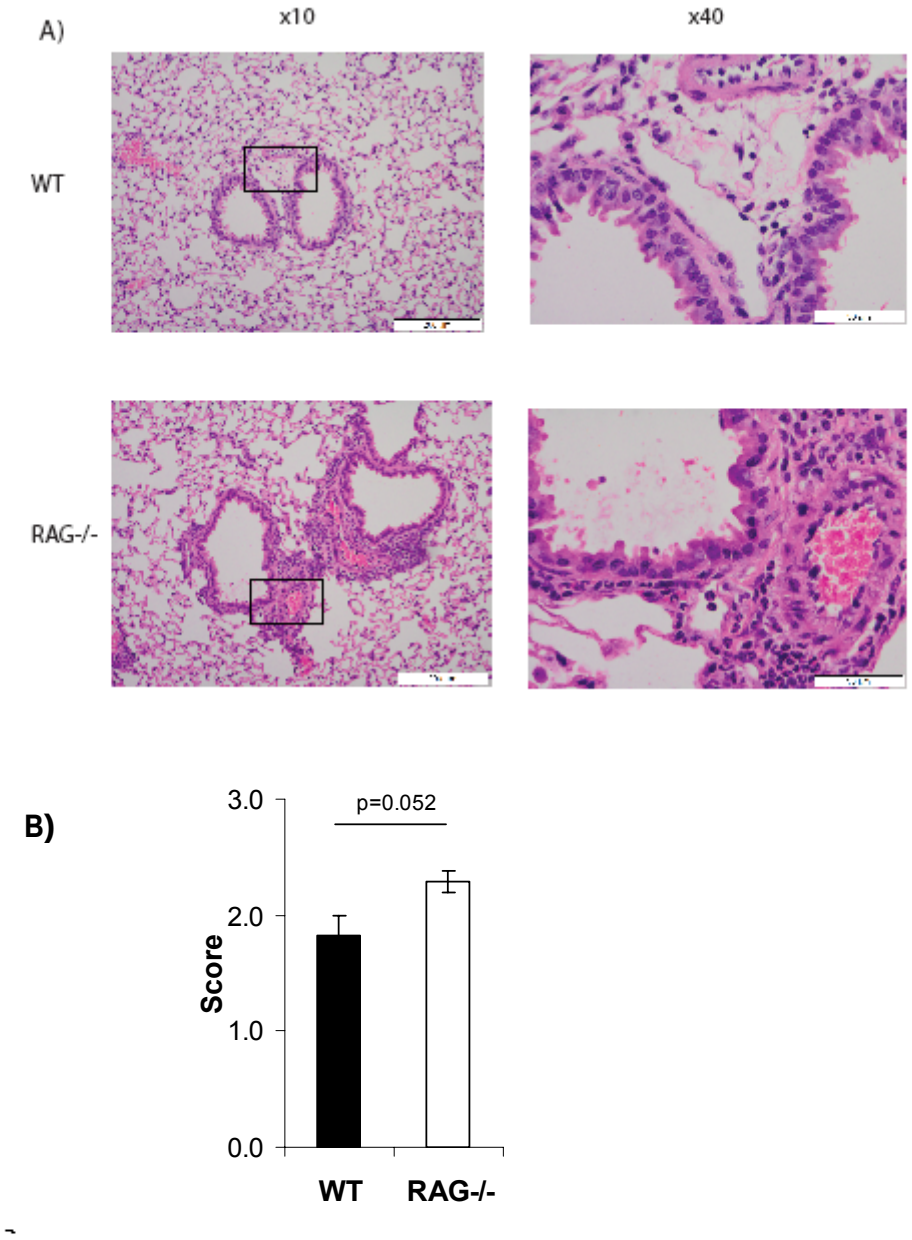


Figure 4.18: The adaptive immune system is not necessary for the generation of lung inflammation in response to intranasal IL-33.
Intranasal IL-33 was administered to wildtype C57BL/6 or RAG$^{-/-}$ mice as per Figure 4.15A. Lungs were fixed in formalin and stained with H&E for eosinophil assessment. A) Representative sections shown at x10 and x40 magnification. B) Eosinophil score assessed as previously described. n=5 per group. t-test p value shown.

In summary, these data show that IL-33 causes a Th2-innate response for which the Th2 cells and adaptive immune system are not an essential component. Some data further indicates the cellular inflammation could be worse in mice with no effective adaptive immune system.

4.4 IL-4 is not required for the response to intranasal IL-33 in mice

Cytokines, in particular IL-4, IL-5 and IL-13, are key in driving Th2-mediated inflammation in asthma and AAI [44,54,68]. When IL-4 or IL-13 is given alone intranasally to mice, they can replicate some of the features of asthma [45,54]. Th2 cells are important sources of these cytokines but other sources include NKT cells, eosinophils, MC and ILCs. Results described in this thesis similarly show IL-33 alone drives innate-type pathology with features of asthma. Furthermore, it is clear that inflammation is not exerted by a direct action of Th2 cells, although they may contribute to cytokine production indirectly. In considering the cytokine hierarchy, analysis of the time course above (Figure 4.9), shows IL-33 drives IL-5 and IL-13 release but limited IL-4, suggesting this latter cytokine is not involved in IL-33 directed effector pathology. However, IL-4 protein can be difficult to measure in BAL and levels are often low [35]. To further elucidate the role of IL-4 and ascertain if is redundant in IL-33 mediated inflammation, experiments were performed in IL-$4^{-/-}$mice. For consistency the protocol in Figure 4.15A was followed; however, the IL-$4^{-/-}$mice are on a BALB/c background.

When IL-33 was administered intranasally to WT and IL-$4^{-/-}$ BALB/c mice, the absence of IL-4 had no impact on the total or eosinophil cell count in the bronchoalveolar lavage (Figure 4.19 A and B) or lung (Figure 4.19 C and D). With regard to cytokines and chemokines in the BAL and serum, no differences were identified between the groups. No IL-4 was measured in any compartment in the IL-$4^{-/-}$ mice (data not shown).

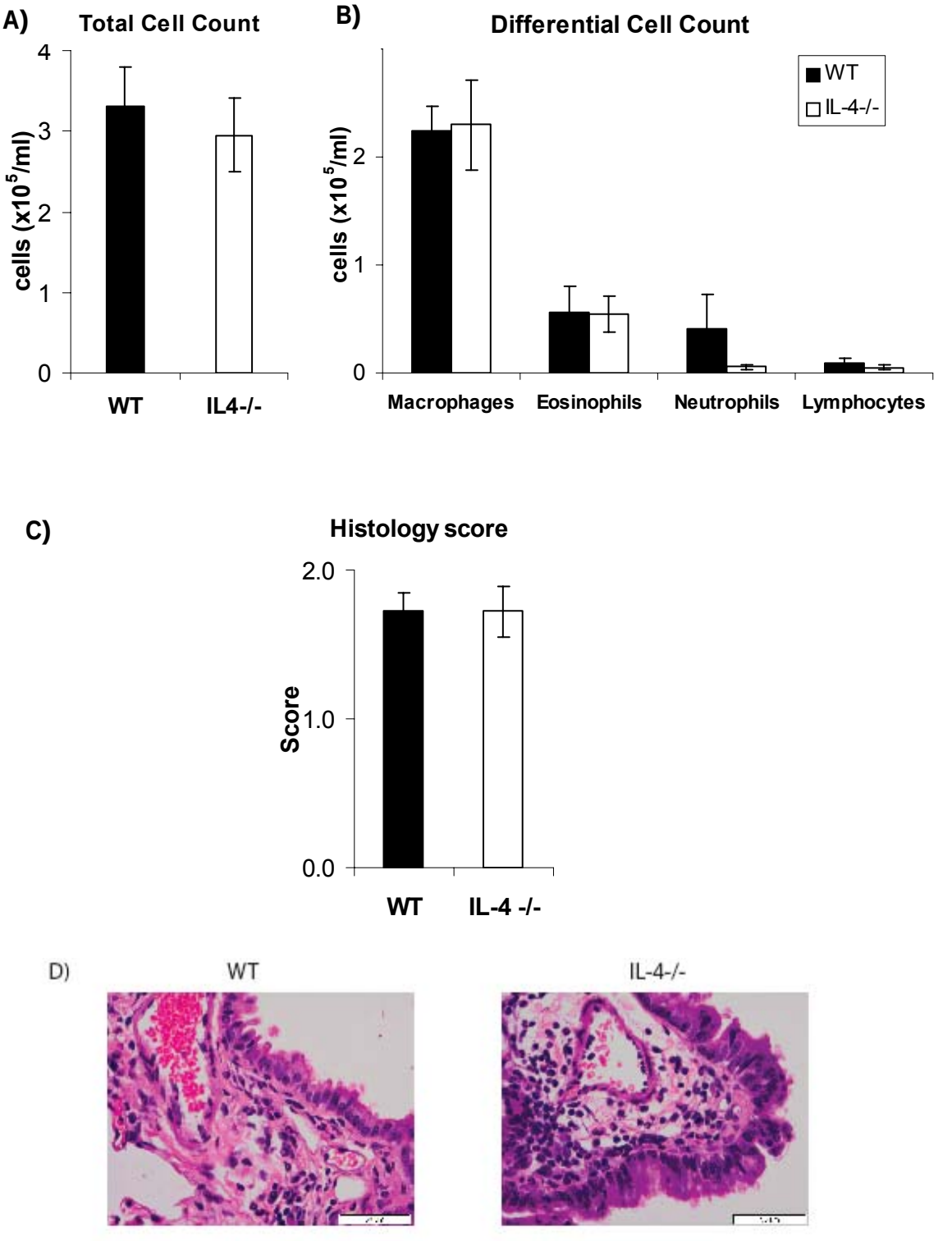


Figure 4.19: IL-4 is not necessary for the generation of IL-33 induced airway and lung inflammation
Intranasal IL-33 was administered to wildtype BALB/c or IL-4$^{-/-}$ mice as per Figure 4.15A. A) Total and B) differential cell counts were performed on the BAL, C) Eosinophil score assessed as previously described on lungs fixed in formalin and stained with H&E. D) Representative histology sections x40 magnification. Results shown are the mean of each group of mice +/- SEM. n=5-7 per group. Data are representative of 3 experiments.

This confirms IL-4 is not critical for the generation of responses to IL-33. Duplicate experiments in IL-5 and IL-13 mice may yield further information in cytokine hierarchy.

4.5 IL-33 mediated eosinophilic inflammation is partially eotaxin dependent

From experiments detailed above (Figures 4.9 and 4.13), a number of secondary mediators were produced in particular IL-5, IL-13, eotaxin/CCL11 and eotaxin-2/CCL24 and mediate IL-33-induced inflammation. Further understanding of the role of these secondary mediators and the identification of cell types releasing them is warranted. The development of BAL eosinophilia coincided with the appearance of BAL eotaxin/CCL11 and eotaxin-2/CCL24 and the strikingly sustained release of these eotaxins resulted in continued eosinophil recruitment for many days following IL-33 administration. To further investigate the role of eotaxins, the use of CCL11 and CCL24 neutralising antibodies in the experimental protocol of IL-33-induced airways inflammation was chosen (Figure 4.15A). These antibodies were chosen following pilot studies testing siRNA and a range of neutralising antibodies. As described in Figure 4.12, inflammation continues to rise following IL-33 exposure and eotaxin levels remain elevated (Figure 4.13), therefore antibody was administered for a further 3 days as shown in Figure 4.20A.

Co-administration of anti-eotaxin antibodies resulted in a reduction in IL-33-mediated airway and tissue inflammation when compared to control mice treated with control IgG. Specifically, anti-CCL11 caused a significant reduction in the total cell count by reducing both the eosinophil as well as macrophage cell count by 90% and 50% respectively (Figure 4.20 B-D). Anti-CCL24 administration had no significant effect on the total or macrophage cell count but resulted in an 80% reduction in the number of eosinophils in the BAL indicating some functional redundancy between the eotaxins (Figure 4.20 B-D). Cytokine analysis revealed reduced levels of BAL IL-5 in mice receiving either neutralising antibody (Figure 4.20E). No changes were seen in serum IL-5 levels and levels of IL-4 and IL-13 in BAL or serum were very low or undetectable (data not shown). Overall the levels of cytokines were low in this model compared to

previous experiments in keeping with a proportionately less intense inflammatory response. This protocol was developed in the C57BL/6 strain rather than BALB/c mice used here, which although Th2 biased, do not to develop as severe inflammation with the same dose of IL-33 as described in Figure 4.15 above.

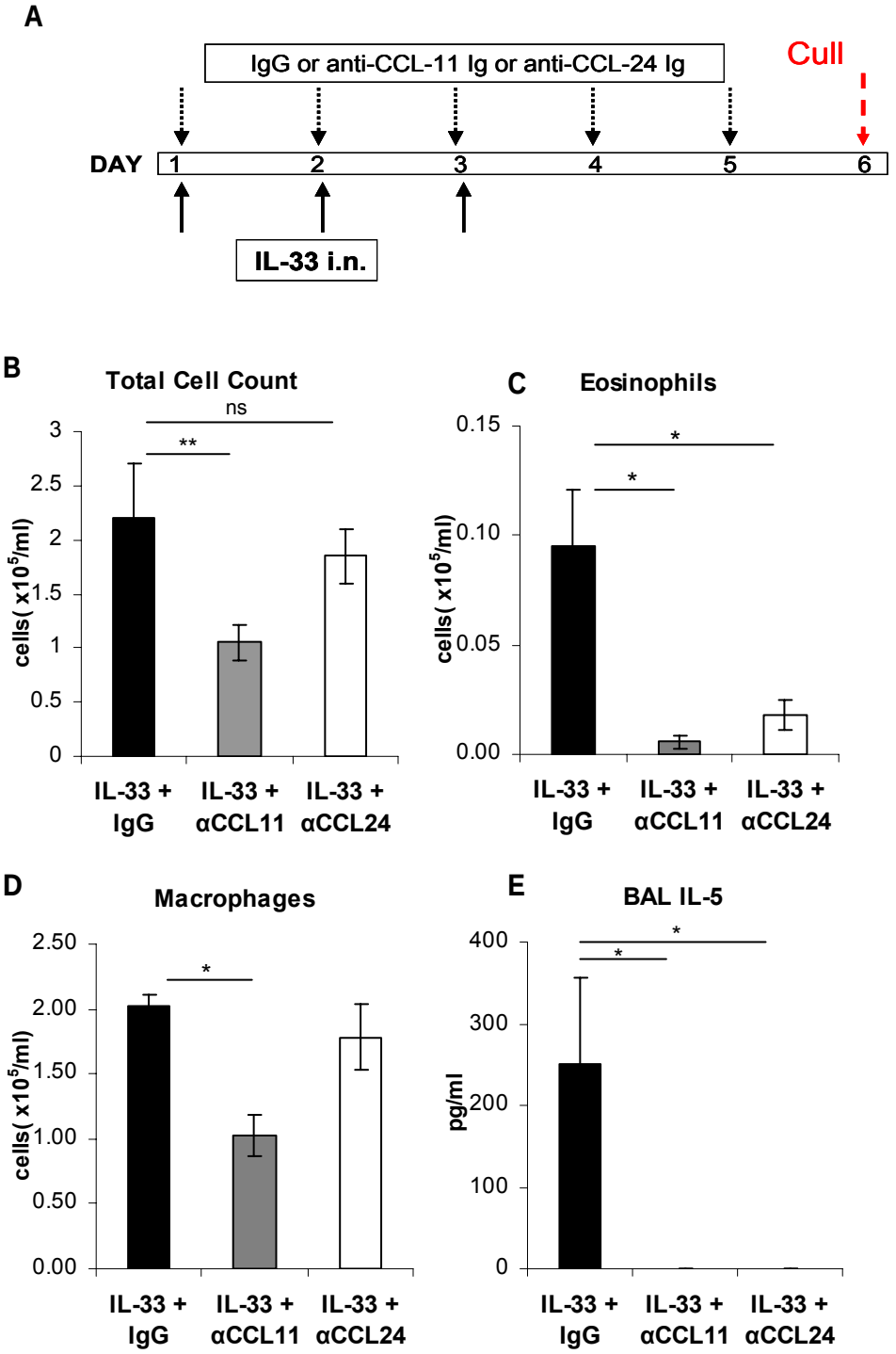


Figure 4.20: CCL11 and CCL24 mediate IL-33 induced airway inflammation
BALB/c mice received 4µg hIL-33 for 3 days. Mice additionally received neutralising anti-CCL11, anti-CCL24 or control antibody intranasally for 5 consecutive days (A). Total B) and differential cell counts (C-D) were performed on the BAL. IL-5 was measured in the BAL by ELISA (E). Results

shown are the mean of each group of mice ±SEM. n=5 per group. Data are representative of 2 experiments. ns p= not significant, *p<0.05, **p<0.01 of IgG versus antibody treated mice.

Anti-CCL24 reduced and anti-CCL11 reduced tissue eosinophilia, the latter not reaching statistical significance (Figure 4.21).

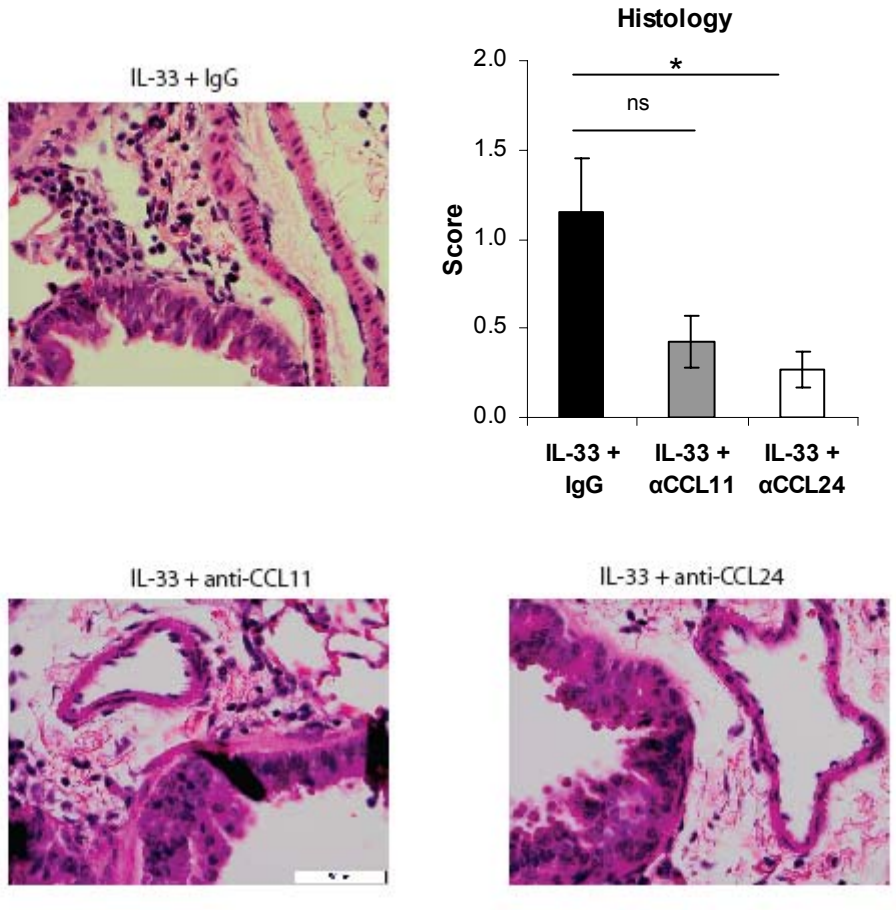


Figure 4.21: CCL11 and CCL24 mediate IL-33 induced lung inflammation
Lungs were harvested and histology score performed as previously described. Results shown are the mean of each group of mice +/- SEM. n=5 per group. H&E panels are representative of each group x40 magnification. Data are representative of 2 experiments. ns p= not significant, *p<0.05, of IgG versus antibody treated mice.

4.6 The effect of IL-33 on airway hyperresponsiveness

IL-33 generates lung and tissue eosinophilia, goblet cell hyperplasia, and inflammatory mediator changes that are in keeping with features of allergic asthma. At the outset of this work, it was not know whether IL-33 alone could replicate other features of asthma, in particular the defining feature of airflow obstruction caused by AHR. By using neutralising antibodies or direct application of cytokine; studies in mice show AHR in allergic airways disease can

predominantly be attributed to the actions of IL-13 [54]. IL-13 is one of the predominant cytokine outputs of IL-33 induced inflammation so it seemed plausible that it could be the mechanism of AHR demonstrated. Enhanced pause (Penh), a non-invasive method of whole body plethysmography, was used to measure AHR [284].

4.6.1 Three doses of IL-33 is insufficient to generate AHR in mice

Firstly, the presence of AHR in C57BL/6 mice treated using the attenuated 3 day IL-33 protocol (Figure 4.15A) described above was assessed. No difference was seen in response to increasing concentrations of nebulised methacholine between the PBS and IL-33 treated groups of mice (Figure 4.22A).

It is well known that differences in inflammation as well as AHR exist between strains of mice often used in asthma models [287,507,508]. Therefore AHR in mice on the BALB/c background, which typically have greater levels of reactivity to methacholine than C57BL/6 mice, was assessed [287].

BALB/c mice treated with IL-33 showed a trend towards induction of AHR at 50 mg/ml of methacholine in comparison to PBS controls. There was no difference seen in AHR between PBS-treated WT BALB/c mice and $ST2^{-/-}$ BALB/c mice (Figure 4.22B).

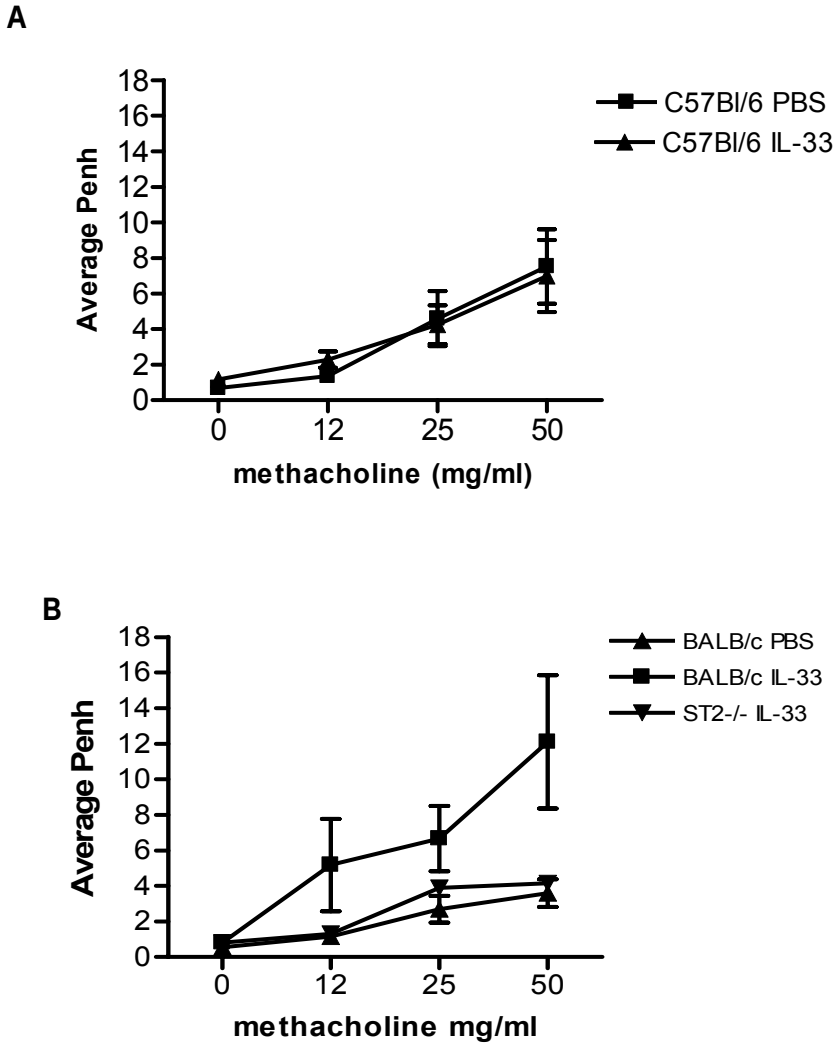


Figure 4.22: 3 doses of intranasal IL-33 does not induce AHR in mice
Mice were treated with intranasal IL-33 or PBS as per protocol in Figure 4.13A. On day 6, mice were exposed to increasing concentrations of methacholine and Penh measured in A) C57Bl/6 and B) BALB/c mice. Results are mean of each group of mice ± SEM. n=4-5.

4.6.2 Seven doses of IL-33 induces AHR in BALB/c but not C57BL/6 mice

There is an apparent dichotomy in mouse strain between airway eosinophilia and AHR. The aim of these experiments is to assess the contribution of the adaptive immune system to IL-33-induced effects including AHR using the $RAG^{-/-}$ mice on C57BL/6 background; therefore the ability to generate AHR in WT C57BL/6 mice was explored. Based on results in Figure 4.20, AHR was assessed in a protocol of maximal inflammation using 7 consecutive daily doses of IL-33 as in Figure 4.1. These assessments were also performed in BALB/c mice to confirm the trend identified in Figure 4.22, and sample size was increased based on those observations.

The Penh assessment was undertaken 24 hours following the last dose of IL-33 in line with standard airway assessment protocol, rather than after 72 hours due to the possibility that these mice could recover from any generated AHR [500].

Results are shown in Figure 4.23. BALB/c mice demonstrated a robust AHR in response to intranasal IL-33 confirming the earlier interpretation. However, IL-33 treated C57BL/6 mice did not show increased Penh measurements in response to increasing doses of methacholine compared to PBS treated mice. This is despite a very significant level of airway inflammation (Figure 4.23C), confirming the dichotomy of AHR and eosinophilia in these mice. At 7 days there was no significant difference between the strains of mice and level of airway inflammation, suggesting that the BALB/c mice had overcame the earlier reduction seen in Figure 4.16 by continued treatment, and both strains of mice reached a maximal tolerable inflammation.

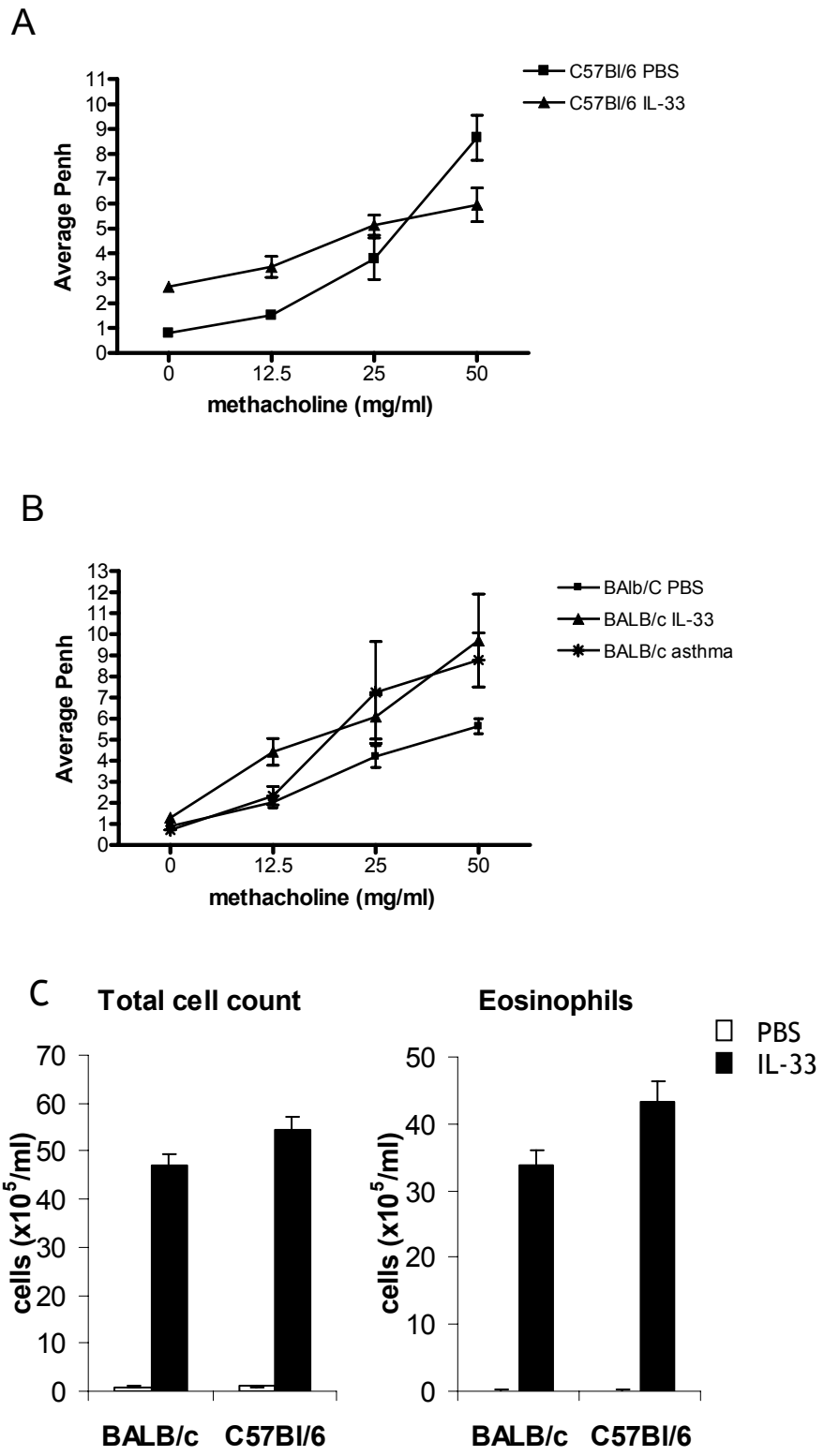


Figure 4.23: 7 doses of IL-33 induces AHR in BALB/c but not C57BL/6 mice despite maximal airway inflammation

Mice were treated with intranasal IL-33 or PBS or as per protocol in Figure 4.1. On day 8, mice were exposed to increasing concentrations of methacholine and Penh measured in A) C57BL/6 mice and B) BALB/c with an additional OVA-induced AAI (asthma) control group. Results are mean of each group of mice +/- SEM. n=8-12. Two way ANOVA BALB/c PBS vs IL-33 $p<0.001$.

4.6.3 Rapid assessment of AHR in C57Bl/6 mice following IL-33 challenge

C57BL/6 mice do not develop IL-33-induced AHR as measured by Penh following final airway challenge. This study protocol was designed to measure eosinophilic inflammation; despite maximal inflammation and standard assessment (24 hours) AHR was not found although it was demonstrated concurrently in BALB/c mice. Bronchial hyperresponsiveness and eosinophilic inflammation are distinct features and it is known that there are differences in inflammatory and AHR features between these two strains of mice [287]. C57BL/6 mice can develop AHR but it does not persist in comparison to BALB/c mice in AAI models [282]. Therefore, it seemed possible that the time point of Penh measurement was incorrect in this model; thus it was hypothesised in direct innate IL-33 inflammation, the appropriate time point would be shorter. Studies have measured changes in respiratory dynamics from 15 minute following a toxic challenge [509]. Experiments to measure Penh were performed 1.5 hours after each of 3 sequential doses of IL-33. This time point was chosen to allow the mice to fully recover from the anaesthetic and intranasal dosing [510].

These results show that measuring Penh 1.5 hours after 1, 2 or 3 daily doses of IL-33 in C57BL/6 mice and at increased doses of methacholine, did not elucidate any IL-33 generated AHR (Figure 4.24).

Since AHR was not generated in C57BL/6 mice, the background on which the $RAG^{-/-}$ mice were available, it was not possible to assess the importance of the adaptive immune response in IL-33-induced AHR. This could be the subject of further work using $RAG2^{-/-}$ on the BALB/c background.

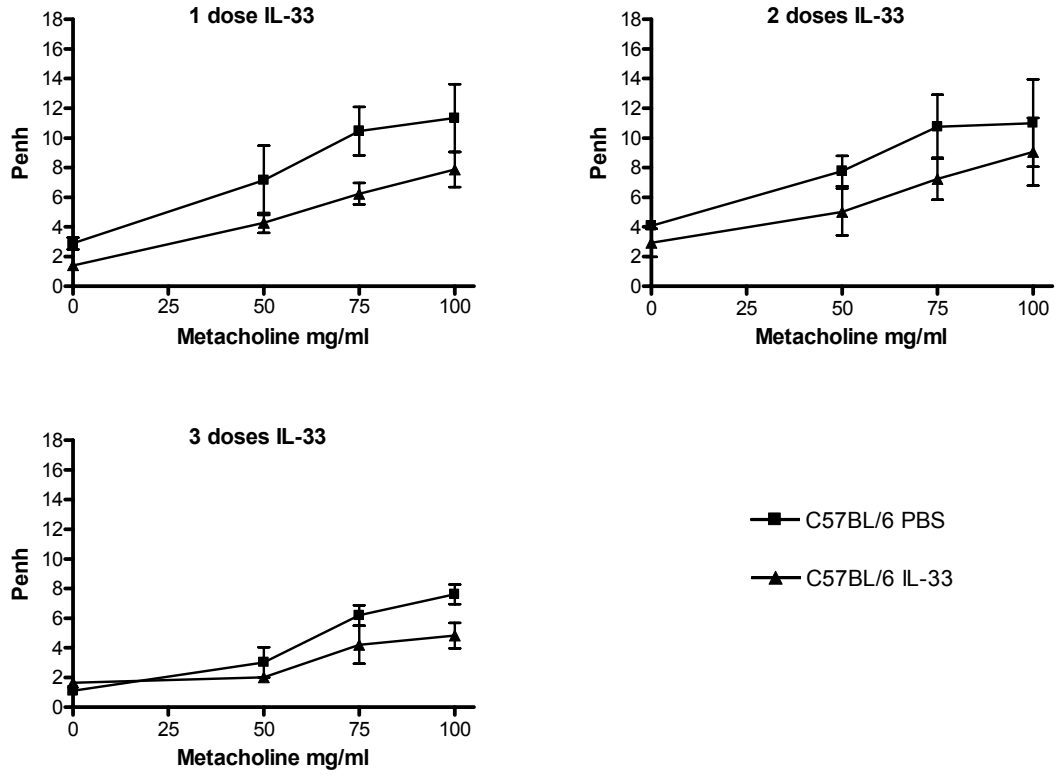


Figure 4.24: IL-33 does not induce AHR in C57BL/6 mice with short measurement protocol
Mice were treated with intranasal 2 µg IL-33 or PBS for 1, 2 or 3 days. On day 6, mice were exposed to increasing concentrations of methacholine and Penh measured 1.5hr after final airway challenge. Results are mean of each group of mice ±- SEM. n=5. 2 way ANOVA not statistically significant.

4.7 Conclusions

The data presented in this chapter clearly demonstrates profound inflammatory effect of intranasal IL-33 on the airway and lung parenchyma, features that are in common with allergic asthma. The predominant cell type in the bronchoalveolar lavage was the eosinophil in keeping with the now well established characteristic effect systemic of IL-33 as shown initially by Schmitz in the peritoneum [293], and subsequently in the lungs by our group and others [335,496]. A rise in numbers of other innate inflammatory cells was also shown. Further examination of the cellular content of the BAL fluid from these experiments by FACS (by Dr Mariola-Kurowska-Stolarska) correlated with the differential cell counts classified by morphological characteristics on cytological staining by the Romanovsky method demonstrating the validity of this technique [281]. More detailed studies on effect of IL-33 on eosinophils, macrophages and neutrophils within the lungs have been undertaken [311,496,511].

Within the lung itself, marked eosinophilia is further demonstrated histologically and is of a predominantly peri-vascular and peri-bronchial pattern consistent with published findings [335,422,496]. The lung cellular composition analysed by FACS identified a mixed population of resident ST2^{+ve} cells capable to respond to directly to IL-33. Subpopulations were identified as macrophages, mast cells and Th2 cells as might be expected from the ST2 receptor protein expression profile previously described. Other populations of ST2^{+ve} cells probably included eosinophils, B cells, and neutrophils. Other unidentified cells ST2^{+ve} cells were found that are capable of responding to IL-33 directly. Possibilities include structural cells, DC, NK cells and others. This group will also include type-2 ILC2, the recently described novel populations of IL-33 responsive non-T, non-B lymphoid cells including NHC, nuocytes and Ih2 cells [157-159]. Structural cells are ideally positioned to respond initially to IL-33 if released as an alarmin by necrotic cells. ST2 mRNA is present in murine fibroblasts [291,412], epithelial cells [318] and human endothelial cells [320,321,413]. The possibility that epithelial cells might drive the initial response to IL-33 is further investigated in chapter 5.

IL-33 recruits or drives proliferation of ST2^{+ve} cells in the lungs in particular macrophages, CD4^{+} cells, mast cells and probably eosinophils and neutrophils. These results indicate IL-33 can directly, without antigen, activate the adaptive as well as innate immune system. The granulocyte results presented here should be repeated with more robust cell surface combined CCR3/SiglecF staining[311]. Cherry *et al* showed eosinophils but not neutrophils express ST2 after activation [309]. Other members of our group have now definitively demonstrated ST2 expression at low levels on eosinophils [311] and neutrophils [312], significantly upregulated by IL-33.

In order to further investigate how IL-33 may bring about this inflammatory profile directly, a series of experiments to look in detail at the cellular accumulation while measuring the cytokine and chemokine levels at a large number of time points was performed. IL-33 induces sustained airway eosinophilia i*n vivo* but eosinophils are actually not detected until 24 hours following the 3rd dose of IL-33. Unstimulated eosinophils express low levels ST2 and studies show IL-33 can promote eosinophilia by directly stimulating bone marrow progenitors in an IL-5 dependent manner[311], but the delay in their appearance in the BAL suggests a more indirect effect *in vivo*. When mature

eosinophils appear in the airway, expressing higher levels of ST2L, they can perpetuate IL-33 driven inflammation directly[311]. Eosinophil recruitment into injured tissue is regulated by different cytokines and chemokines including IL-3, IL-4, IL-5, IL-13, GM-CSF, and chemokines eotaxin/CCL11 and RANTES[512]. IL-5 is probably the most specific and affects function, differentiation, proliferation, recruitment and survival and as such has been extensively investigated, including blocking antibodies and clinical trials discussed in chapter 1. Data here show IL-5 was not significantly present in BAL until 24 hours after the eosinophils, suggesting that IL-33 driven eosinophilia is not, at least not initially, driven by IL-5. Eosinophils themselves are a source of IL-5 and once recruited may further enhance eosinophilia in an autocrine and paracrine manner. Another cytokine noted in significant amount in the BAL and also the serum of IL-33 treated mice was IL-13. Similar to IL-5, IL-13 did not appear until after eosinophilia in the BAL. IL-13 induces goblet hyperplasia and mucus secretion when applied directly to the airway [53]. These changes were apparent in lung histological sections shown here and therefore most likely explained by IL-33 induced IL-13 production. Others have shown systemic IL-33 causes similar lung changes in WT but not in IL-13$^{-/-}$ mice, corroborating endogenous IL-13 is the mechanism of this feature [422]. Consistent with this, blocking IL-13 in the i.n. IL-33 model with neutralising antibodies reduced eosinophilic AI confirming a role for IL-13 in IL-33-mediated effects [513].

The potential effects of innate IL-33 have been further investigated in transgenic mice overexpressing IL-33 and in IL-33$^{-/-}$ mice. In the transgenic mice, cleaved mature 18kDa protein was released into the lung (BAL) and the mice developed spontaneous airway inflammation, mucus hypersecretion and cytokine production mirroring exogenous IL-33 treatment [514]. IL-33 deficient mice did not show any phenotypic abnormalities as result of the loss of endogenous IL-33. However, in response to protease allergen in non-sensitised mice, IL-33 deficiency resulted in attenuated type-2 responses which was not seen in WT or RAG$^{-/-}$ mice in agreement with a role of IL-33 in innate responses [515].

Eotaxins potently recruit eosinophils. The profile of BAL eotaxin/CCL11 and eotaxin-2/CCL24 production correlated with eosinophilia detected after the 3rd IL-33 dose. This is in agreement with a known synergistic role for eotaxins and airway eosinophilia [516]. Eotaxin-1 and -2 also synergise with IL-5 and IL-13 in

eosinophil recruitment [516-518]. This suggests these chemokines are important in mediating initial IL-33 driven recruitment of eosinophils to the airway and lung, in keeping with the respective dominant function of eotaxin-2 and eotaxin [56,516]. Thus, the possibility of abrogating the effect of IL-33 by using eotaxin neutralising antibodies was explored. Data here show both eotaxin and eotaxin-2 are partially responsible for this inflammation. To further explore this mechanism, it is useful to consider which cellular source may be responding to IL-33 directly or indirectly to produce the eotaxins. Eotaxin-2/CCL24 sources in the lungs are resident macrophages, endothelial cells and epithelial cells[519]. As macrophages are resident and accumulate in the BAL immediately following IL-33 administration, they are a candidate of producing eotaxin-2 in direct response to IL-33. Indeed, a sub-population, the alternatively activated macrophages (AAM or M2 or M(IL-4)) which play an important role in type 2 immunity and are induced by IL-4, IL-13 or IL-21 on stimulation produce of eotaxin-2 and TARC [109,520]. Work within our group shows that IL-33 can also enhance polarisation of M2 macrophages. Moreover, *in vitro* and *in vivo* depletion of alveolar macrophages results in attenuated IL-33 induced airway inflammation [513]. IL-33 induction of M2 macrophages has been reported by others[521] and is shown to be involved in reduced obesity [436], suppressing murine encephalitis and autoimmune uveitis models [449,522] as well as promoting lung fibrosis [523]. IL-4 does not appear to be important in development of M2 macrophages in IL-33-driven inflammation *in vitro* or *in vivo* and that IL-13 is the predominant IL-4Rα stimulus [513]. CD4 Th2 cells, mast cells and type-2 ILCs are candidate sources for IL-13 in the airways [58,293,496,524,525] with studies suggesting the ILCs an essential source of initial IL-13 [335,526-528].

Sources of eotaxin/CCL11 production in the lung include structural cells; smooth muscle, bronchial epithelial cells, vascular endothelial cells and fibroblasts [67,196,230] as well as immune cells; macrophages, eosinophils T-cells [529]. CCL11 was not released from the M2 macrophages on IL-33 stimulation [513] and there is no description of IL-33 driven CCL11 production from isolated T cells. Although IL-33 up regulates the eotaxin receptor, CCR3, on eosinophils it does not enhance their eotaxin production [311]. In relation to structural cells, the response of epithelial cells to IL-33 is further considered in chapter 5. To date, IL-33 driven CCL11 production from endothelial cells is not described. *In vitro*, it has

now been demonstrated fibroblasts can produce CCL11 in response to IL-33 which is enhanced by IL-13 suggesting these cells could be the source of eotaxin in this model [530]. Levels of eotaxins remained significantly many elevated days following last exposure to IL-33 in keeping with a sustained local source. Neutrophils which can acquire CCR3 expression, could be recruited by this mechanism [531]. It is not clear if the source(s) of IL-33-driven eotaxin is direct or via type 2 cytokine production such as IL-4/13 which are known to regulate eotaxin production via a stat-6 pathway [226]. A cascade of mediator production is initiated by IL-33 so it is perhaps unsurprising that single chemokine blockade would fail to 'switch-off' the inflammatory response and suggests these are downstream mediators.

A small but significant amount of MCP-1 was measured in the BAL that peaked on day 4. In response to IL-33, mast cells have been shown to produce MCP-1 which primarily recruits monocytes [419]. IP-10 (CXCL10) is involved in the chemoattraction of macrophages and T cells. Surprisingly, in these studies MIP-1α was not measured in the BAL of IL-33 treated mice. This has been shown to be present in the BAL after IL-33 treatment by others in our group (Kewin PK, 2007, PhD Thesis, University of Glasgow, and Mirchandani AS, 2012, PhD Thesis, University of Glasgow, 2012) and by myself in similar experiments; the difference perhaps explained by a technical issue or mouse strain.

The long established finding that ST2 as a selective marker for CD4 T cells has lead to an association with allergic airways disease. *In vitro* polarised murine Th2 cells produced IL-5 and IL-13 in response to IL-33 [293]. Smithgall and colleagues went on to show this in antigen- dependent and -independent routes in humans cells from allergic donors [326]. Surprisingly, they were not the source of abundant levels of BAL IL-5 and IL-13 in this model of AI. Data presented here shows that following IL-33 treatment a significant expansion in the population of CD4 cells was seen within the lung. Using $RAG^{-/-}$ mice, data here demonstrate that Th2 cells and furthermore, all lymphoid cells of the adaptive immune system, are not essential for IL-33 induced airway inflammation experimentally and hence this model must be dependent on the innate immune system. These findings were corroborated by subsequently published work by Kondo and colleagues although a different genetic background strain of $RAG^{-/-}$ was used [422,527]. The possible worsening of inflammation in the mice used in data

presented here actually suggests a loss of regulation provided by the adaptive immune response which is supported by subsequent studies [515]. As well as T cells, $RAG^{-/-}$ mice are also deficient in B and NKT cells and the effect of IL-33 on other potentially protective pathways in these cells could result in a net worsening of inflammation. Evidence from a cardiac transplant model shows IL-33 can promote development of a population of $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs)[532]. Although indicating an immunoregulatory role for IL-33, these studies are performed in the disease context where IL-33 is disease-protective, and thus extrapolation to Th2-type diseases is made with caution, however Tregs are known to be involved in resolution of lung injury in mice [533].

Similarities to the innate mechanism of action of IL-33 must be drawn with IL-25, another epithelial cell derived cytokine. IL-25 induces a type-2 cellular, antibody and cytokine response including IL-4 production. Like IL-33 this effect is not dependent on the adaptive immune response and $RAG^{-/-}$ mice also display worse inflammation with IL-25 treatment. Furthermore, the source of IL-5/13 in this model is dependent on a lineage negative population *in vivo* [161].

CD4 Th2 cells are not critical for IL-33-induced AI, but there is evidence IL-33 has an enhancing role in CD4 responses. Interleukin-33 upregulates the inflammatory potential of human Th2 cultures [534] and IL-33 stimulated ILC cells can enhance CD4 T cell Th2 cytokines *in vitro* and host CD4 T cell responses to antigen in the lungs *in vivo* [535]. In this way, IL-33 may act to exacerbate already present effector CD4 Th2 cell population in established disease states. Candidates for innate cells that produce IL-33 driven type 2 cytokines in this model include: mast cells which secrete IL-13 but also IL-5 [330,419]; basophils secreting IL-4, IL-13 and IL-5 [326]; and eosinophils secreting IL-13 [311]. Also, ILCs have been identified as an abundant source of IL-5 as well as IL-13 in IL-33 enhanced antigen or innate airways inflammation [58,335,526]. Furthermore, ILC are essential in viral exacerbation caused by influenza virus in complete absence of adaptive immunity, which is important if transferable to human studies explaining mechanisms of how viral infections exacerbate asthma [408].

IL-4 is important in Th2 differentiation and B cells isotype class switching to produce Th2 associated antibodies IgG1 and IgE in asthma [42]. Schmitz and colleagues treated *in vitro* IL-4 polarised Th2 cells with IL-33 which produced IL-

5 and IL-13 without a change in the already high concentrations of IL-4. *In vivo*, IL-33 treated mice developed systemic Th2 type inflammation with increased levels of IgE [293]. While this IgE finding could by related to increased IL-4 production, IL-4 was not detected in the serum, although increased levels of IL-4 mRNA were measured. Intriguingly, in the time course experiments presented here, only insignificant amounts of IL-4 were measured in the BAL in response to IL-33. However, IL-4 can be difficult to measure in this compartment[35,287]. Using IL-$4^{-/-}$mice, data here show for the first time that the Th2 cytokine IL-4 is not essential for the development of innate IL-33 Th2/type-2 responses *in vivo*. As discussed, the adaptive immune response is not essential for the innate response triggered by IL-33 so an IL-4 independent mechanism is in keeping with this. Also in keeping with this data is the discovery that IL-33-mediated effector responses in the lungs are dependent on ILCs and this occurs in part via their production of IL-5 and IL-13 but not IL-4 [335]. In the context of allergic airways disease the generation of T cell and antibody responses at sensitisation *in vivo*, the mechanism of action of IL-33 could vary. Extension of the work of this thesis in our laboratory shows *in vitro* IL-33 can polarise a subset of CD4 T cells (antigen driven) cells to IL-5/13 producing CD4 T cells in the absence of IL-4 [496]. Furthermore, IL-33 exacerbates AAI, independent of IL-4 (further discussed in chapter 6) [496]. A requirement for IL-4 is described for IL-33 mediated mast cell degranulation and IgE amplification [536]. In addition, in an innate acute colitis model that is associated with type-2 cytokine dysregulation, IL-33 exacerbated disease in an IL-4 dependent manner [447]. CXCL10/IP-10, VEGF and IL-17 were also increased in this colitis model similar to results in the AI model here but not in agreement with elevated CXCL9/MIG, IL-6 and IL-4 in the colitis model. In contrast, IL-33 enhanced cutaneous fibrosis was found to be IL-4 independent [537]. In IL-33-induced eosinophil activation, IL-4 independent and dependent mechanisms are both described indicating the mechanisms for IL-33-mediated responses are multiple and dependent on environmental situation and surrounding milieu [538].

In the innate IL-33 AI model, despite the preponderance for BALB/c mice to have biased Th2 responses, inflammatory responses were exaggerated in the classically Th1 biased C57BL/6 strain at lower doses of IL-33. The effect of IL-33 was found to be ST2 specific. There is evidence from asthma models that

eosinophilic and neutrophilic cellular inflammation is higher in C57BL/6 mice correlating with higher eotaxin-1 levels [287]. The findings presented in this thesis have since been replicated by a study which showed significant differences between inflammation in innate IL-33 AI following 3 days administration in BALB/c and C57BL/6 mice [515]. However data presented here also show that at higher doses of IL-33 with maximal inflammation, no difference seen between the mice strains in relation to cellular inflammation but IL-33 induced AHR was still dichotomous. These observations confirm differences exist between strains and this must be borne in mind when interpreting data from such models.

AHR is a cardinal feature of asthma and as IL-33 induces IL-13 production which is important for AHR development [53], it seemed plausible that IL-33 could also drive this feature. It has been demonstrated here that IL-33 does induce AHR in BALB/c mice. This is a novel finding and has subsequently been corroborated by other groups where it was associated with increased IL-13 lung mRNA [422,527]. The mechanism of IL-33 induced IL-13 dependent AHR could be verified in IL-13$^{-/-}$ mice. The data here show that dosing and assessment protocols as well as mouse strain influence this IL-33 driven effect. AHR was demonstrated only at highest levels of methacholine in BALB/c mice treated in shorter 3 day protocol despite significant inflammation (or peaking) at this time. This could in part be explained by the chosen protocol, where a delay of 72 hours following final administration to time of assessment could allow the time of maximal AHR to be missed. Although evidence from asthma models shows AHR persists at least up to 7 days or weeks following final aerosolised antigen challenge in BALB/c mice the profile in non-antigen sensitised mice could be different [273,282]. Measurement of AHR 24 hours following a maximal inflammation 7 day protocol more robustly demonstrated AHR in these mice compared to those treated with PBS, at all concentrations of methacholine. It is recognised that there is dichotomy between eosinophilia and AHR and that the most commonly used mouse strains in these asthma studies, differ in their generation of these features [282,287]. BALB/c mice have significantly greater airways responses to inspired methacholine compared to C57BL/6 mice and that appears to be replicated in the data here. Despite adjustments in protocol timing, it was not possible to generate AHR in the C57BL/6 mice. It is not clear whether IL-33 simply does not cause AHR in these mice or whether the protocol or assessment

method was not appropriate for detecting it. One study reported in abstract publication only suggests high dose IL-33 could induce AHR in C57BL/6 mice measured by invasive techniques[539]. Appreciation of these apparent strain differences may reflect different phenotypes in the response to IL-33 and further study could inform asthma clinical phenotypes.

It was not possible to go on to assess the contribution of CD4 T cells to AHR as the $RAG^{-/-}$available were on the C57BL/6 background. Using $RAG2^{-/-}$ mice on a BALB/c background, others have shown AHR like eosinophilia, was more severe in the $RAG2^{-/-}$ suggesting a loss of regulation by adaptive immune response in the direct response to IL-33 [422]. A further important study confirmed the induction of AHR in these mice and the mechanism in this innate context was via NKT and NH (ILC) cell production of IL-13 [527]. In the context of AAI, maintenance of AHR, measured invasively, was shown to be dependent on the IL-33/ST2 pathway via Th2 cells in BALB/c and but not C57BL/6 mice suggesting different mechanisms whereby IL-33 can mediate AHR [282].

Whilst the conclusions regarding possible different phenotypes in IL-33 induced AHR are interesting, it should be appreciated that there are some limitations on the use of Penh as a measurement of AHR and hence interpretation of results using it. Enhanced pause is a dimensionless index that reflects change of amplitude of pressure waveform and expiratory time and thus has been criticised as a derived parameter not reflecting real respiratory function [285]. Gold standard whole body plethysmography assessment would be necessary to confirm the findings presented here in relation to AHR in mice. However, regarding AHR in mice models in general, as mice do not exhibit spontaneous airway hyperresponsiveness any extrapolations to the role of IL-33 in AHR in asthma must be guarded.

In conclusion, data in this chapter demonstrate that IL-33 applied directly to the airway in the absence of antigen, acts in a ST2-dependent manner to induce eosinophilia and AHR. This is associated with increased production of type-2 cytokines and chemokines. In particular, eotaxins CCL11 and CCL24 are found to be partially responsible for the IL-33 driven response but the cytokine IL-4 is not required. Furthermore, this response is not dependent on lymphoid cells although they may enhance the response which suggests innate cells play a more

crucial role. This finding may help to explain non-allergic forms of asthma that develop independently of Th2 cells.

5 The role of ST2/IL-33 on epithelial cells

5.1 Introduction and Aims

Asthma is classically considered as a Th2 inflammatory and overactive allergic antibody response. Whilst undoubtedly important in the pathogenesis of asthma, trials targeting this pathway have been underwhelming aside from anti-IgE therapy which has been beneficial in a select group of patients. This heterogeneous response has led to the understanding that there are multiple asthmatic phenotypes with distinct pathological mechanisms and to consideration of the contribution of other pathways, in particular structural cells [540]. Epithelial cells (ECs) at the airway mucosal surface are the first line of defence and this barrier is impaired in asthma [185]. They are partly, responsible for early non-specific innate immunity and release early danger signals such as HMGB-1 [541]. ECs also induce Th2 adaptive immunity by a variety of mechanisms [188]. Genetic studies identify links between aberrant epithelial genes and asthma [542]. Dysregulation of the repair process in asthma leads to sustained inflammation and subsequent remodelling. ECs have a role in promoting this by acquiring mesenchymal characteristics (epithelial-mesenchymal transition or EMT), releasing inflammatory mediators and promoting angiogenesis [175].

Results in chapter 4 show that within the lung, there is a population of non-immune cells expressing ST2L, capable of responding to IL-33; the number of which increased substantially after IL-33 treatment. In the absence of pre-existing inflammation, it seems plausible that other unidentified $ST2^{+ve}$ cells are the initial responder to IL-33 and resident ECs are a potential candidate. Weak ST2L mRNA expression has been shown in stimulated epithelial cells and soluble ST2 mRNA in both unstimulated and stimulated cells [306,314]. In chapter 3 the presence of ST2 protein was demonstrated within ECs, predominantly within in the nucleus. Furthermore, IL-33 induced inflammation is partially dependent on eotaxin and the cellular source of this is unknown; ECs may be a possible source.

Therefore, it is hypothesised that airway ECs express ST2L and trigger IL-33 driven inflammation and remodelling by releasing cytokines and chemokines including eotaxin. Experiments in this chapter aim to identify the presence of ST2L on ECs and to study the effect of IL-33 on these cells *in vitro* and *in vivo*.

Using this information, downstream effects on remodelling in airway models both *in vivo* and *in vitro* will be examined.

5.2 ST2 expression on airway epithelial cells

Before attempting to establish primary or differentiated cell cultures, the expression of extracellular ST2L using a human bronchial epithelial cell line (HBEC), subsequently referred to as normal human bronchial epithelial (NHBE) cells was examined. These are immortalised cells originally derived from donor airways (gifted by Dr C McSharry, Glasgow, UK).

5.2.1 Bronchial epithelial cells express ST2L

Following on from the description of ST2 expression in the lungs in chapter 3, ST2 expression was assessed in the NHBE cells. Cells were grown in 4 chamber culture slides and ST2 expression performed using anti-ST2 antibody (Atlas) using the ImmPRESS (Vector) kit staining system. Figure 5.1 shows spindle shaped epithelial cells with predominant cytoplasmic ST2 staining in growing cells (Figure 5.1 A-B). No obvious difference was seen in ST2 expression in cells grown in culture medium supplemented with IL-33 (10-100 ng/ml)(Figure 5.1 C-D).

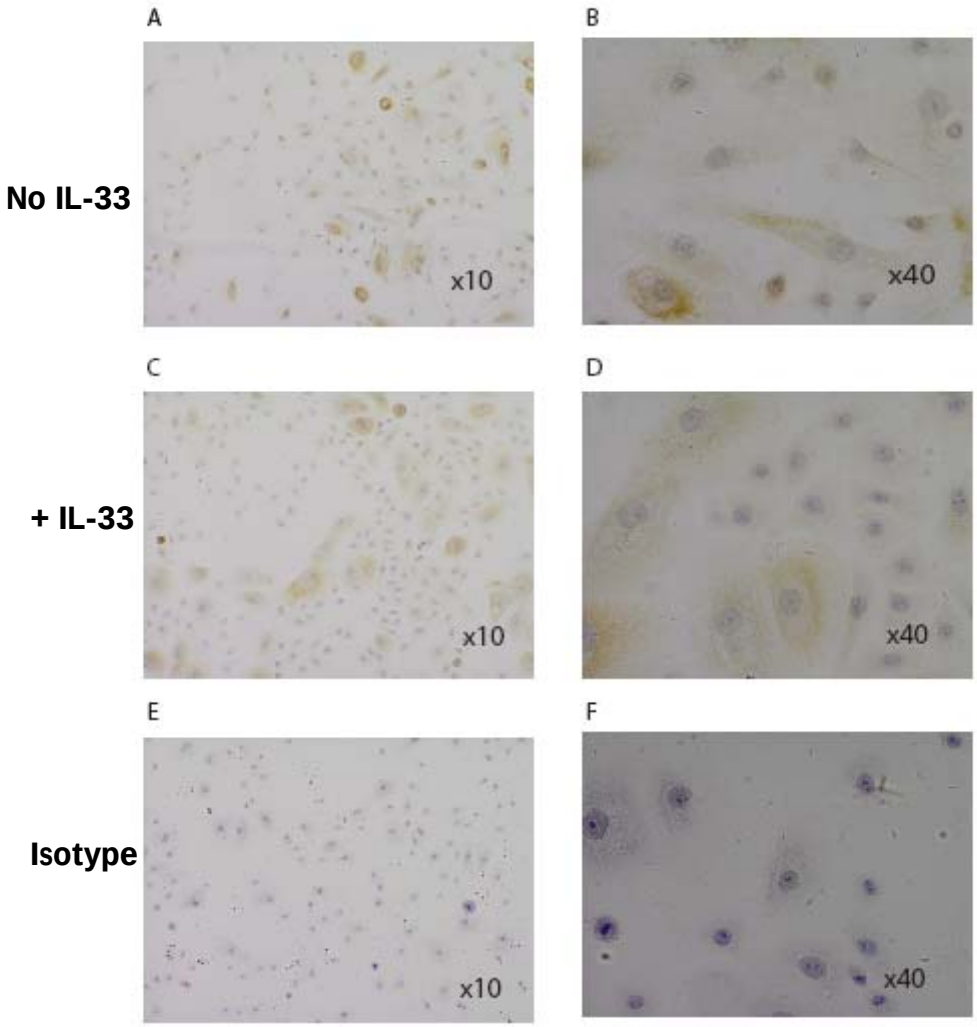


Figure 5.1: ST2 expression in NHBE cells
NHBE grown on culture slides and stained with anti-ST2 antibody (Atlas) or isotype. Panels (A-D) show ST2 staining in cells grown in culture medium (A-B) and medium supplemented with IL-33 100 ng/ml C) and D). Isotype control in E) x10 and F) x40 magnification.A), C), E)x10 C) and B), D), F) x40 magnification D).

NHBE cell ST2 expression was further analysed by FACS. Cells were grown in media in 75cm^2 flat bottomed culture flasks and analysed when 70-80% confluent. Figure 5.2B demonstrates that 12-28 % of NHBE cells are ST2$^+$ve (13.45 ±1.12, mean ± sem). Cytokeratin (CK) filaments are cytoplasmic skeletal structures found specifically in ECs [543]. Cytokeratin expression on NHBE was assessed with anti-pan Cytokeratin antibody (abcam, ab42460). CK expression in the NHBE cells was 98% positive with variation in expression intensity (Figure 5.2C). Following on, co-staining of CK and ST2 was attempted but proved technically difficult with ST2 surface expression affected by intracellular staining methods undertaken to assess CK expression. To validate ST2 staining, ST2 expression was also assessed on a human mast cell line, HMC-1, an

immortalised cell line known to highly express ST2. ST2 expression was verified as demonstrated in Figure 5.2D.

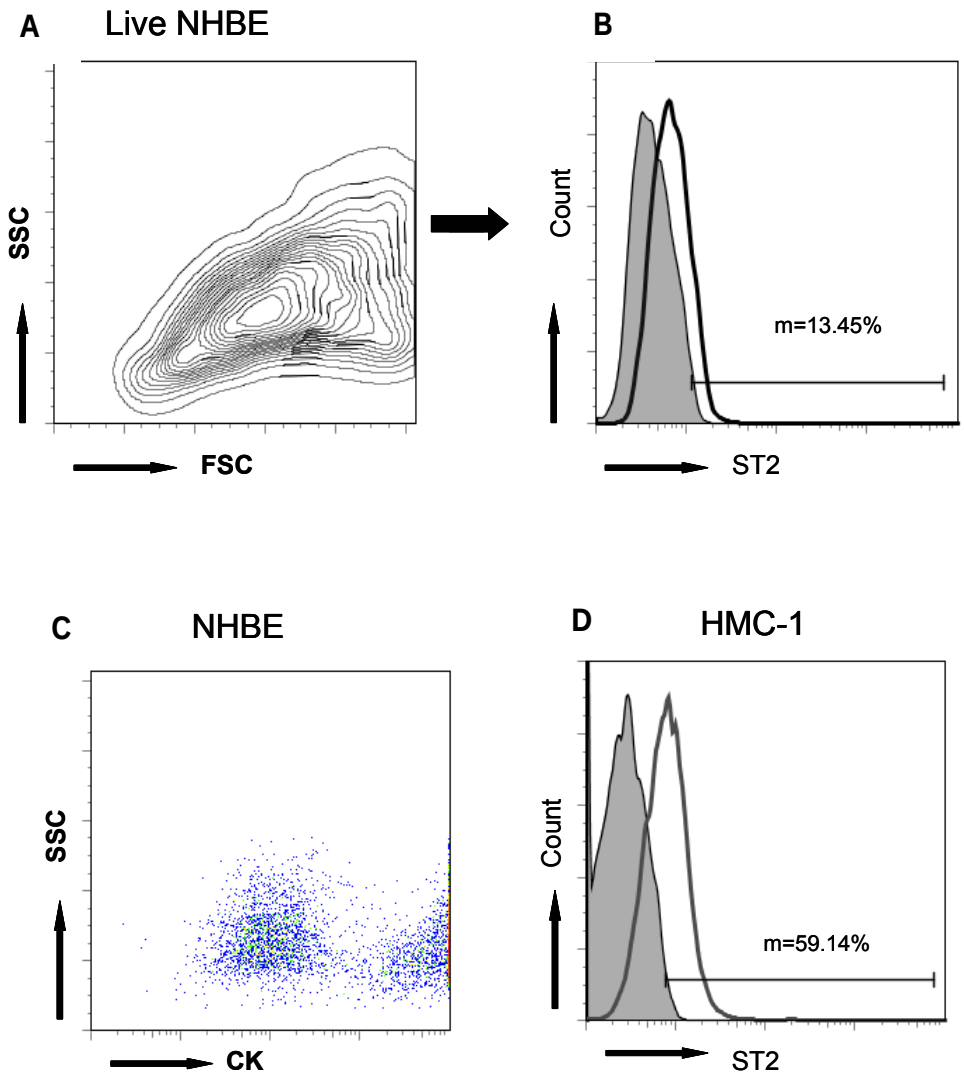


Figure 5.2: Normal human bronchial epithelial cells express ST2
NHBE were cultured, collected and stained with ST2 (unshaded) or Istoype (shaded) antibody. A) Live (7-AAD^{-ve}) NHBE were gated on FSC^{hi} and SSC^{med} parameters. B) ST2 expression in selected live NHBE cells C) Intracellular cytokeratin (CK) NHBE staining following cell fix and permeabilsation. Panels A)-C) are representative of at least 3 experiments D) HMC-1 mast cells surface ST2 expression ST2 (unshaded) or Istoype (shaded). Panel representative of duplicate samples from one experiment. Isotype gate <2%.

5.2.2 NHBE ST2L expression is unaffected by IL-33 treatment

To assess the effect of IL-33 on ST2 expression in these cells, NHBEs were cultured at 60-70% confluence with added IL-33 for 24 hours when ST2 expression was assessed. IL-33 did not appear to affect the proportion of ST2^{+ve} cells (Figure 5.3).

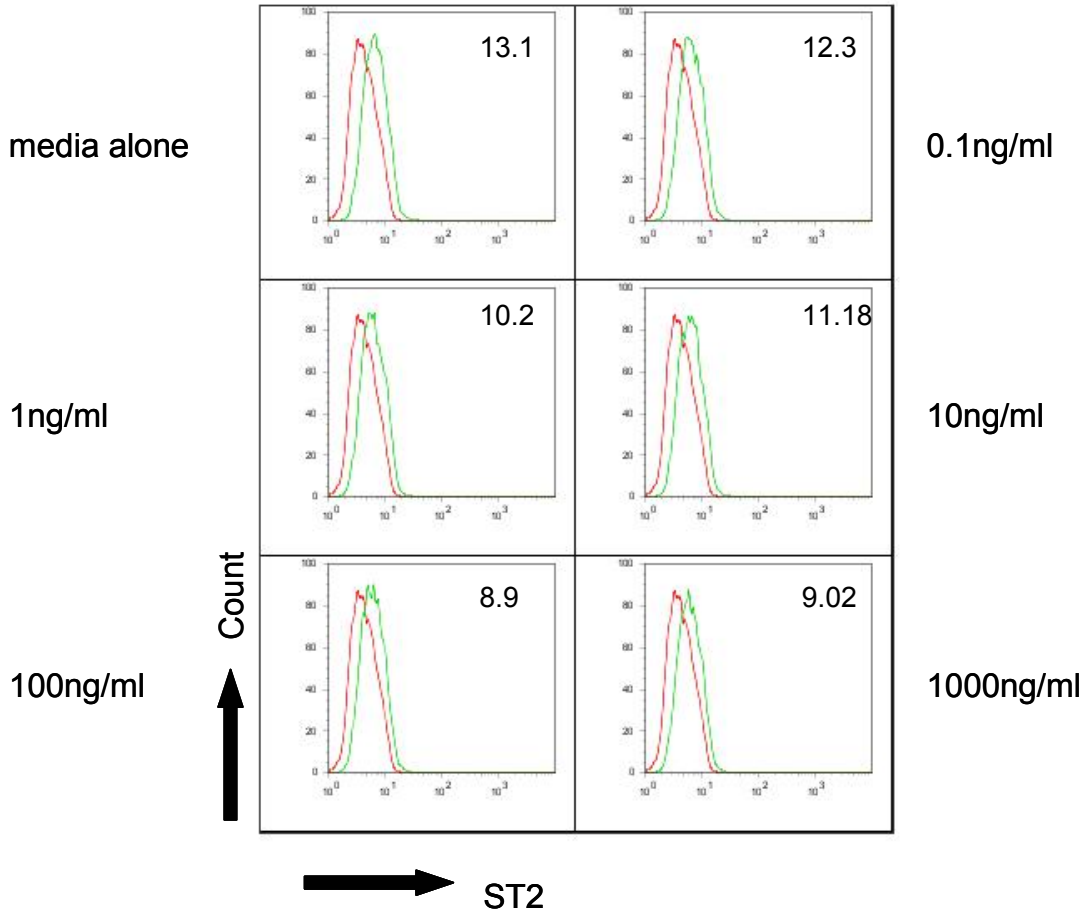


Figure 5.3: NHBE cell ST2 expression is unaffected by IL-33 treatment
NHBE were grown 6 well tissue culture plates. When 60-70% confluency was reached, hIL-33 (0.1-1000ng m/l) was added for 24 hours. Cells were enzymatically disrupted and stained for ST2 expression as Figure 5.2. Panels represent NHBE ST2 expression at increasing concentrations IL-33 noted.Red=Isotype, green=ST2.

5.2.3 Primary murine tracheal epithelial cells express ST2L

Following on from the studies above, it was important to assess the ST2 expression in more physiologically appropriate primary airway ECs. Optimally, *ex vivo* human material is used to evaluate ECs [544]. However, as human tissue was not directly available, the decision was made to isolate murine airway ECs. Successful culture techniques of these cells are difficult to establish. Using methodology by Davidson *et al*, cells were isolated from digested tracheas of BALB/c mice and cultured as described in more detail in chapter 2 [453]. When analysed for CK staining this cell population reveal a sub-population of CK^{+ve} ECs (Figure 5.4).

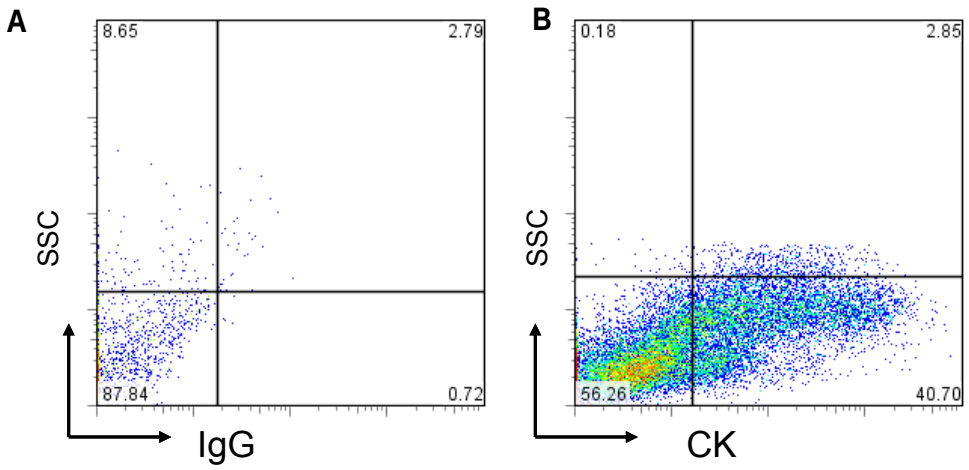


Figure 5.4: Isolation of murine tracheal epithelial cells
Murine tracheas were removed and incubated *ex vivo* in dissociation media for 60 minutes. The cell suspension was rested in Petri dishes to remove more adherent cells. The suspension was then washed, resuspended in FACS medium, counted and stained with Isotype A) or B) CK-PE antibody. The number of epithelial cells was determined by expression of intracellular CK.

Further analysis by surface ST2 staining appears to show that approximately 24% of ECs are $ST2^{+ve}$ (Figure 5.5) .

Unfortunately the yield was approximately 1 $x10^5$ cells per mouse, 50% of the published yield. Although the technique could potentially be further optimised, for culture purposes, the decision was taken to purchase and use primary human ECs.

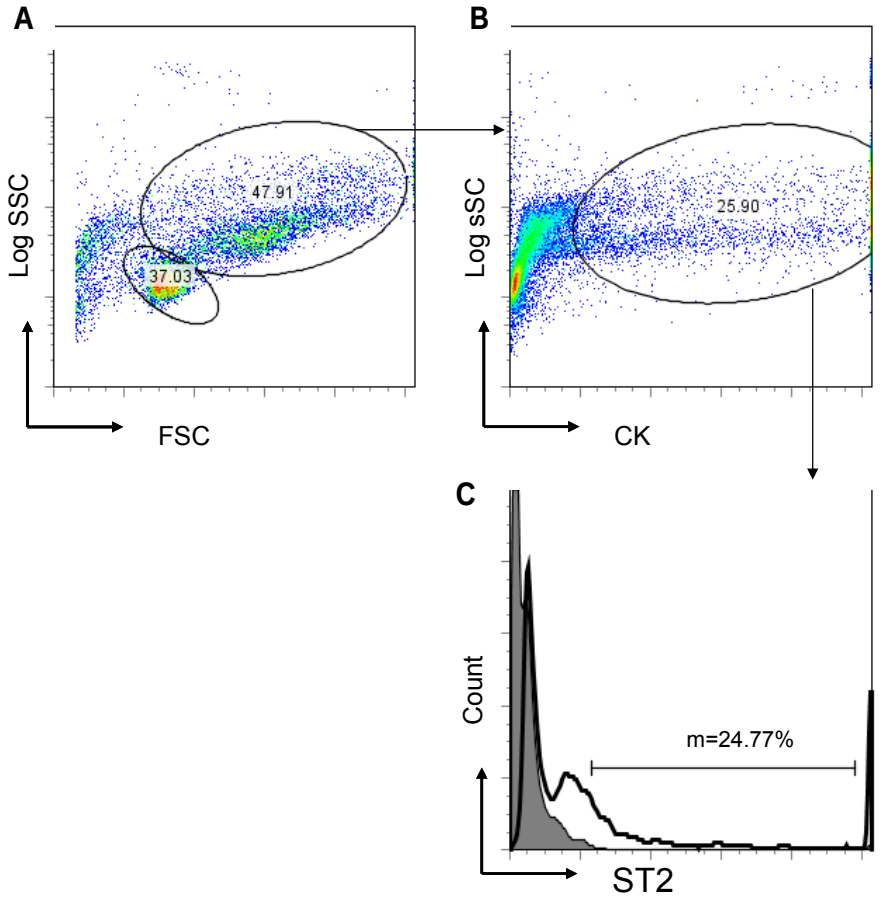


Figure 5.5: The expression of ST2L in primary murine tracheal epithelial cells
Murine tracheas were removed and cells dissociated *ex vivo*. (A-B) Using SSC and FSC parameters A) and CK staining B), EC were gated. C) The level of ST2 expression on the CK^{+ve} cells, ST2 (unshaded) or isotype antibody (shaded). Isotype gate <2%.

5.2.4 Primary human bronchial epithelial cells express ST2L

Commercially available primary human bronchial epithelial cells (pHBE) (Promocell, Germany) were subsequently assessed for ST2L expression. Similar to the primary murine cells, these are isolated from donor airway epithelial tissue taken from above the bifurcation of the lungs. 19.6% of pHBE cells had detectable surface ST2 (Figure 5.6).

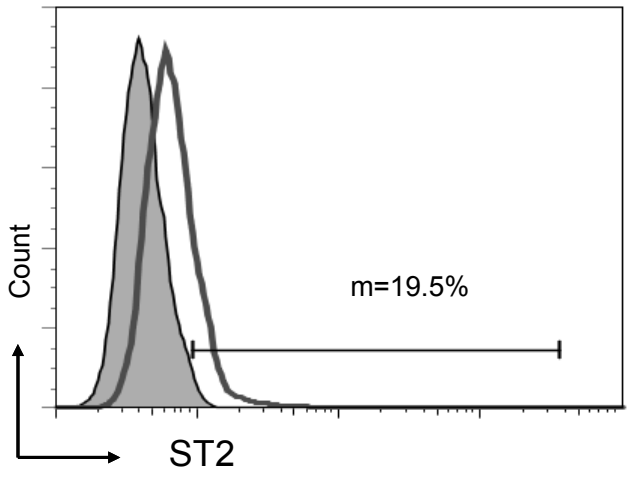


Figure 5.6: The expression of ST2 on primary human bronchial epithelial cells
Primary human bronchial epithelial cells were labelled with human ST2 (unshaded) or isotype antibody (shaded) and expression analysed by FACS. Representative FACS plot of n=2 experiments. ST2 percentage expression 19.5 +/- 2.9.

These data confirm the presence of ST2L on murine and human bronchial and lung epithelial cells.

5.3 The effect of IL-33 on epithelial cells

Having established epithelial cells express ST2L, the effect of IL-33 on cultured epithelial cells and on differentiated cells at an air-fluid interface, an *in vitro* airway model, was determined.

5.3.1 IL-33 induces cytokine and chemokine production from NHBE cells

ECs can initiate and perpetuate innate and adaptive immune responses by adhesive interactions, expression of cell surface receptors but also by selective release of chemokines. Epithelial-derived cytokines are not as clearly discerned but some novel examples TSLP and IL-25, are implicated in the pathogenesis of asthma [244,252,256]. To assess the contribution of epithelial cells to IL-33 induced inflammation in the airway, firstly NHBE IL-33-stimulated chemokine and cytokine production was assessed. NHBE were harvested when they reached 70% confluence and stimulated in 6-well culture plates with increasing concentrations of IL-33. Supernatants were collected 24 hours later and subsequently ELISA or multiplexed immunoassay was performed. Concentrations

of commonly produced epithelial chemokines (IL-8, MIP1α/CCL3, MCP-1/CCL2 and RANTES/CCL5) and cytokines (IL-6, IL-1β and TNFα) were analysed. Given the eosinophilic profile of IL-33 *in vivo* described in chapter 4, eotaxins, Th2 and Th1 cytokines along with growth factors were also measured.

Production of IL-8 (CXCL8) is an important function of epithelial cells recruiting inflammatory neutrophils. Upon stimulation with IL-33 the NHBE cells produced significantly increased levels of IL-8 (Figure 5.7).

Eotaxin was partially responsible for the IL-33 driven eosinophil recruitment as demonstrated in chapter 4. A very low baseline level of eotaxin was detected in NHBEs culture supernatants but there was no change in the eotaxin concentration with IL-33 treatment (Figure 5.7). Eotaxin-2 levels were also assessed in the supernatants by ELISA and none was detected (data not shown).

Other IL-33 driven chemokines produced, with the ability to direct movements of various cell populations include MCP-1/CCL2 and RANTES/CCL5 as well as MIP1α/CCL3, MIP1β and IP-10 at lower but significant amounts (Figure 5.7). These results show epithelial cells can attract a number of immune cells in particular neurophils, monocytes/macrophages and T cells in response to IL-33.

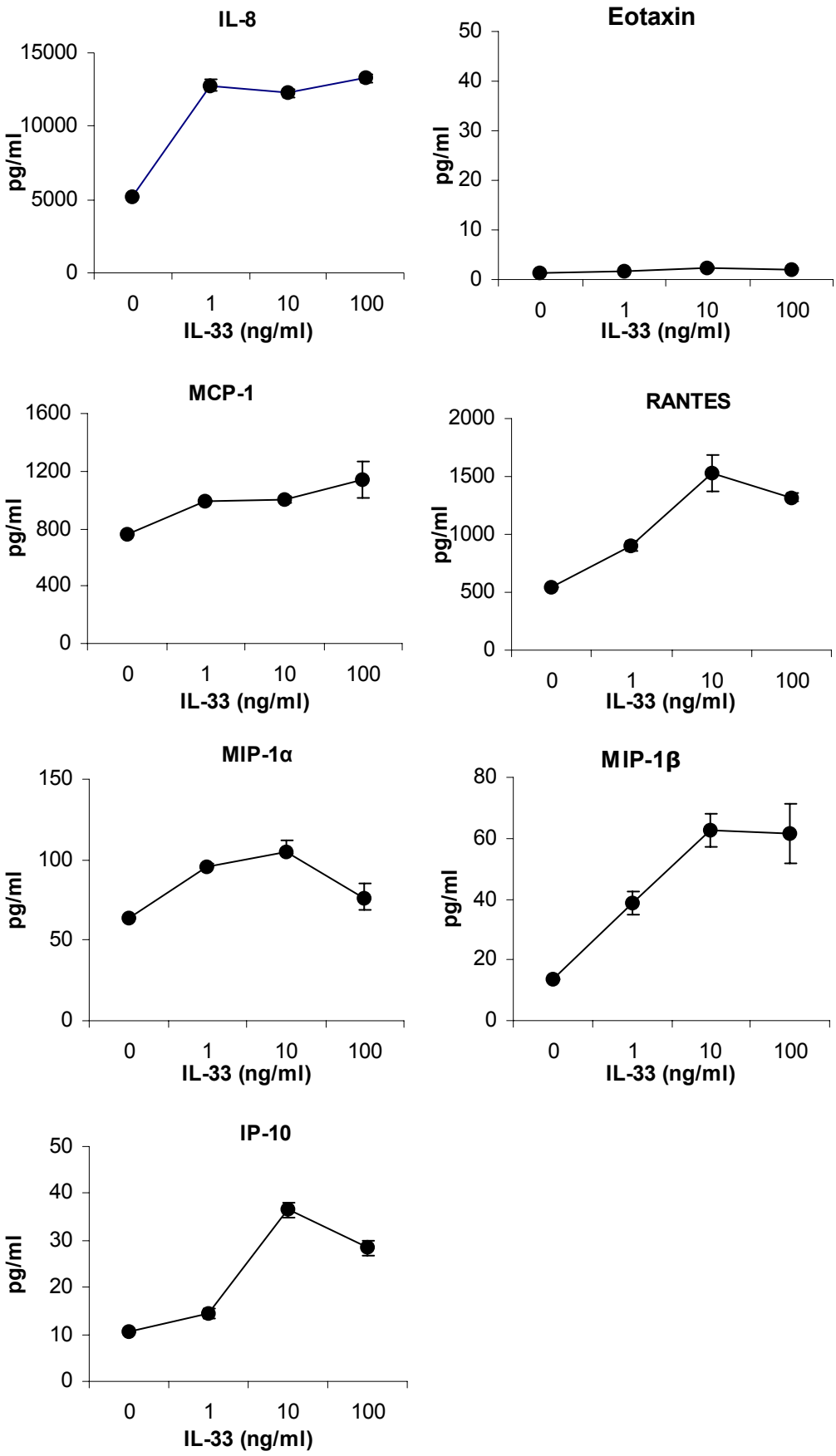


Figure 5.7: See over for legend

Figure 5.7: NHBEs produce chemokines in response to IL-33
NHBE were cultured until 70% confluent then cultured in 6 well tissue culture plates for 24 hours with increasing doses of recombinant hIL-33 (axxora). Culture supernatants were collected and cytokine and chemokine profile assessed on multiplex or elisa. Results shown are mean of technical duplicates (± SD) analysis wells from the same experiment. One way anova for the effect IL-33 on IL-8 $p<0.001$; eotaxin p=ns; MCP-1 $p<0.01$; RANTES $p=0.01$; MIP-1α $p<0.01$; MIP-1β $p<0.01$; IP-10 $p<0.001$. Results are representative of 2 or more experiments. p=non-significant.

Of the pro-inflammatory cytokines measured, IL-6 and TNFα results are shown in Figure 5.8. Production of IL-6 from ECs was increased with IL-33 treatment. TNFα was produced in small amounts but this was unaffected by IL-33. The anti-inflammatory Interleukin-1 receptor antagonist (IL-1RA), was produced in significant amounts by the ECs but this was unchanged with IL-33 treatment and no IL-1β was detected in the supernatant (data not shown).

IL-12p40/p70 production was induced by IL-33 but IFNγ or other type 1 cytokines were not produced. NHBE cells produce increased levels of IL-2R (Figure 5.8) however no IL-2 was detected in the supernatants following IL-33 treatment (data not shown). Soluble IL-2R is elevated in patients with epithelial cell tumours but the clinical significance is unclear [545].

With regard to type-2 cytokines, an increase in IL-4 production was observed but this was at low levels (Figure 5.8). Neither IL-5 nor IL-13 were produced (data not shown).

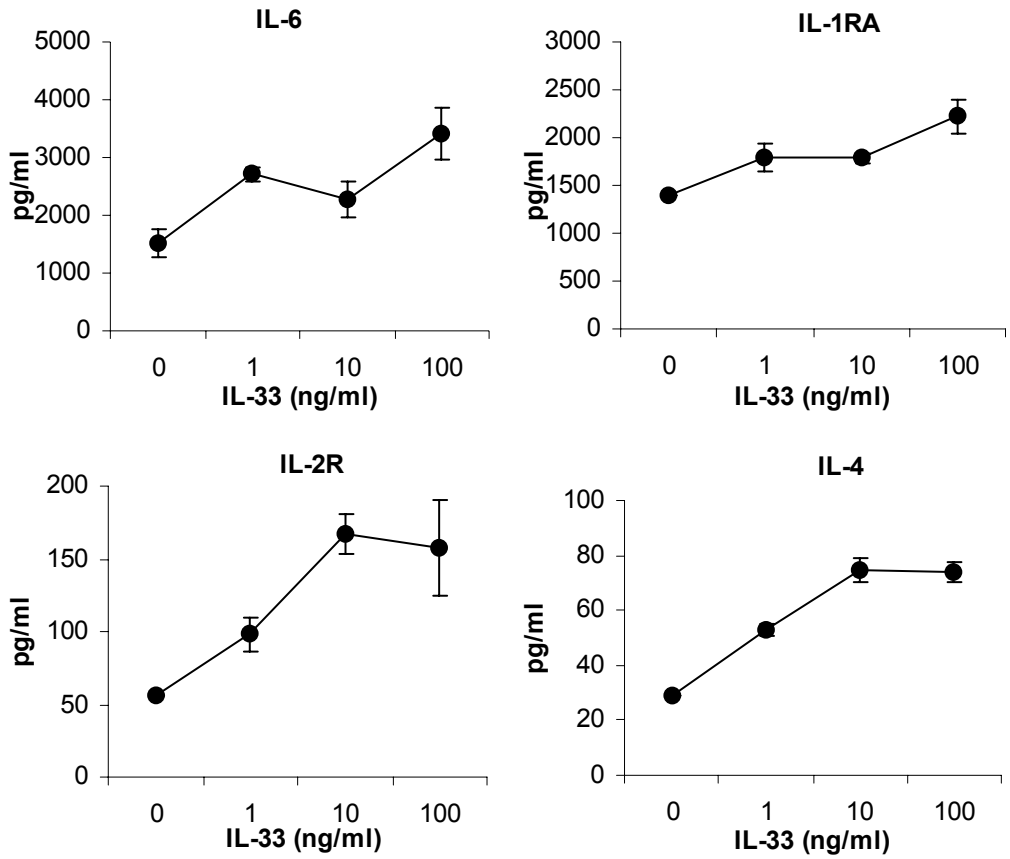


Figure 5.8: NHBEs produce cytokines in response to IL-33
NHBE were cultured with IL-33 as described. Culture supernatants were collected and cytokine profile assessed on multiplex immunoassay or ELISA. Results shown are mean of technical duplicates ± SD from the same experiment. One way anova for the effect IL-33 on IL-6 $p<0.01$; IL-1RA p=ns; IL-2R $p<0.05$; IL-4 $p<0.01$. Results are representative of 2 or more experiments. p=non-significant.

Epithelial cells also produce a range of growth factors. Of those measured, vascular endothelial growth factor (VEGF), a potent angiogenesis stimulant was induced with IL-33 treatment. Cytokine growth factors GCSF and GMCSF were produced in small amounts with the change in GCSF in response to IL-33 reaching statistical significance. Basic fibroblast growth factor (or FGF-β) which promotes fibroblast proliferation and angiogenesis was also released (Figure 5.9).

Mediators produced by epithelial cells showing some increases but at low levels are MIG, IFNα, HGF and IL-17. Other cytokines and chemokines measured by multiplex assay or ELISA and not induced by IL-33 in epithelial cells are IL-7, IL-10, IL-15 and EGF (data not shown).

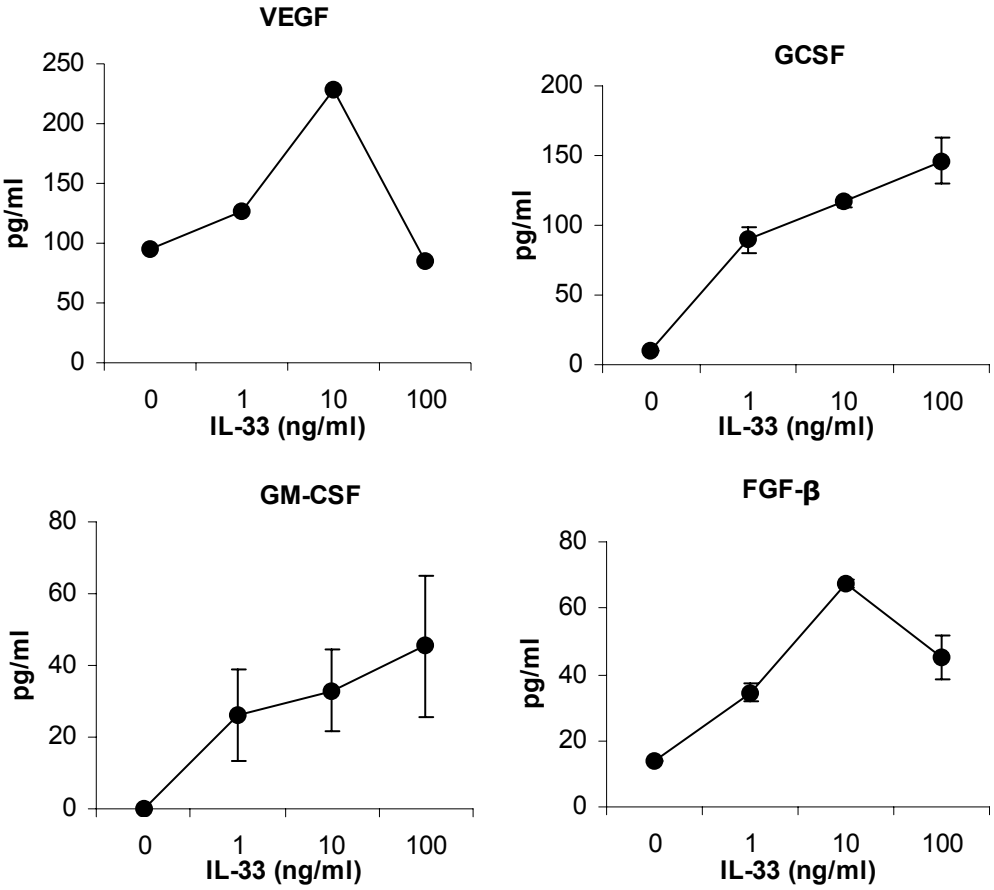


Figure 5.9: NHBEs produce growth factors in response to IL-33
NHBE were cultured with IL-33 as described. Culture supernatants were collected and cytokine profile assessed on multiplex or elisa. Results shown are mean of technical duplicates (± SD) from the same experiment. One way ANOVA for the effect IL-33 on VEGF $p<0.01$; G-CSF $p<0.001$; GM-CSF p=ns; FGFβ $p<0.001$. Results are representative of 2 or more experiments. ns=non-significant.

5.3.2 IL-33 induces dose dependent production of IL-8 and VEGF from NHBE cells

To further investigate the IL-33 induced IL-8 and VEGF production, dose response experiments were performed. From screening immunoassays and ELISA, an increase in mediator secretion appeared to occur at 10 ng/ml IL-33 and possibly inhibited at higher concentrations of 100ng n/ml and 1000ng/ml (1000ng/ml data not shown). To determine an optimal dose for IL-33 treatment a more detailed dose response was characterised by using 0.01-100 ng/ml IL-33 with IL-8 and VEGF concentrations measured by ELISA after 24 hours IL-33 treatment. A dose response seen in Figure 5.10 was obtained for IL-8 and VEGF with 10ng/ml and 100 ng/ml respectively the optimal concentrations reflecting different dynamics of release.

Next, the effect of corticosteroids on this response was determined. Further groups of cells were treated with 10 ng/ml of IL-33 with or without the potent steroid dexamethasone. The release of IL-8 was inhibited by the co-administration of dexamethasone. However, the release of VEGF was unaffected by the addition of dexamethasone demonstrating distinct regulation in IL-33 induced mediators induced in epithelial cells.

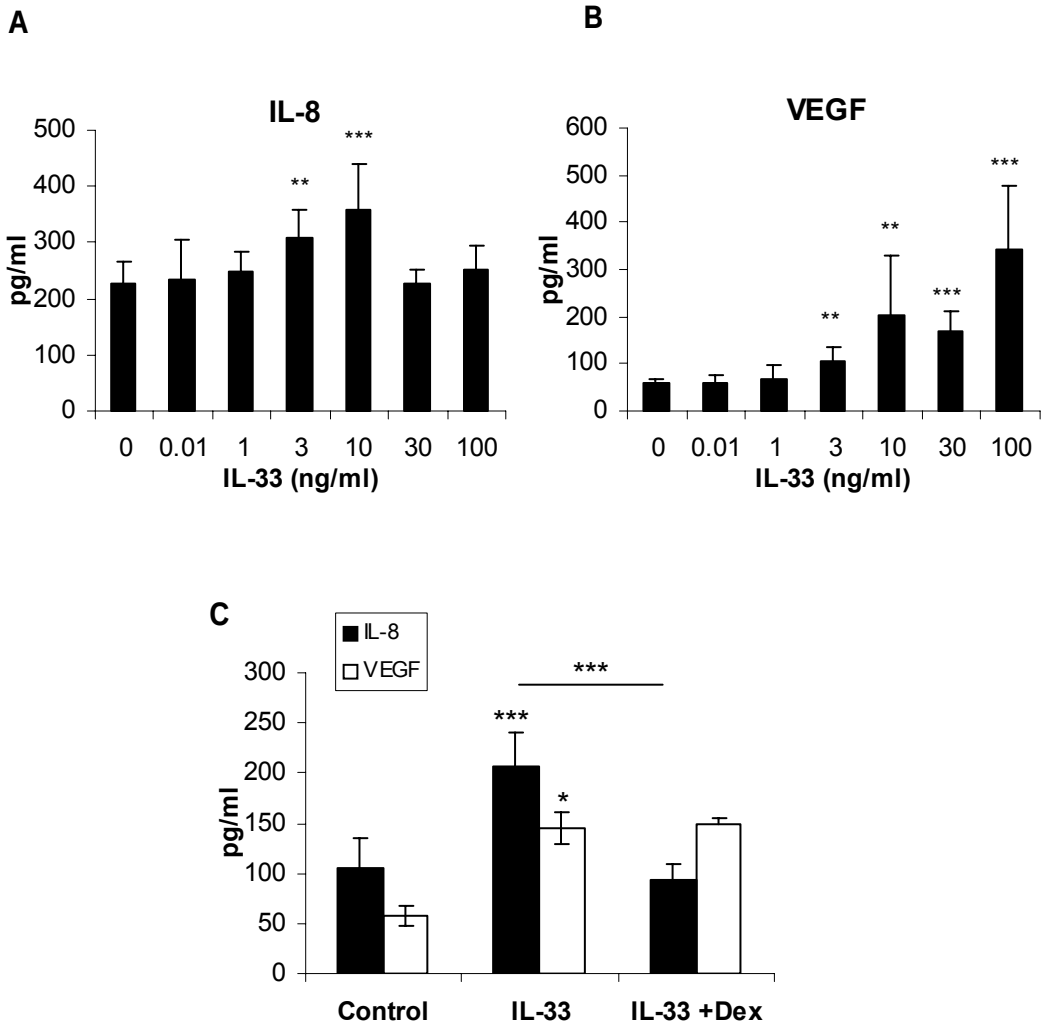


Figure 5.10: IL-8 and VEGF production by NHBE cells is dose dependent and differentially corticosteroid sensitive
NHBE cells were cultured as before and cells treated with increasing concentrations of IL-33. After 24 hours culture supernatants were removed and IL-8 and VEGF assessed by ELISA. A) Dose response graphs of A) IL-8 and B) VEGF. C) Further culture wells were treated with IL-33 10ng/ml with or without Dexamethasone (100nM) (Dex). Results shown are mean ± SD for 2-10 culture replicates. *p<0.05, **p<0.01,*** p<0.001 for IL-33 vs control in A and B or otherwise as shown.

5.3.3 The effect of IL-1β, TNFα and IFN γ on IL-33 induced cytokine release from NHBE cells

The above data demonstrate a range of mediators produced from epithelial cells in response to IL-33 treatment. In the EC microenvironment common inducers of cytokine responses from ECs are the pro-inflammatory cytokines IL-1β, TNFα and IFNγ. In combination these cytokines or "cytomix" is often used experimentally to replicate this environment [546]. Therefore, a set of experiments was performed to assess whether IL-33 would have any additive effect on that of cytomix on the EC production of IL-8 and VEGF. It would be expected that cytomix would induce release of IL-6 so this was also measured.

NHBE were plated out in 24 well culture plates and different combinations of IL-1β, TNFα and IFNγ as well as IL-33 added to the culture wells. The concentrations of IL-1β, TNFα and IFNγ used were based on published experiments with a final concentration of 10 ng/ml in combination with 10 ng/ml of IL-33 as determined by the preceding data [546].

Results are shown in Figure 5.10. When reagents were added individually, concentrations of IL-6 and IL-8 in the supernatants increased most potently with IL-1β or TNF-α. In comparison with IL-33 alone, they induced an approximate 7 fold or 20 fold increases in IL-6 and IL-8 production respectively. The combination of IL-1β and TNFα appeared most synergistic, with IFN-γ not enhancing the IL-1β or TNF-α driven production. However, the cytomix combination was the most potent. IL-33 appeared to have synergistic effect on most combinations but the magnitude of this effect was small.

In contrast, VEGF production was only strongly enhanced when IL-33 added to cytomix. This suggests adding IL-33 into a pro-inflammatory milleu can influence EC-induced angiogenesis potential.

These data indicate that the surrounding environment and presence of other mediators will have implications on the effect of IL-33 on ECs *in vivo*.

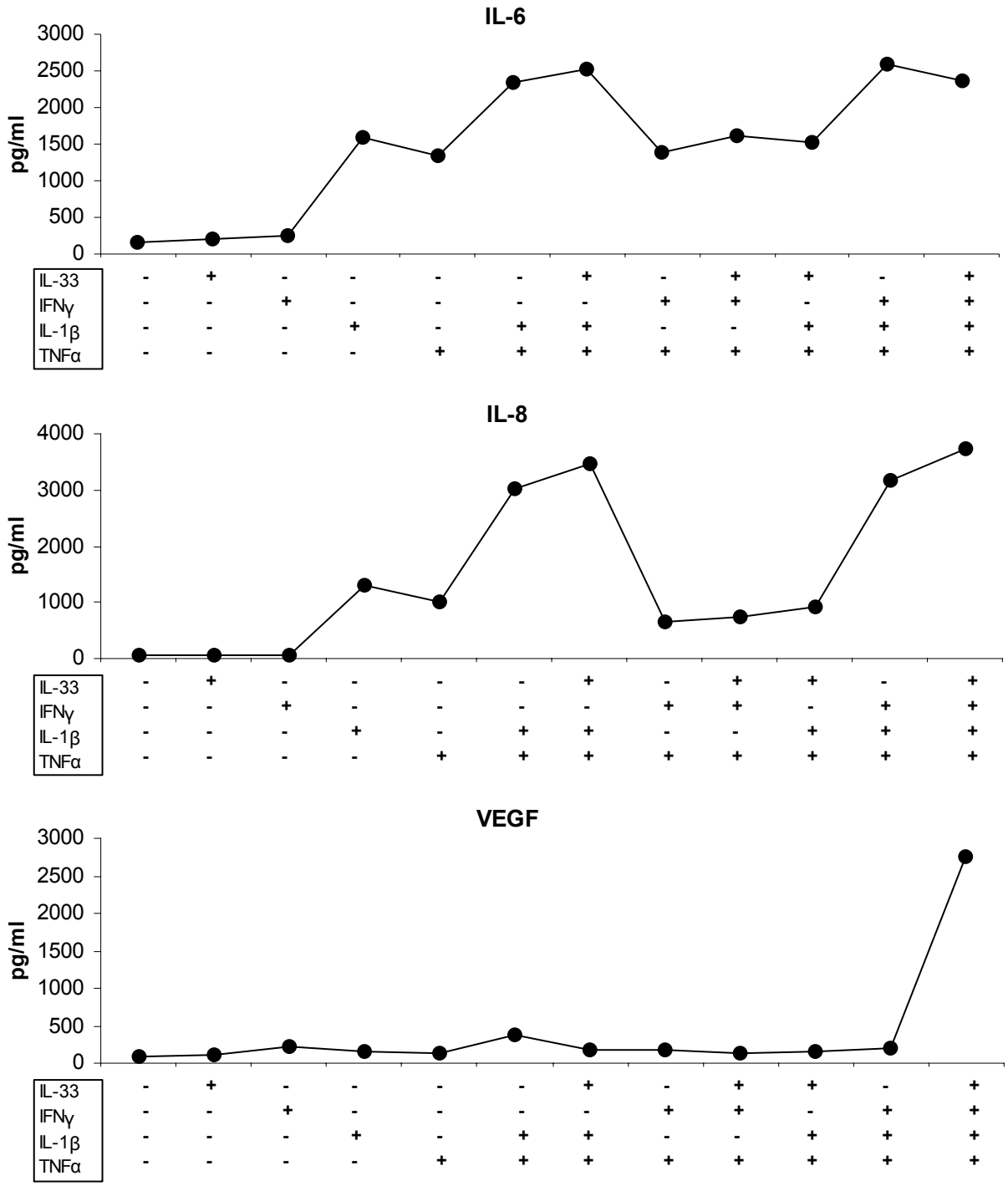


Figure 5.11: IL-33 induces synergistic cytokine and chemokine production with IFNγ, IL-1β and TNFα in NHBE cells
NHBE cells were cultured on 24 well plates and cells treated with fresh media containing combinations of IFNγ, IL-1β, TNFα and IL-33 all at 10 ng/ml as annotated in graph. After 24 hours culture supernatants were removed and IL-6, IL-8 and VEGF concentrations assessed by ELISA. Results shown are of single culture wells.

5.3.4 IL-33 does not induce NHBE proliferation in vitro

Having shown IL-33 enhances chemokine and cytokine production from NHBE cells, in order to assess whether this could be explained by an increase in cell number, cellular proliferation was assessed. NHBE were cultured with IL-33 for 24 or 48 hours and a further group grown in serum free media for 24 hours before IL-33 added to encourage a quiescent state. Proliferation was assessed by the colorimetric assay MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and was unaffected under the various conditions with IL-33 treatment (Figure 5.12A). This was confirmed by measuring ^{3}H thymidine uptake which was unchanged in cells treated with IL-33 for 24 hours compared to control (Figure 5.12B).

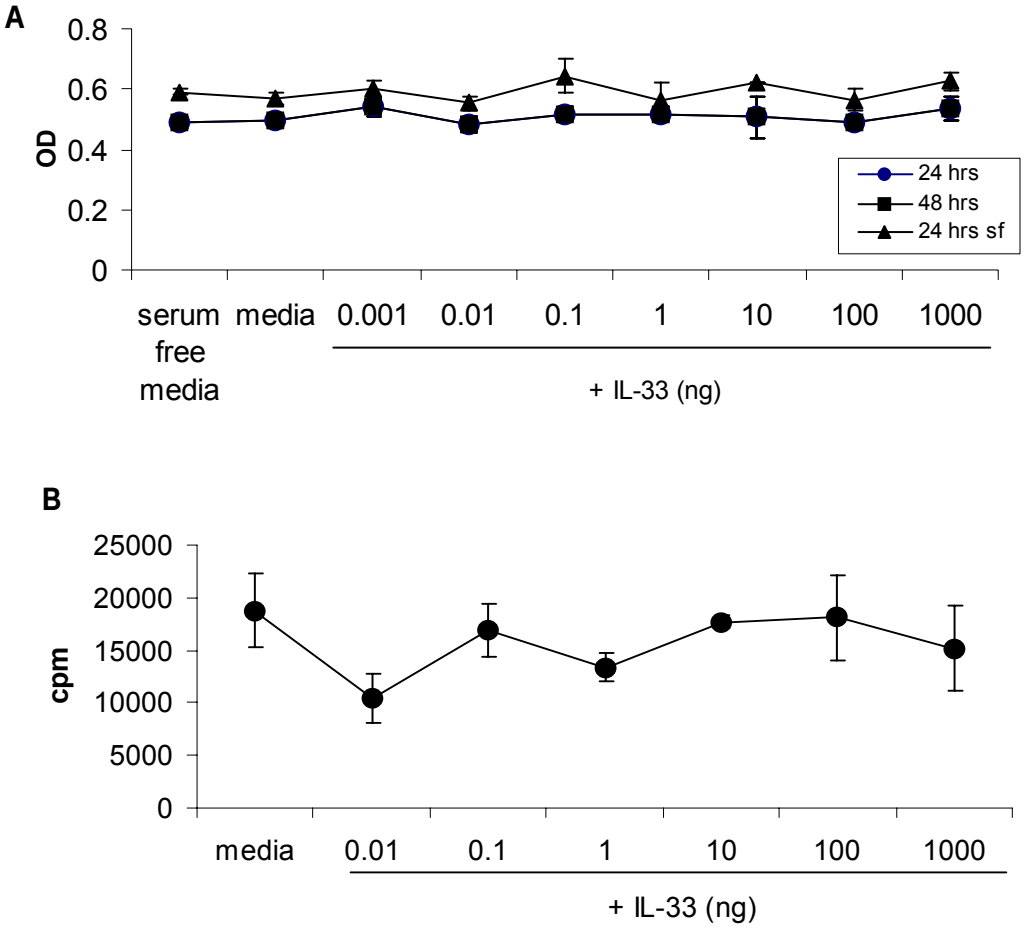


Figure 5.12: Proliferation of NHBE cells is unaffected by IL-33 treatment
Cells were grown in media with increasing concentrations of IL-33 for 24 hours or 48 hours and a further group of cells were kept in serum free media for the 24 hours preceding IL-33 treatment. Proliferation was measured by colorimetric assay, MTT following manufacturers instructions and A) or B) by ^{3}H Thymidine incorporation (for last 8 hours of culture) in the 24 hour treatment group. Results are counts +/- mean of triplicate wells. One way anova for the effect of IL-33 on proliferation p=non-significant. Representative of 3 experiments.

5.3.5 The effect of IL-33 on primary human epithelial cells

Having determined the ability of IL-33 to induce cytokine production in NHBE cells, the effect on pHBE was assessed. The effects on the cells of epithelial stimulant cytomix, was also determined. Direct microscopy of growing pHBE cells is seen in Figure 5.13.

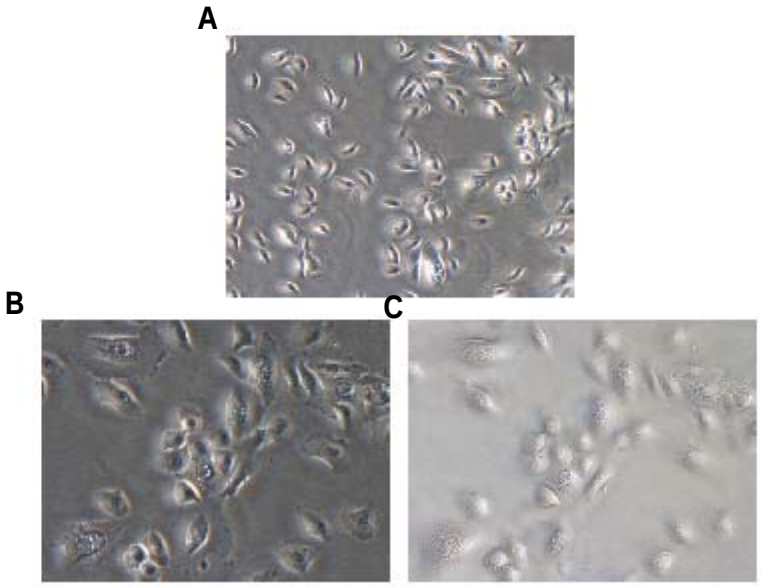


Figure 5.13: Morphology of primary human bronchial epithelial cells
pHBE grown per manufacturers instructions on 75 cm^2 culture flasks. Cells viewed x10 A) and x20 B) magnification by light microscopy. C) Phase contrast microscopy x20 magnification. Non-confluent monolayer of primary epithelial cells demonstrating spindle shape and early cobblestone morphology.

These cells were treated *in vitro* with IL-33 or in combination with cytotmix (IL-1β, TNFα and IFNγ, all 10 ng/ml). As anticipated, in agreement the with NHBE data, IL-33 significantly induced the production of IL-8 from the pHBEs in a dose dependent manner after 24 hour culture (Figure 5.14B). A further set of experiments were undertaken co-treating the pHBE with cytomix and IL-33, with cell culture supernatants taken at 6, 12, 24 and 48 hours to determine any time dependent effect. Cytomix stimulated the cells to produce large quantities of IL-8. Like the NHBE, IL-33 appeared to be synergistic with the cyotmix over time but overall the magnitude of the effect of IL-33 was less than cytomix stimulation (Figure 5.14B). A similar pattern was seen with IL-6 (data not shown).

VEGF production from the pHBEs was similarly dose dependent. In contrast to the NHBE, these cells produced a substantial quantity of VEGF in response to the cytomix. This was enhanced by IL-33 by 24 hours (Figure 5.14C) (6,12 hour data not shown).

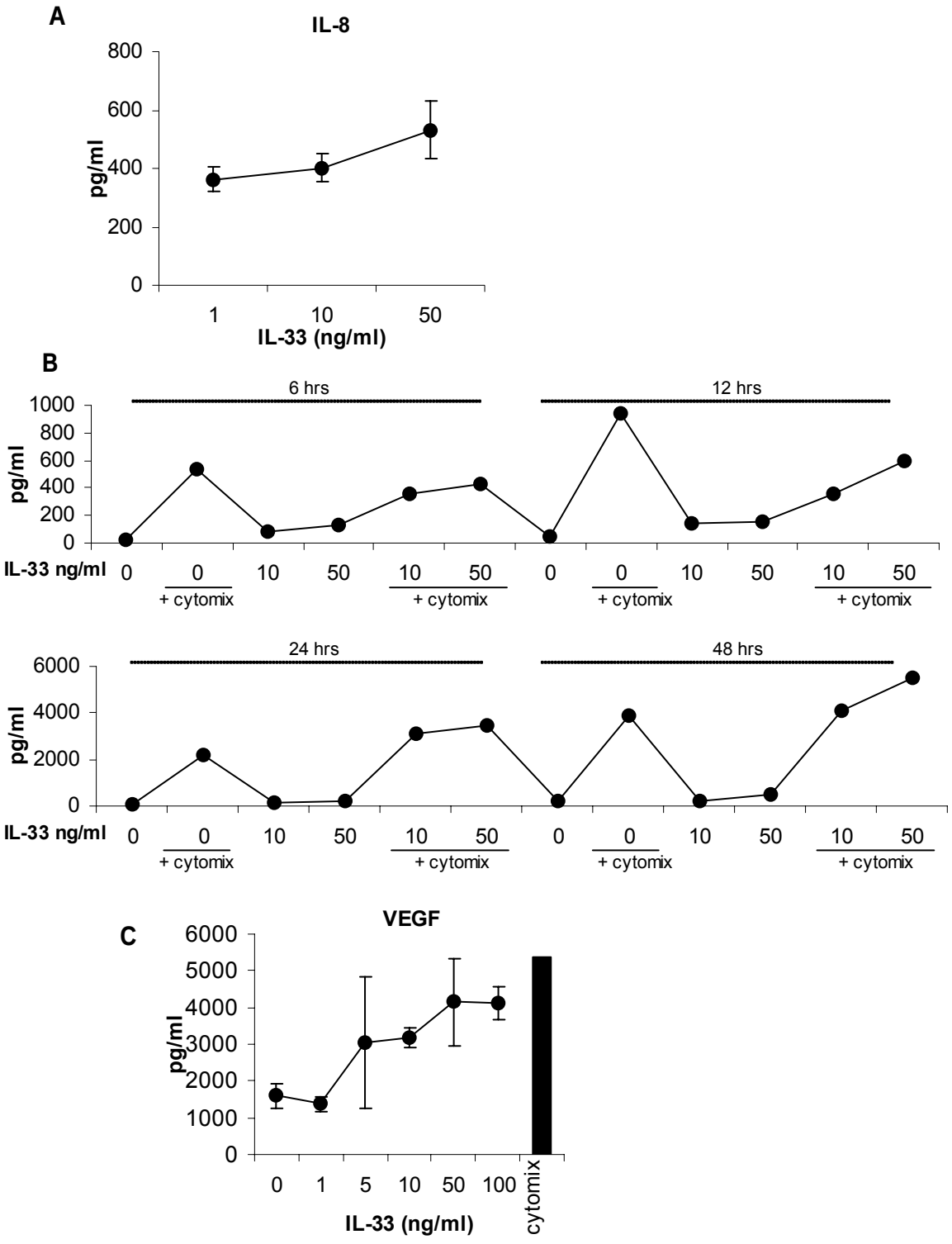


Figure 5.14: IL-8 and VEGF are released by primary bronchial epithelial cells in a dose dependent manner and enhanced by cytomix

Primary HBE were cultured as previously described and plated onto 12 well tissue culture plates (density 10,000/well) for 12, 24 or 48 hours with increasing concentrations of IL-33. Some wells had co-treatment with cytomix (IFNγ, IL-1β, TNFα at 10ng/ml) and supernatants were analysed for IL-8 and VEGF concentrations by ELISA. A) Dose response curve IL-8 B) Time course IL-8 C) 24 hours dose response VEGF. Results shown are mean of duplicate experimental wells +/- SD except for cytomix experiments where results are single samples. One way ANOVA for effect of IL-33 on IL-8 $p < 0.05$; and on VEGF $p<0.05$.

The epithelium can trigger innate responses by recognising PAMPS through the TLRs[185]. Polyinosinic-polycytidylic (poly(I:C)) is a synthetic analogue of double stranded RNA (dsRNA), a molecular pattern shown associated with viral infection and recognised by TLR3. TLR3 activation is a mechanism through which viral infections contribute to exacerbations of asthma [547]. To directly compare the effects or TLR3 and ST2 activation *in vitro*, pHBE cells were treated with IL-33 or Poly (I:C). Poly (I:C) induced stimulation of IL-8, IL-6 (and MIP1α) and had a far greater effect than IL-33 on the pHBEs. VEGF levels were comparable further suggesting the most important output from epithelial cells in response to IL-33 is VEGF.

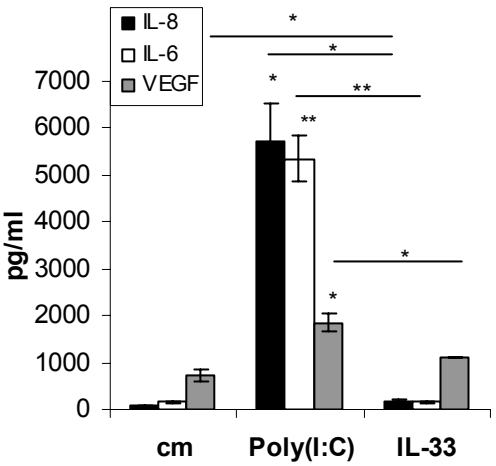


Figure 5.15: Primary epithelial cell cytokine and chemokine production is greater with TLR3 than ST2 stimulation
Primary NHBE were cultured as previously described and plated onto 12 well tissue culture plates (density 10,000/well). Cells were treated with Poly (I:C) (10µg/ml) or IL-33 (10ng/ml) for 24 hours. Supernatants were analysed for IL-8, IL-6 and VEGF concentrations by ELISA. Results shown are mean of triplicate experimental wells +/- SD. cm=complete media. *p <0.05, **p<0.01.

5.3.6 The effect of IL-33 on polarised epithelial cells

In vivo, the epithelium is composed of differentiated epithelial cells, ciliated or non ciliated (secretory and basal) cells separated from underlying mesenchymal cells by a basement membrane (BM). BM's are made of type IV collagen and other materials [548]. Cells are arranged in a pseudostratified manner with all cells in contact with the BM but only some reaching the luminal surface. Having

characterised the effect of IL-33 on epithelial cell lines and pHBE cells, in order to further investigate the role of IL-33, it was important to use a system that could better mimic the characteristics of these *in vivo* differentiated epithelial cells [549,550]. For *in vitro* models, differentiation is commonly achieved by growing the cell cultures at an air-liquid interface (ALI) [549]. Cells are usually grown on a permeable membrane of tissue culture inserts with collagen support, allowing the generation of apical and basal layers.

Without regular access to donor primary human cells, the aim was to develop primary culture from mouse tracheal cells based on a novel method growing murine airway ECs on a native basement membrane [454]. The basal membrane is generated by Madin-Darby canine kidney (MDCK) cells [455] and contains normal components of an epithelial basal lamina including type IV collagen [551] and as such more representative of the *in vivo* state. These methods are more fully described in chapter 2. However, in pilot experiments a large number of mice were required to obtain a significant amount of cells (see above). Therefore, the decision was taken to extend these methods to use the NHBE cells in the same native BM model.

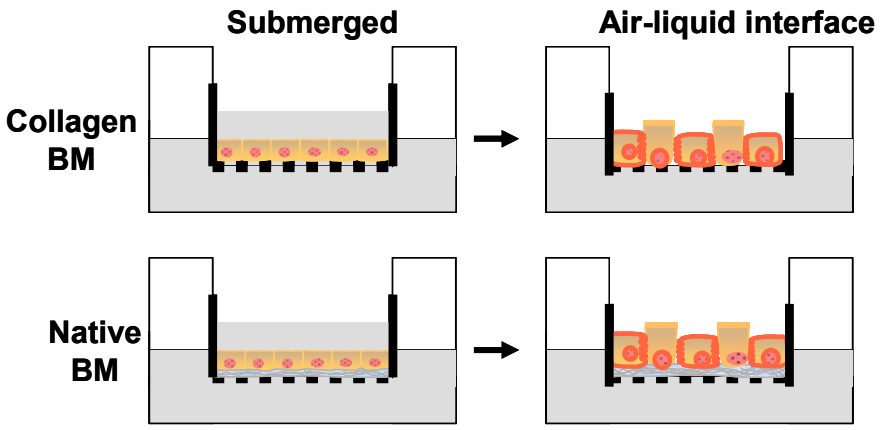


Figure 5.16: Generating an *in vitro* model of differentiated respiratory epithelium
Transwell tissue culture inserts (0.4µm or 8 µm) with collagen on apical surface were placed in 6 well culture plates. Collagen BM is collagen-only basement membrane on 0.4 µm culture inserts. Native BM was formed by applying Madin-Darby canine kidney (MDCK) cells to the apical surface of the collagen gel on 8µm transwells for 3 weeks when the cells were removed by osmotic and detergent lysis. NHBE were grown in submerged culture on the collagen BM or native BM until confluence was reached after which they were exposed to an air-liquid interface for differentiation.

Electron microscopy (EM) assessment of the airway epithelial cell morphology is the gold standard for confirmation of a ciliated, pseudostratified epithelium [455]. Other features of differentiation include the development of stable

transepithelial electrical resistance (TEER) reflective of development of tight junctions and so without EM, the TEER was used to confirm differentiation in these cultures [552] (Figure 5.16). The morphology of the cells on the native BM is shown in Figure 5.17.

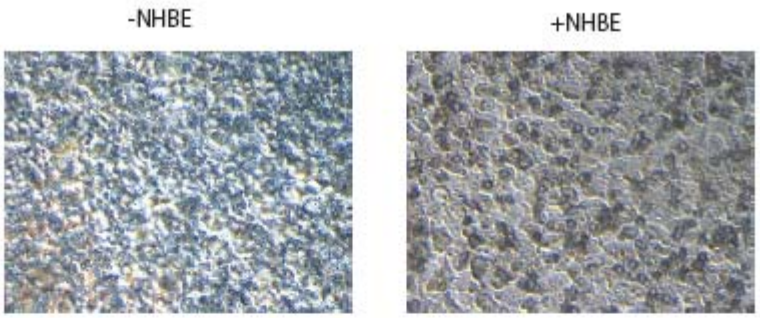


Figure 5.17: Morphology of NHBE on native collagen/MDCK derived BM
Phase contrast image of transwell culture inserts with and without cells at ALI on the surface. Sheets of tightly packed epithelial cells are seen with a cobblestone appearance. Magnificaion x 20. TEER measured on the transwell 140-150 ($\Omega.cm^2$)

To assess whether these differentiated cells had similar angiogenic profile of mediator production as NHBE and primary cells, IL-33 was added to the basal surface culture medium. Cells grown on collagen only BM showed small increases in production of IL-6, IL-8 and VEGF at low levels of IL-33 stimulation (Figure 5.18A). Higher concentrations of IL-33 (≥100 ng/ml) were possibly inhibitory (data not shown). There was no significant production of eotaxin, MIP-1α or IL-17 (data not shown). Cells grown on the native BM produced increased VEGF higher concentrations of IL-33. In contrast, IL-6 and IL-8 production did not increase with IL-33 treatment (Figure 5.18B).

The results in this section represent preliminary data because of time constraints on this project further experimentation was not possible. A number of limitations were encountered; the technique is novel so further work required to optimise and validate it; the development of each *in vitro* culture system takes several weeks (3-6); inter-experiment comparison was difficult with varied levels of mediator production and the reasons for this are unclear. However, it does seem that VEGF is reproducibly produced from differentiated epithelial cells in response to IL-33. Further work includes consolidating the technique and performing a scanning EM to identify ciliated cells as well as optimising IL-33 concentrations and treatment duration. Following this, using primary cells a

robust, differentiated model to fully elucidate the effects of IL-33 on airway epithelial cells can be used. Subsequently, this could be modified with 'diseased cells' and cells transfected with virus to mimic *in vivo* states.

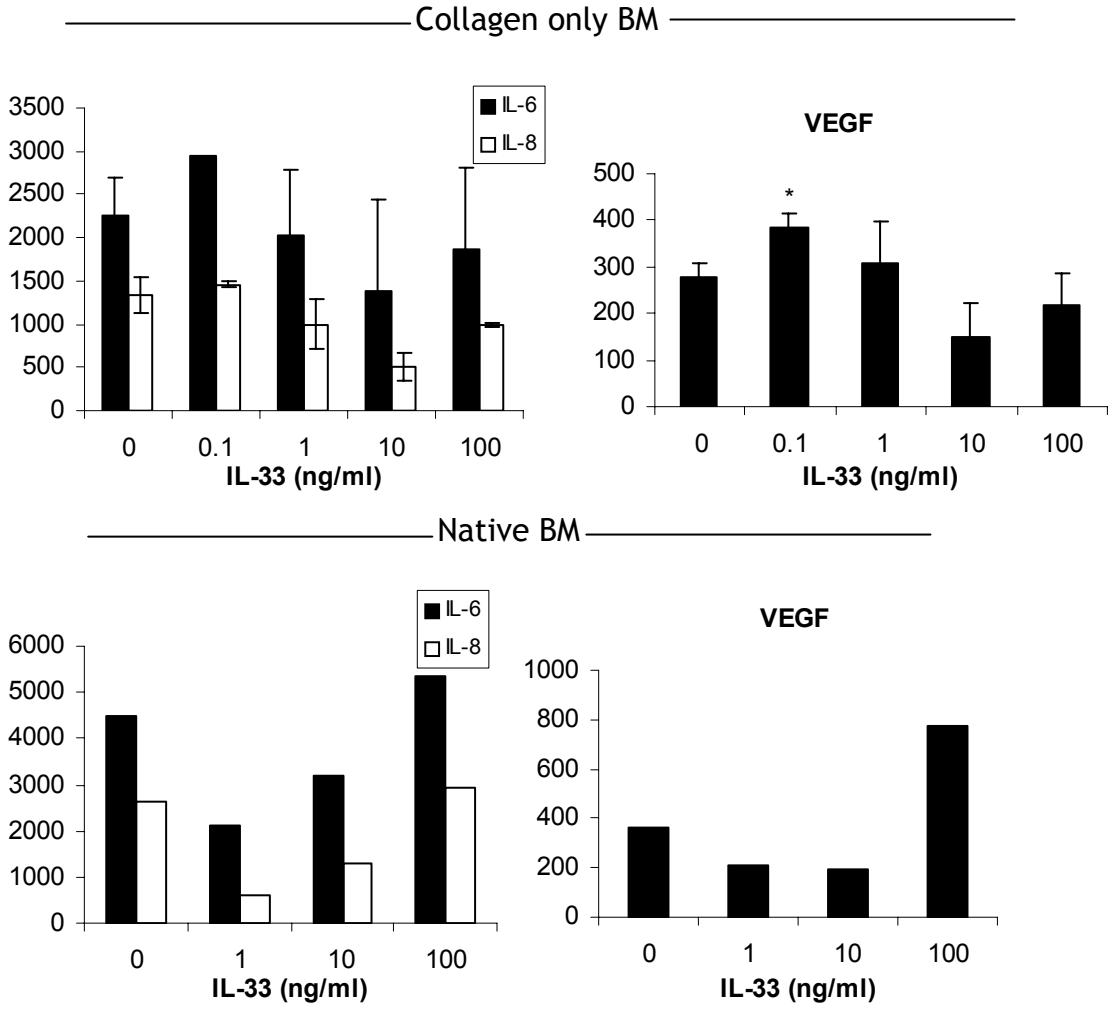


Figure 5.18: Cytokine and chemokine production from differentiated NHBE cells
NHBE were grown on either A) on collagen gels 0.4µm transwell inserts (collagen only BM) or on B) a MDCK derived membrane deposited on collagen gel on 8µm transwell inserts (native BM) When cells reached confluence, they were exposed to an air-liquid interface for differentiation. 7 days later increasing concentrations of IL-33 were added to the culture medium and 24 hours later supernatants collected and concentration of mediators measured by ELISA. Results shown are mean +/- SD of 2 independent experiments in A) and representative of 3 independent experiments in B).

5.4 IL-33 induces angiogenesis and vascular remodelling in the lung

In chapter 4, following exogenous treatment of mice airways with IL-33, an increase in the production of the pro-angiogenic factors VEGF and KC was seen within 24 hours, suggesting resident cells were responsible. One type of resident cell, the epithelial cell expresses ST2L and moreover, IL-33 drives production of VEGF and IL-8 from these cells. Hence to further investigate the effect of these IL-33 promoted pro-angiogenic factors, evidence of a subsequent angiogenesis and remodelling effect was sought *in vivo*.

5.4.1 IL-33 increases endothelial cells in lung

Angiogenesis is the growth of new blood vessels from existing ones and remodelling involves structural changes in the vessels with the formation of new ones [553]. Features of pulmonary vascular remodelling include changes in all layers of the vessel wall with fibroblasts, smooth muscle cells as well as endothelial cell proliferation involved [554]. VEGF is an endothelial-specific mitogen and involved in angiogenesis [206]. Therefore to ascertain if IL-33 might result in endothelial cell changes through VEGF or other mechanisms, whole lung digests from mice treated with IL-33 were analysed for the presence of $CD31^{+ve}$ cells, a marker for endothelial cells.

Figure 5.19 shows lungs treated with IL-33 have increased numbers of endothelial cells. To ascertain if like epithelial cells, endothelial cells can respond directly to IL-33 and amplify the response among structural cells, co-expression of ST2 should be measured. Unfortunately, the available CD31 antibody was labelled with the same fluorochrome as the only ST2 FACS antibody so a direct or indirect effect could not be determined.

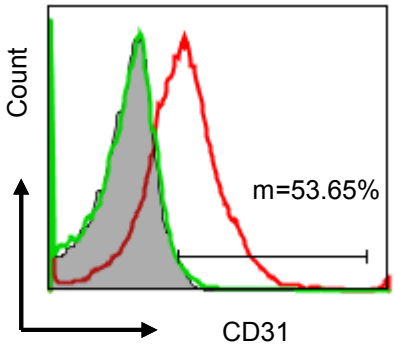


Figure 5.19: IL-33 increases endothelial cells in the lung
BALB/c mice were given PBS or 2 µg IL-33 or PBS intranasally for 7 days. Lungs from individual mice were collected, digested and single cell suspensions prepared as previously described. Cells were stained with CD31 or isotype antibodies. A histogram of CD31 expression is shown. Filled histogram isotype control and unfilled CD31 expression in PBS (green line) and IL-33 (red line) treated mice. Data shown is representative plot. CD31 expression in PBS treated mice 4% and in IL-33 treated mice 53.65 %.

5.4.2 IL-33 increases in vascular remodelling in lungs

Next, the ability for IL-33 induced BAL VEGF and KC to lead to vascular remodelling in the lungs was assessed. Using the i.n. IL-33 model (Figure 4.1-2) lungs of individual mice were fixed in formalin and then histological sections stained for von Willebrand factor (vWF) using immunohistochemical methods. VWF is made within endothelial cells which line vessels and is crucial in the formation of blood clots. An angiogenic index was developed for assessment: the numbers of vessels were identified by the presence of a clear structural lining and positive staining for vWF; 10 fields that included both airway and vessel were then assessed per slide and index calculated using imaging software (see methods chapter 2). Two blinded assessors quantified the changes.

IL-33 treated lungs had significantly increased number of blood vessels identified by vWF staining. (Figure 5.20 A-D). This increase in blood vessels resulted in a trend of increased in percentage lung area covered by vessel but this did not reach statistical significance.

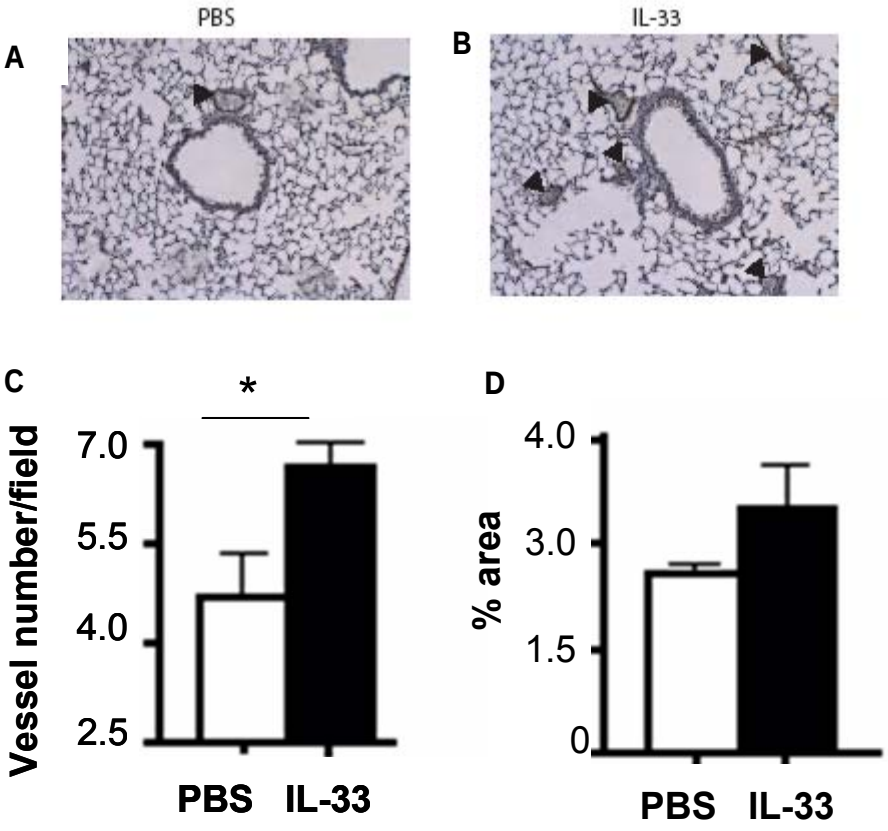


Figure 5.20: IL-33 drives vascular remodelling in the lung
In mice treated with intranasal IL-33, lungs removed were fixed and stained with vWF. Representative sections are shown in A) PBS and B) IL-33 treated lungs. The sections were analysed for C) vessel number and D) vessel area as a percentage of overall area. Results shown are mean each group +/-SEM, n= per group. Arrowheads represent vessels. *p<0.05.

The increase in vascular remodelling driven by IL-33 is hypothesised to be mediated by the release of VEGF from the ECs. To conclusively demonstrate this, neutralisation of the growth factor with resultant inhibition of angiogenesis is required. Using the conditioned media from experiments in Figure 5.10, human endothelial cells (HUVEC) formed tubule with increased branching points in an *in vitro* angiogenesis assay. This effect was eliminated when the conditioned media was pre-incubated with VEGF neutralising antibody (assay performed by Dr M Shepherd) (Figure 5.21).

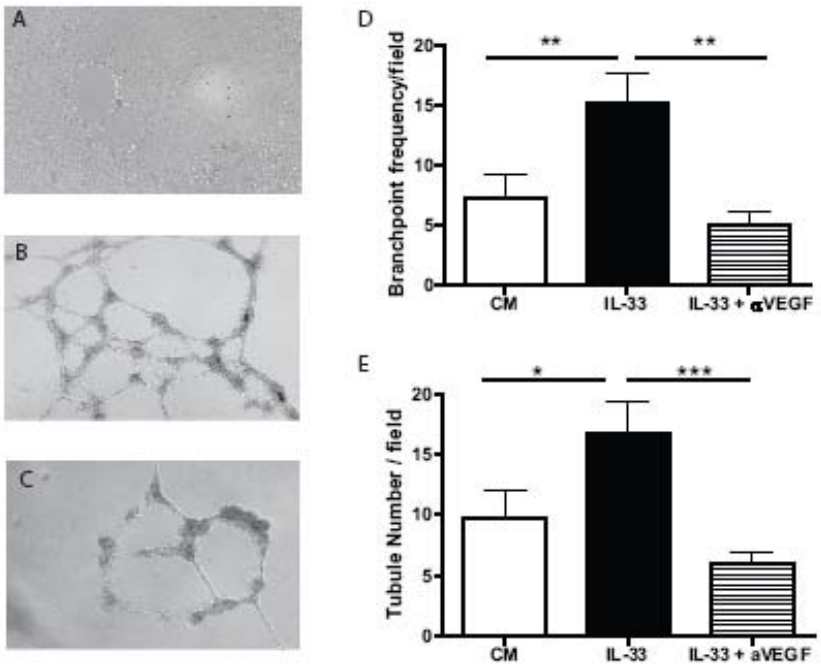


Figure 5.21: IL-33 treated NHBE promote VEGF dependent angiogenesis
Endothelial cells grown on collagen plugs in culture medium enriched with 50% NHBE conditioned medium (CM) A) or IL-33 treated NHBE conditioned medium (IL-33) B). Some medium was pre-treated with anti-VEGFantibody before addition to endothelial cells C). Branchpoint frequency D) and tubule number E) were assessed.

5.5 Conclusions

The work presented in this chapter aims to address the role of ST2 on airway epithelial cells and endothelial cells. In summary, ST2L is present on airway epithelial cells and that its activation by IL-33 leads to release of inflammatory and pro-angiogenic mediators which in turn drive vascular remodelling in the lung.

The cell(s) initially responsive to IL-33 in the airway is not clearly elucidated. In chapter 4, a significant number of unidentified resident cell ST2^{+ve} cells were found in healthy lungs.

Activated epithelial cells weakly express ST2L mRNA [306]. ST2 protein by immunohistochemical analysis has been demonstrated in chapter 3. The abundance or expression of membrane bound ST2 or its function on airway epithelial cells was not known. These data clearly shows that resident epithelial cells in healthy lungs, epithelial cell lines and primary airway epithelial cells express surface ST2L capable of responding to IL-33 *in vivo*. More recently, ST2

mRNA and protein expression has been verified in airway ECs [320,555] and also endothelial cells but not other resident tissue cells, rested fibroblasts or smooth muscle cells [320]. Data here show the presence of IL-33 did not affect the expression level of ST2 on EC in *in vitro* experiments. Subsequent to this work, others have shown different factors may influence ST2 expression on ECs with IL-4 enhancing expression of ST2 in NHBEs at a mRNA and protein level [320]. More recently, morphology of ECs found to be unaltered in IL-33 treatment and ST2 expression was enhanced by IL-13 in differentiated goblet cells but not ciliated cells [556].

The role, if any, of ST2 in the development in epithelial cells has not been considered here but is not known to be involved in the development of Th2 or mast cells[338]. ST2 forms a receptor complex with IL-1RAcP with NFκB and MAPK signalling induced by IL-33. The signalling pathways in ECs was not specifically addressed here but others have shown IL-33 induces ERK, JNK but not p38 phosphorylation in NHBEs [320,555] and collaborative data from our laboratory using cells generated from experiments presented here agree with this data (Mirchandani A, unpublished results).

The effector function of IL-33/ST2 on epithelial cells was considered and when treated with IL-33 *in vitro*, EC produced a range of pro-inflammatory and pro-angiogenic mediators were produced including IL-8, IL-6, and VEGF. Interleukin-8 was released from NHBEs and pHBEs. This was dose dependent at physiological concentrations of IL-33 and inhibited by CS in agreement with later published studies [320]. IL-8 (CXCL8) is a neutrophil chemotactic factor and is an important mediator in the acute innate immune response, often secreted by cells following TLR activation. It also triggers phagocytosis and is also a potent promoter of angiogenesis [557]. As described, asthma phenotypes include those with a neutrophilic predominance but it is unclear if IL-33 role has a role in this less well investigated phenotype. Concentrations of other CXCL family neutrophil attractants; epithelial neutrophil attaractant-78 (ENA-78)/CXCL5, GROα/CXCL1, and GROβ/CXCL2 were not measured but would be useful to investigate the role of IL-33 in neutrophil recruitment.

In chapter 4, eotaxin/CCL11 was identified as partially responsible for the IL-33 driven eosinophil recruitment; ECs are hypothesised as the source. However,

little or no eotaxin was measured in the samples from any of the cell types measured here. It is possible a second co-stimulus is required for eotaxin release *in vivo*. In concordance, TNFα induces eotaxin production from ECs, which is further enhanced by Th2 cyotkines [558]. However, it is most likely that ECs are not the main source of eotaxin in IL-33 driven eosinophilia. Potential candidates are fully considered in chapter 4 with fibroblasts the most likely source [530]. In keeping with the view that ECs have the capacity to recruit immune cells in response to a danger or inflammatory signal, IL-33 induces the release of MCP-1, RANTES, MIP1α and MIP1β, predominantly attracting monocytes, T cells and eosinophils.

IL-6 production from epithelial cells was enhanced by IL-33. IL-6 is pleiotropic cytokine and as an anti-inflammatory cytokine it inhibits TNF-α and IL-1 while activating IL-1Ra and IL-10 [559]. Interestingly, as IL-6 can regulate CD4 T cell responses, promoting IL-4 production during Th2 differentiation and inhibiting Th1 differentiation, it could be a further mechanism of IL-33 mediated Th2 responses [221].

With regard to Th2 cytokines, only low levels of IL-4 were produced indicating EC are not a significant source of IL-5 and IL-13 measured *in vivo* in response to IL-33. ECs can produce Th2 cytokines but are more likely to influence mast cell or T cell production of these cytokines by the release of secondary mediators. IL-12 was produced, promoting Th1 and antagonising Th2 differentiation but other type-1 cytokines such as IFNγ were not produced. Inducible IL-12 production from ECls has been reported in response to viral infection. Interleukin-12 also promotes anti-angiogenic activity directly and by promoting anti-angiogenic factor IP-10, which was produced here at a low concentration in NHBEs [560]. This suggests some homeostatic anti-Th2 or anti-angiogenic activity. The surrounding microenvironment in which these mediators are produced is important in determining whether levels produced translate to a clear effect *in vivo*. Cytokine growth factors G-CSF and GM-CSF release are often stimulated from epithelial cells by bacterial endotoxins or IL-17 [561,562]. These growth factors increase bone marrow stem cell release and neutrophil and eosinophil survival [512,563]. GM-CSF can also act as a true chemotaxin for eosinophils at higher concentrations [564]. Hence, EC could contribute to IL-33-induced eosinophilia.

Basic fibroblast growth factor (bFGF) is a strong mitogen for fibroblasts at low concentrations and a chemoattractant at high concentrations. Proliferation of lung fibroblasts has been shown to play a role in the induction of airway remodelling[565]. In the asthmatic airways EMT contributes to fibroblast population and a population of sub-epithelial myofibroblasts accumulates [566]. The role of IL-33 induced bFGF from ECs could contribute to this pathology but requires further investigation. Latest studies suggests IL-33 can directly promote remodelling via fibroblast production of collagen and firbronection [491]. At another mucosal surface, the skin, IL-33 promotes fibrosis in an IL-13 dependent manner with up-regulation of genes involved in ECM formation including collagens and MMPs [537].

Of particular note, the production of VEGF from epithelial cells was found consistently through the cell lines, primary cells and polarised airway model. VEGF has been detected in tissue and samples from asthmatics and is related to severity [205,567]. VEGF is a multifunctional angiogenic regulator that contributes to asthma pathology predominantly by angiogenesis but also stimulates epithelial cell proliferation and endothelial cell survival [568]. VEGF promotes vascular permeability and so the early rise in VEGF *in vivo* in response to IL-33 shown in chapter 4 will also promote plasma leakage and inflammation. Angiogenesis occurs acutely with allergen challenge [569] and in an allergen environment, IL-33 driven VEGF could potentially contribute to this. Other VEGF producing cells include macrophages and mast cells but neither appear to produce VEGF in response to IL-33 directly but rather it augments release in the case of mast cells [445,570]. IL-33 enhanced VEGF production *in vivo* is seen in a colitis model in mice [447].The secreting cells are not identified but given a similar mucosal surface, the EC is a candidate. Beyond the vasculature, VEGF has further functions including Th2 inflammation which will also be enhanced by IL-33 induced VEGF [206].

The observation that VEGF production is uninhibited by CS is significant, suggesting any clinically driven IL-33-induced VEGF-mediated angiogenesis would continue unchecked regardless of a diminished inflammatory response. This finding of CS insensitivity here goes against published studies of diminished VEGF production with CS [571]. IL-33 expression on airway smooth muscle cells has also been shown to be resistant to CS, therefore IL-33 release and effector function could be beyond the scope of current asthma treatments [399].

To mimic proinflammatory conditions, NHBEs or pHBEs were also exposed to a cytomix (TNFα, IFNγ and IL-1β) or poly(I:C) to mimic viral infection. As would be expected, IL-8 and IL-6 production were increased under these conditions and although this was enhanced by IL-33 the amplification of these cytomix factors outweighed that of IL-33. Release of VEGF was noticeably increased when cells were treated with IL-33 alongside cytomix and the amount produced in response to poly (I:C) was only modestly more than with IL-33. This suggests VEGF may be a more specific IL-33 driven response. Interestingly, with similar stimulation release of sST2 but not IL-33 has been detected from primary human cells [316].

In contrast to the data presented here, subsequent similar results published describe only IL-8 being produced from primary NHBEs from 2 donors. There was no IL-6, IL-4, MCP-1, IP-10, IL-12 and in agreement with data here no IL-5, IL-10, IL-13, TNFα, IL-1β [320]. Other growth or angiogenic factors were not measured. Results from different donors may vary but these published results are more in keeping with the results presented here from the polarised cells.

A number of EC cell types were used in these experiments looking at ST2 expression and IL-33 mediated effects and so a number of limitations need to be considered. The majority of experiments were performed in a NHBE cell line, originally from a primary donor, subsequently immortalised. A range of EC techniques are described and successfully performed in HBECs [572]. Although cell lines provide a useful tool readily accessible for investigation they may have undergone significant changes to avoid senescence which could affect biology. Other cell lines should be used in repeat experiments for comparison including BEAS-2B and the alveolar epithelial cells A549.

The murine cells used for ST2 expression were tracheal rather than bronchial in origin. One study in humans showed a high level of similarity between cells of tracheal and bronchial origin within and between different human donors in a genome-wide expression profile comparison [550]. Although a good potential source of primary cells, the main limitation found using the murine tracheal cells is in the number of cells produced. Although there is scope to optimise this protocol, to overcome this problem an alternative technique could be to use the murine nasal septum, which has been shown to be a good source of murine respiratory epithelium [573]. FACS analysis of CK (intracellular) and ST2 (surface) was

consistently difficult to interpret when combined despite straightforward single antibody analysis. ST2 binds non-specifically to dead cells and despite appropriate washes and exclusion for these, this may affect interpretation. Identification of ECs from whole lung digests has subsequently been identified using a robust gating strategy of $FSC^{hi}SSC^{med}$, $EpCAM^{+}$ (epithelial cell adhesion molecule), $CD45^{-}$ and $MHCII^{-}$ [574]. Alternate markers of EC including MUC5AC (goblet cells) [575], acetylated α-tublin [556] and E-cadherin [576] could be attempted by FACS or using immunohistochemistry techniques.

In an attempt to imitate the *in vivo* polarised nature of epithelial cells, an *in vitro* culture differentiated culture system was established. Compared to *in vivo* studies these models have advantages of flexibility, controlled experimental conditions, and opportunity for intervention. Overall the mediator output from these cells appeared more muted, with VEGF being the most reproducible mediator. High baseline levels of IL-8 in these cells meant any additional effects generated by IL-33 was hard to distinguish. Recently, using a system of collagen only BM and differentiated NHBE (primary donor), Tanabe *et al* also found no IL-33 mediated increase in IL-8. However, differentiated EC subtype goblet cell, achieved by supplementing media with IL-13, did secrete apical IL-8 via a ST2-ERK pathway [556].

Because of the limitations in availability of primary cells, NHBEs were used in establishing the system. Some cells lines are reported to differentiate and develop tight junctions less well than primary cells, which could explain some of the findings [577]. Using commercial primary cells is unlikely to eliminate this limitation as donor-to-donor variability in development of differentiation exists [577]. Primary murine respiratory epithelial cells from the nasal septum from inbred mouse strain grown on a native BM could provide a novel solution to this variability, using this alongside primary human cultures when available for comparison. It is also important to consider the EC in asthma as its inherent dysfunctional nature could respond differently to IL-33. A fully validated culture system such as that described will be invaluable in studying more fully the effects of IL-33 on epithelial cells function. This requires confirm of differentiation where EM to identify cilia, secretion of mucus and development tight junctions or *zona occludens* demonstrated by stable transepithelial resistance or immunoflourescene for zo-1 could be used.

In asthma, there is an increase in airway vessel number and size [578]. The number of endothelial progenitor cells (EPCs) correlates with an increase in vessel number [579]. The generation of new vessels is suggested to cause swelling and stiffening of the airway wall and hence affect airflow obstruction as well as remodelling effects. IL-33 drives the production of VEGF from epithelial cells, therefore its role in angiogenesis in the airway and considering features comparable to asthma were investigated. The number of endothelial cells in the lung was increased in the lungs of mice treated with IL-33. It was not possible to say whether this increase was created by proliferation of resident cells or influx of EPC recruited directly or indirectly. Recruitment of EPCs appears to be dependent on IL-8 and IL-8 receptor (CXCR2) knockout attenuates bronchial angiogenesis in pulmonary ischaemia [580]. In support of this, data here shows IL-33 induces IL-8 as well as other angiogenic factors RANTES, FGF, MCP-1 in response to IL-33 [581]. Along with VEGF these factors aid promotion of an angiogenic switch akin to asthma [579]. In humans, ST2 mRNA expression has been described in endothelial cells and is proliferation dependent (in HUVECs) thus IL-33 could also exert a direct effect [306,315,320,582]. Endothelial cells secrete IL-8 and IL-6 in response to IL-33, a further source of these mediators *in vivo* [582]. The direct effects of IL-33 on endothelial cells were further investigated by Choi *et al*. They showed the number of $CD31^+$ cells in tissue, by immunofluoresence, were increased by IL-33 treatment with endothelial cell chemotaxis and proliferation also enhanced. These findings were dependent on IL-33 and appeared to be mediated by nitric oxide (NO) production in the endothelial cells [413].

Angiogenesis is a pathologic component of asthmatic airway vascular remodelling and also occurs acutely with allergen challenge [569]. This correlates with the finding in chapter 4 of increase in BAL VEGF within 24 hours of airway challenge. The effect of IL-33 on remodelling was ascertained using an *in vivo* model. There was a significant increase in the number of VWF positive blood vessels following exposure to IL-33. The mechanism appeared to be via the release of VEGF as *in vitro* VEGF blockade resulted in reduction of microtubule vessel formation. Extending this work by measuring VEGF, KC and GRO-β /CXCL2 levels in lung homogenate and *in vivo* blockade of VEGF to confirm this mechanism would be of value.

Further work examining other aspects of remodelling would be of interest including: identification of collagen (Sirius red) and quantification of total collagen in lung tissue; identification of peri-bronchial smooth muscle thickness (α-smooth muscle actin); identification of proliferating mesenchymal cells (p-anca) and assessment of mucin producing goblet cells (PAS). In addition, examining any differential effects in a chronic asthma model such as that using HDM which does not show resolution of remodelling, by assessing the effect of a neutralising antibody to IL-33 [74]. Impaired barrier function along with delayed wound healing in the gut in response to IL-33 has been described and this could also be the case in the airway [583]. Regulation of adhesion molecules, particularly VCAM-1 ICAM-1 and e-selectin should be assessed.

IL-33 was originally described as a nuclear factor and acts as a repressor of gene transcription. Nuclear IL-33 is present in EC [584] but, our studies also reveal further cytoplasmic AEC IL-33 (chapter 3). Dispersal of cytoplasmic proteins upon cell death or active secretion could release IL-33 into the lumen. Much research focus has been on the release of IL-33 from epithelial cell as a main source of IL-33 in the airway [186,469] and its relationship with other epithelial derived cytokines including IL-25 and TSLP [185]. IL-33 appears to be the most upstream [585]. Data presented here now show in addition EC are also capable of responding to IL-33 in a paracrine and autocrine fashion.

In summary, the airway epithelium is now appreciated as an 'epimmunome', a key cell in triggering and perpetuating innate and adaptive immunity [586]. EC release IL-33 and initiate Th2 inflammation and furthermore, express ST2L, responding to IL-33 in releasing mediators perpetuating inflammation and acting as a bridge between innate and adaptive immune systems. Details of interplay between ECs and immune cells are not yet fully understood. Improved culture models, cell specific genetically-modified mice and analysis of human biopsies should inform potential mechanisms. The airway epithelium is central in driving pathological changes in remodelling the asthmatic lung. Data presented here shows that in addition to initiation of effector type-2 responses, IL-33 also drives angiogenesis and vascular remodelling that typifies chronic asthma. Treatments for the associated airway remodelling in asthma are lacking and thus identification of molecules underlying this is a priority.

6 The role of ST2 and IL-33 in adaptive airway inflammation

6.1 Introduction and Aims

From the data described in chapter 4, it can be seen that innate cells in the airway produce type-2 cytokines IL-5 and IL-13 but not IL-4, in direct response to the cytokine IL-33. This results in features which replicate murine AAI and human asthma. Th2/type-2 cytokines play a crucial role in allergic airways disease and can also be produced by CD4 Th2 cells in a well studied mechanism [1,20]. The generation of allergen sensitisation is dependent on IL-4; IL-4 regulates the commitment of T cells to this phenotype and the development of allergen-specific immunoglobulins [42]. However, there is also evidence for IL-4 independent sensitisation pathways in asthma [46]. IL-33 stimulates Th2 cells to produce IL-5 and IL-13 but not IL-4 [293,422] and *in vivo,* IL-33 exacerbates the effector response in murine model of AAI (Kewin PK, 2007, PhD Thesis, University of Glasgow). Taken together, a role for IL-33 in the adaptive immune responses in allergic airways disease is proposed.

A role for ST2 in asthma and AAI has been considered given its expression on Th2 and mast cells, both of which are important in the generation and persistence of human and murine asthma [19,40]. Previously, it had been demonstrated that $ST2^{-/-}$ mice develop attenuated inflammation in selected models of AAI [344,496] and application of soluble ST2 may be also beneficial in this model [341].

To further assess the role of IL-33/ST2 in the generation of adaptive Th2 responses and in particular the contribution of IL-4, the allergen sensitisation phase in a murine model of allergic airways disease will be studied using WT, $IL\text{-}4^{-/-}$ and $ST2^{-/-}$ mice.

6.2 IL-33 at allergen sensitisation

6.2.1 Choosing a model to study the effects of IL-33 at allergen sensitisation

There are a number of different techniques used to model asthma in experimental settings. The most widely used approaches are: allergen sensitisation with an adjuvant to boost responses followed by allergen challenge;

transfer of allergen-specific lymphocytes; transient gene transfer; viral infection; and using transgenic mice [500].

6.2.1.1 Adoptive transfer

To study the effects of IL-33 at sensitisation, the adoptive transfer of allergen-specific lymphocytes was chosen. It has the advantages of being able to control and adjust sensitisation and challenge conditions separately as well as the ability to study the transferred lymphocytes using different mouse genotype-specific FACS markers.

TCR transgenic mice CD4 D011.10 cells express major histocompatibility (MHC) class II restricted TCR specific for a peptide derived from ovalbumin (OVA), OVA peptide (ovapp). Cells from these mice were stimulated *in vitro* towards a Th2 phenotype in the presence of skewing cytokines and transferred to naïve WT mice as in Figure 6.1A. These cells maintain their characteristics after being injected and express the cell marker KJ-126. In pilot experiments (data not shown) cells or PBS were given i.v. followed by 3 i.n. OVApp or PBS challenges. No response was seen in the PBS group so these controls were not routinely repeated in subsequent experiments. Furthermore, the transfer of 0.5 $x10^6$ or 1 $x10^6$ cells in mice challenged with OVApp developed a small amount of eosinophilia so optimal dose experiments were completed as in Figure 6.1. Results in Figure 6.1B and C indicate 2 $x10^6$ cells are sufficient to generate airways inflammation. A further dose response is seen with the transfer of 5 $x10^6$ cells however, $2x10^6$ cells was chosen for further experiments to minimise resources required to generate cell production. Transfer was confirmed by presence of KJ 126^+CD4^+ D011.10 cells in the BAL by FACS analysis (by Dr Kurowksa-Stolarska, data not shown).

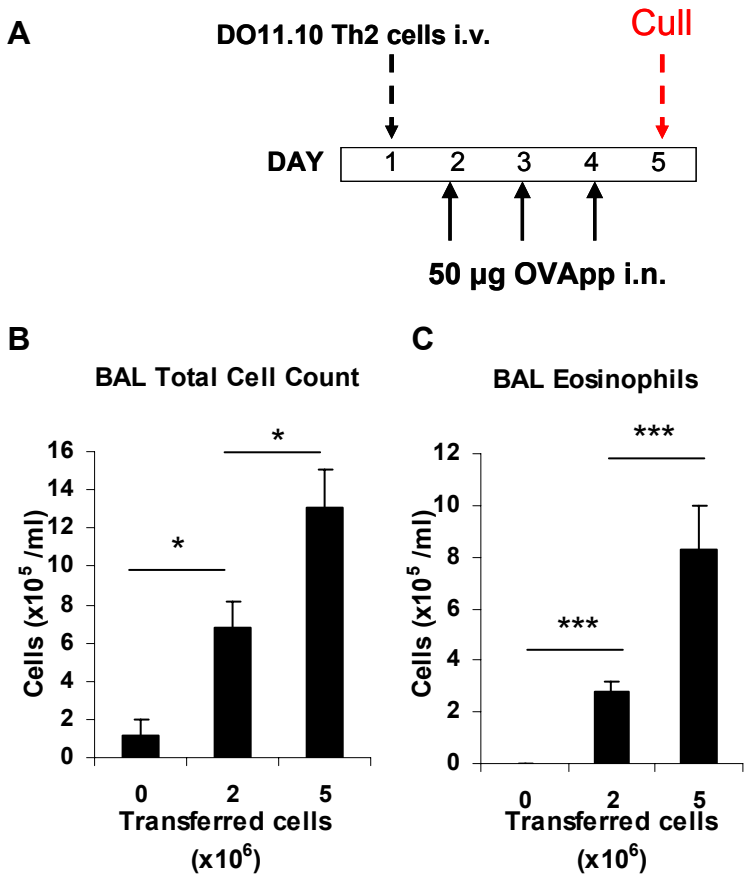


Figure 6.1: Testing adoptive transfer model using OVA specific T cells
A) WT mice were challenged i.n. with 10µg of ova peptide (OVApp) on 3 consecutive days following transfer of 0, 2 or 5 x10^6 DO11.10 CD4 Th2 cells. Cells were grown *in vitro* as described in chapter 2. B) Total cell counts and C) eosinophils counts in the BAL. Group size n=2-4, (p * <0.05, p*** <0.001). Representative of 2 experiments.

Following on from the adoptive transfer of the Th2 cells, IL-5 and IL-13 producing $CD4^+$ T cells were transferred i.v. to naïve mice as per Figure 6.1A (generated by Dr M Kurowska-Stolarska). These IL-33 stimulated cells develop in the absence of IL-4 [496]. Following subsequent allergen challenge, low levels of AAI were detected in the BAL by the presence of eosinophilia (Figure 6.2B). Mediators in BAL and serum were at very low levels (data not shown). The amount of AAI was low and it was deemed insufficient to easily detect differences between the groups. In addition, problems generating and transferring large numbers of cells consistently to WT mice occurred. Thus, the decision was taken to assess endogenously generated cells using the more conventional antigen sensitisation and challenge 'asthma model'.

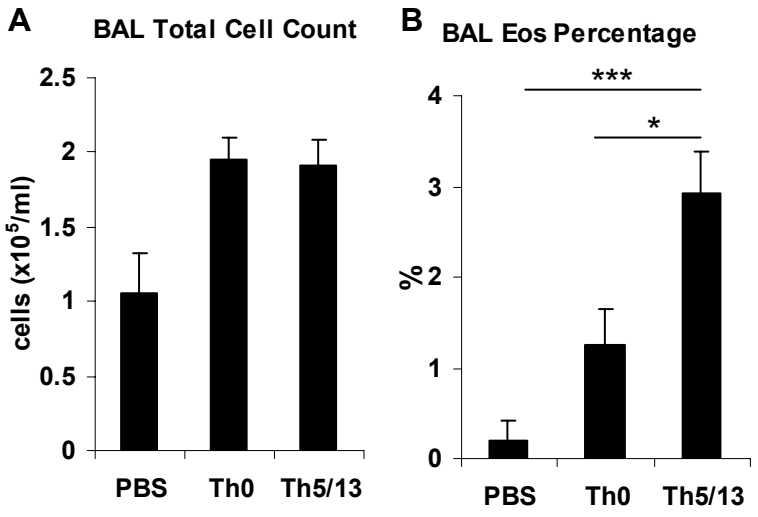


Figure 6.2: Testing adoptive transfer model using OVA specific T cells
WT were challenged with 10µg of ova pp on 3 consecutive days following transfer of 2 x10^6 DO11.10 Th0 or IL-5 and IL-13 producing CD4 cells (Th5/13) cells or PBS on day 1 as per Figure 6.1A). Cells were grown *in vitro* as described in chapter 2. A) Total cell counts and B) eosinophil percentage in the BAL. Group size n=5-8 per group, (*p <0.05, ***p <0.001).

6.2.1.2 Antigen sensitisation and challenge

To study the acute allergic response in this experiment set a shorter 14 day model of allergic airways inflammation (AAI) adapted from Stock et al was chosen in preference to the traditional 28 day model[452]. Briefly, a sensitising i.p. injection of OVA along with an adjuvant is followed 8 days later by 3 consecutive OVA i.n. challenges as per Figure 6.3A. Initially, experiments were performed to robustly test this model. Mice were treated as per protocol in Figure 6.3A and demonstrated significantly increased total BAL total cell counts from 0.6 x10^5/ml in the PBS treated mice to 2.84 x10^5/ml in OVA treated mice (Figure 6.3B). The differential cell count showed a significant increase in all cell types analysed. Eosinophils in particular increased from 0 in PBS group to 0.89 x10^5/ml in OVA treated group (Figure 6.3C).

Th2 cytokine production is classically elevated in murine asthma models and so cytokine levels were measured in the BAL fluid by ELISA (Figure 6.3D). Higher amounts of IL-4 and IL-5 were detected in OVA challenged compared to PBS challenged mice.

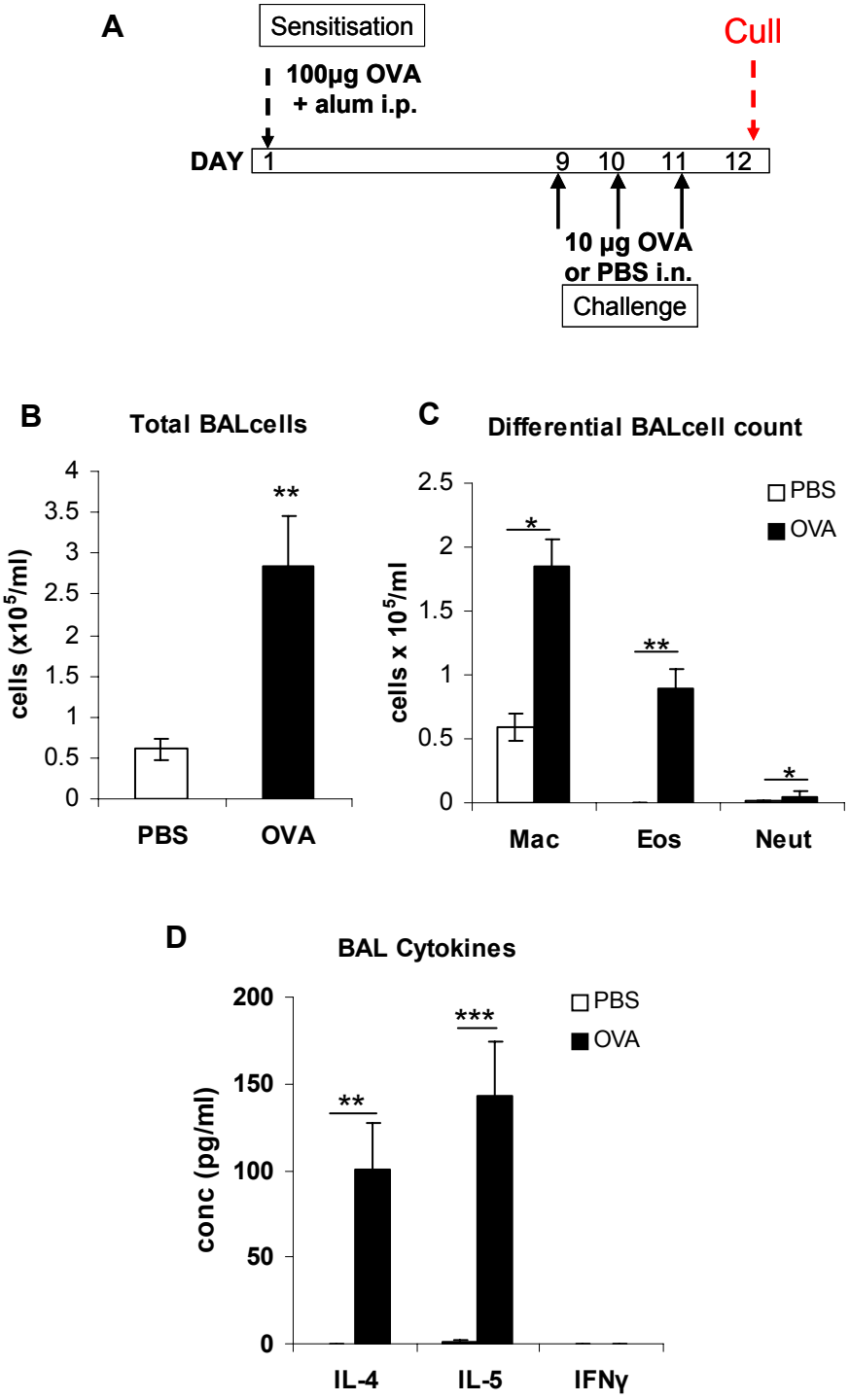


Figure 6.3: The 12 day allergic airways inflammation protocol
A) BALB/c mice were immunised with OVA (100µg) on day 1 and challenged with OVA (10µg) in 30 µl of PBS on day 9, 10 and 11. Control mice were challenged with PBS alone. BAL and further samples were acquired 24 hours following final airway challenge. B) Total and C) differential cell count in the BAL. D) BAL cytokine levels measured by ELISA. Data shown mean ±SEM, n=5 per group. (*p <0.05, **p <0.01 ***p <0.001). Data representative of at least 3 experiments.

The antigen (Ag)-specific recall response in the DLN was examined by culturing freshly isolated cells with OVA *in vitro*. Increased levels of IL-5 and IL-4 were measured in cells from OVA-challenged compared to PBS-challenged mice (Figure 6.4 A and B). Spontaneous and OVA-stimulated proliferation was observed in OVA sensitised but PBS challenged mice however this was significantly increased in the OVA challenged mice (Figure 6.4C). This antigen sensitisation and challenge model was subsequently used for further experiments to investigate the role of IL-33/ST2 at sensitisation.

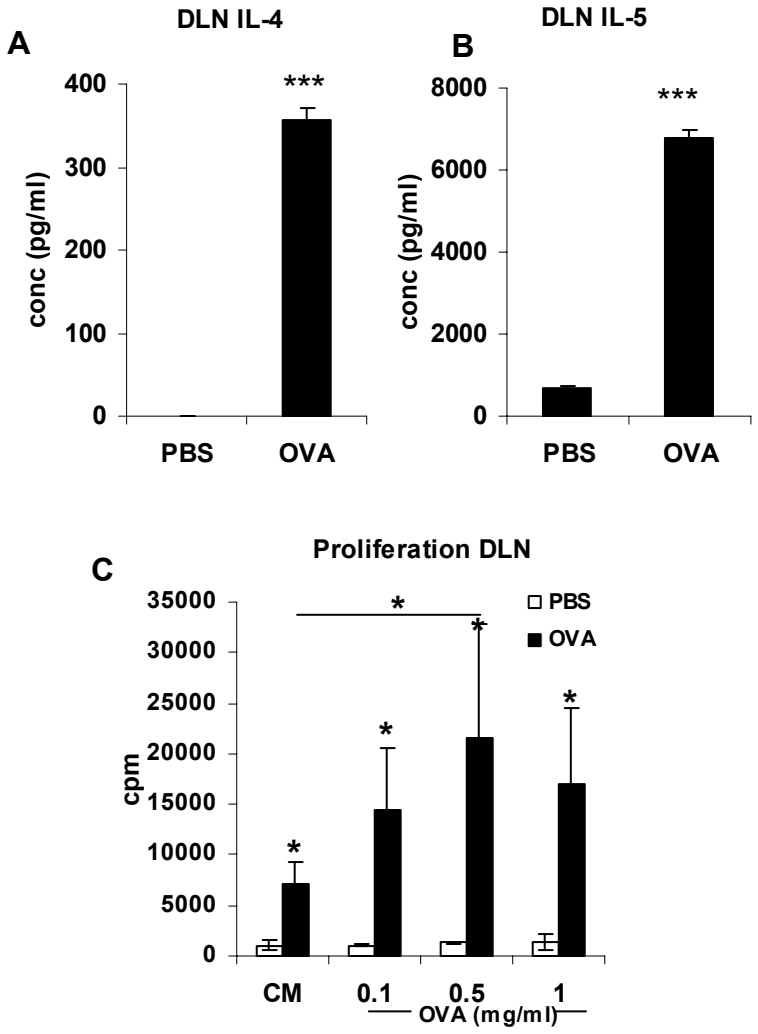


Figure 6.4: Antigen recall responses in 12 day allergic airways inflammation model
BALB/c mice challenged with OVA or PBS as per Figure 6.3A had mediastinal draining lymph nodes (DLN) collected and single cell suspension from individual mice prepared. Cells with final concentration of $1x10^6$/ml were cultured with complete medium alone (CM), or OVA at 0.1/05/1 mg/ml for 72 hours and culture supernatants collected and cytokines measured by ELISA Alternatively the cell suspension was stimulated in triplicate on 96 well plate with ^{3}H-thymidine incorporated for final 8 hours. Proliferation measured using a scintillation counter. Data shown mean ±SD of experimental triplicates of individual mice, n=5 per group. (*p <0.05, **p <0.01 ***p <0.001). Data representative of at least 3 experiments.

6.2.2 IL-33 does not exacerbate allergic airways inflammation in usual ovalbumin model in mice

A number of studies indicate ST2 modulates AAI [322,341]. We have demonstrated ST2 deficiency attenuates the airway inflammation demonstrated in Figure 3.3 [496]. This suggests a role for ST2/IL-33 in allergic airways inflammation, but neither the phase affected nor the mechanisms involved are clear. IL-33 drives production of $CD4^{+}IL\text{-}5^{+}$ cells *in vitro* suggesting it specifically plays a role in driving Ag-specific airway inflammation at sensitisation. To assess this, following on from the experiment in Figure 6.4, this experimental protocol was repeated with the addition of IL-33 administered i.p. at sensitisation as in Figure 6.5.

Mice treated with IL-33 in addition to OVA at sensitisation showed no difference in total BAL cell counts compared to OVA-sensitised mice. There was a non-significant increase in BAL eosinophilia, BAL IL-5, serum IL-13 and a decrease in BAL IL-4 (Figure 6.6 A-F).

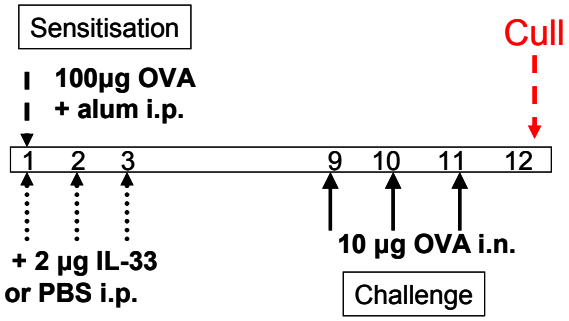


Figure 6.5: IL-33 at sensitisation in 12 day allergic airways inflammation protocol
AAI was induced in BALB/c mice as in Figure 6.3A. Mice were additionally treated with three doses of 2 µg IL-33 or PBS at sensitisation on days 1-3. BAL, serum, draining lymph nodes and lungs were obtained for analysis 24 hours following final i.n. dose.

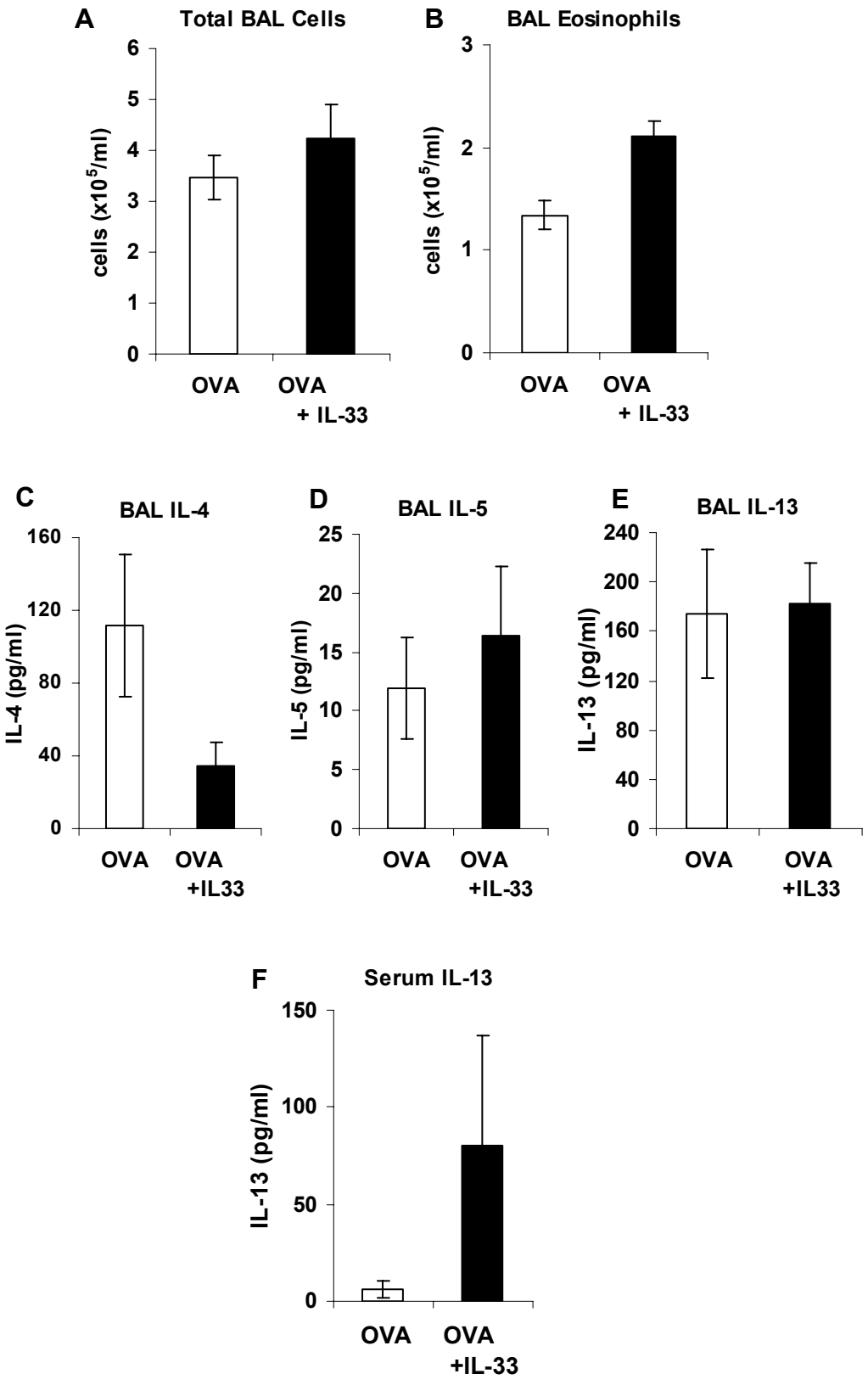


Figure 6.6: BAL cellular inflammation is not affected by IL-33 at sensitisation in 12 day allergic airways inflammation protocol
Mice treated as per protocol in Figure 6.5. A) Total cell counts and B) differential eosinophil counts in the BAL were analysed. Cytokine levels in the BAL C)-E) and serum F) were measured by ELISA. n=5-10 per group. Results shown are mean individual mice ±SEM p=ns for all comparisons. Data shown are representative of 2 experiments.

In the DLN, ST2 and intracellular cytokine expression was analysed by FACS. There was no difference in the expansion of $CD4^+ST2^+$ cells between the groups (6.15% vs. 5.9%). Gating on these cells showed an increase in single positive IL-4^+ and IL-5^+ in the group co-administered IL-33 at sensitisation (Figure 6.7) *In vitro* Ag-recall cultures did not demonstrate any significant changes in cytokine production between the groups (data not shown).

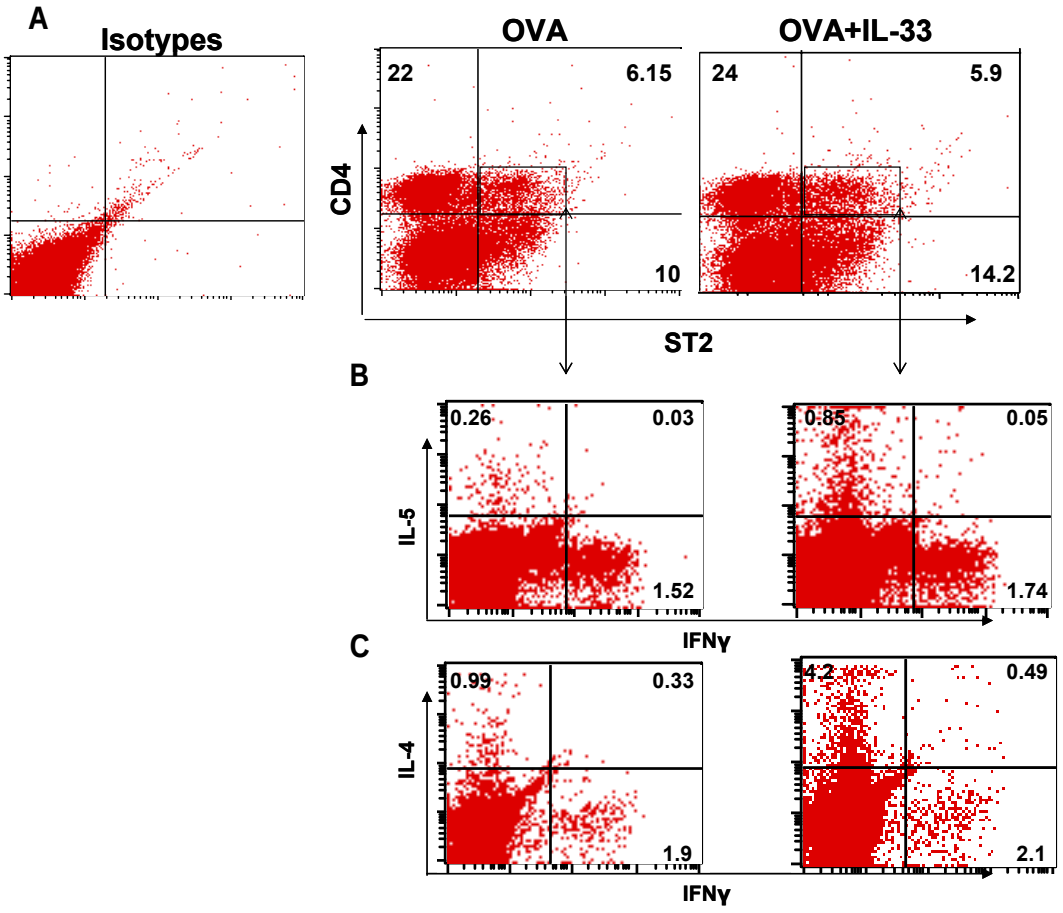


Figure 6.7: IL-33 at sensitisationincreases Th2 $CD4^+$ IL-4^+ as well as $CD4^+$IL-5^+IL-4^- cells
BALB/c mice were treated as per protocol in Figure 3.5A. Draining thoracic lymph nodes were collected and analysed by FACS. A) $CD4^+$ $ST2^+$ cells were identified with B) levels of IL-5^+ and IFNγ and C) IL-4^+ and IFNγ expression determined. Data from 1 experiment.

6.2.3 IL-33 at sensitisation induces $CD4^+IL\text{-}5^+$ cells and exacerbates allergic airways inflammation in a low dose ovalbumin model

The dose of antigen and presence of mediating cytokines are important factors in determining the development of helper T cell subsets. Low dose antigen can induce tolerance but Sakai *et al* demonstrated an increasing sensitising dose actually attenuates all the key model features in an inversely proportional fashion [280]. To determine if any effect of exogenous IL-33 on the ovalbumin model at sensitisation was being obscured by high dose OVA; the dose of OVA was reduced. The sensitisation dose was reduced to 10 µg and the challenge dose to 2 µg (Figure 6.7A).

6.2.3.1 IL-33 exacerbates cellular inflammation in the airway

Using this model with a modified dose of OVA, the IL-33 at sensitisation was administered as shown in Figure 6.7A. Mice given OVA only showed a modest increase in cell numbers compared to PBS sensitised mice but included a significantly increased number of eosinophils (Figure 6.7B and D). In contrast, mice given OVA and IL-33 at sensitisation developed a large influx of cells compared to OVA only sensitised mice (6.04×10^5/ml vs. 1.01×10^5/ml) (Figure 6.7B). The BAL differential count in mive given OVA+IL-33, composed approximately 50% macrophages and 50% eosinophils; a significantly higher proportion of eosinophils compared to the OVA-only sensitised mice (26% eosinophils) (Figure 6.7 C and D). Importantly, control mice given only IL-33 only at sensitisation and challenged with OVA, showed no increase in total or eosinophil cell counts in comparison to PBS treated mice. To demonstrate the specificity of IL-33, further control groups of $ST2^{-/-}$ mice were sensitised with OVA with or without IL-33 co-administration. $ST2^{-/-}$ mice developed attenuated responses consistent with the findings in the normal dose AAI model findings published by our group [496]. No differences were seen between mice co-sensitised with IL-33 in the $ST2^{-/-}$ mice (Figure 6.7 B-E).

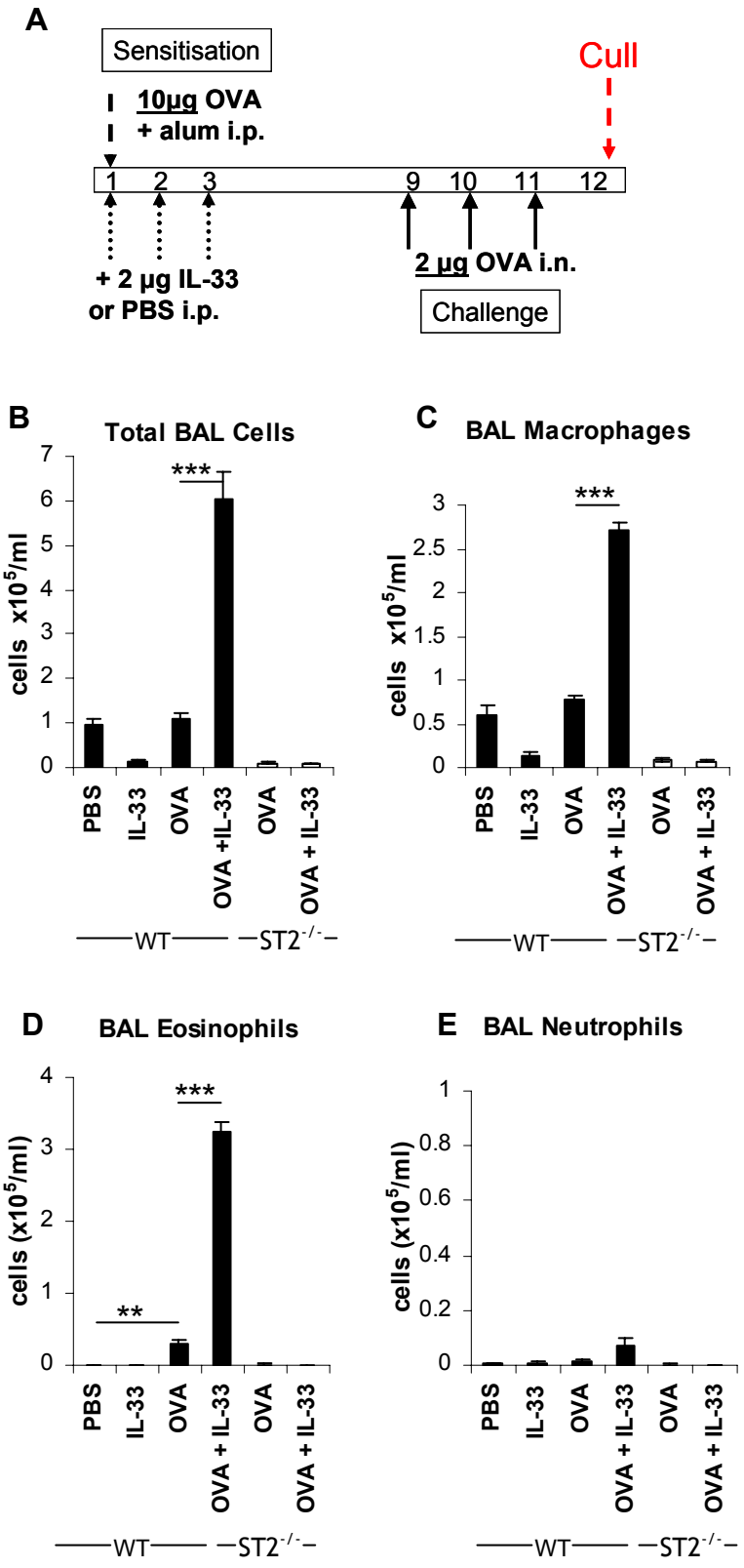


Figure 6.8: IL-33 exacerbates OVA-induced airway cellular inflammation
Allergic airways inflammation was induced in WT or ST2$^{-/-}$ BALB/c mice using low dose 10 µg ovalbumin with 2% alum administered i.p. on day 1. Mice were additionally treated with three doses of 2 µg IL-33 or PBS at sensitisation on days 1-3. All mice were given 3 i.n. challenges on days 9-11 and sacrificed on day 12 as per protocol in A). Further control groups of WT mice sensitised with either PBS or IL-33 alone and challenged with OVA were used as negative controls. BAL, serum, draining lymph nodes and lungs were obtained for analysis. Total cell counts in the BAL were analysed in B) and differential counts in C)-E). Data shown are representative of 2-3 experiments, n=5-10 per group. Results shown are mean of individual mice +/- SEM. **p<0.01 ***p<0.001.

6.2.3.2 IL-33 exacerbates cellular inflammation and goblet cell mucus production within the lung

Next the pathological changes in the lung were examined. Histological changes were examined on H&E stained slides as well as mucus staining on Periodic-acid Schiff (PAS) treated slides. Typical changes of peri-vascular and peri-bronchial eosinophilic inflammation along with epithelial shedding were demonstrated in the OVA sensitised group as shown previously (Figure 6.9B). These appeared less profound that usual dose OVA AAI model in keeping with the more modest BAL inflammation demonstrated in this low dose OVA model. The combination of OVA and IL-33 at sensitisation significantly enhanced the inflammatory infiltrate compared to OVA-only sensitised mice as shown in Figure 6.9A and C. IL-33 at sensitisation appeared to enhance the mucus production in goblet cells compared to OVA-only sensitised mice (Figure 6.9 D-G).

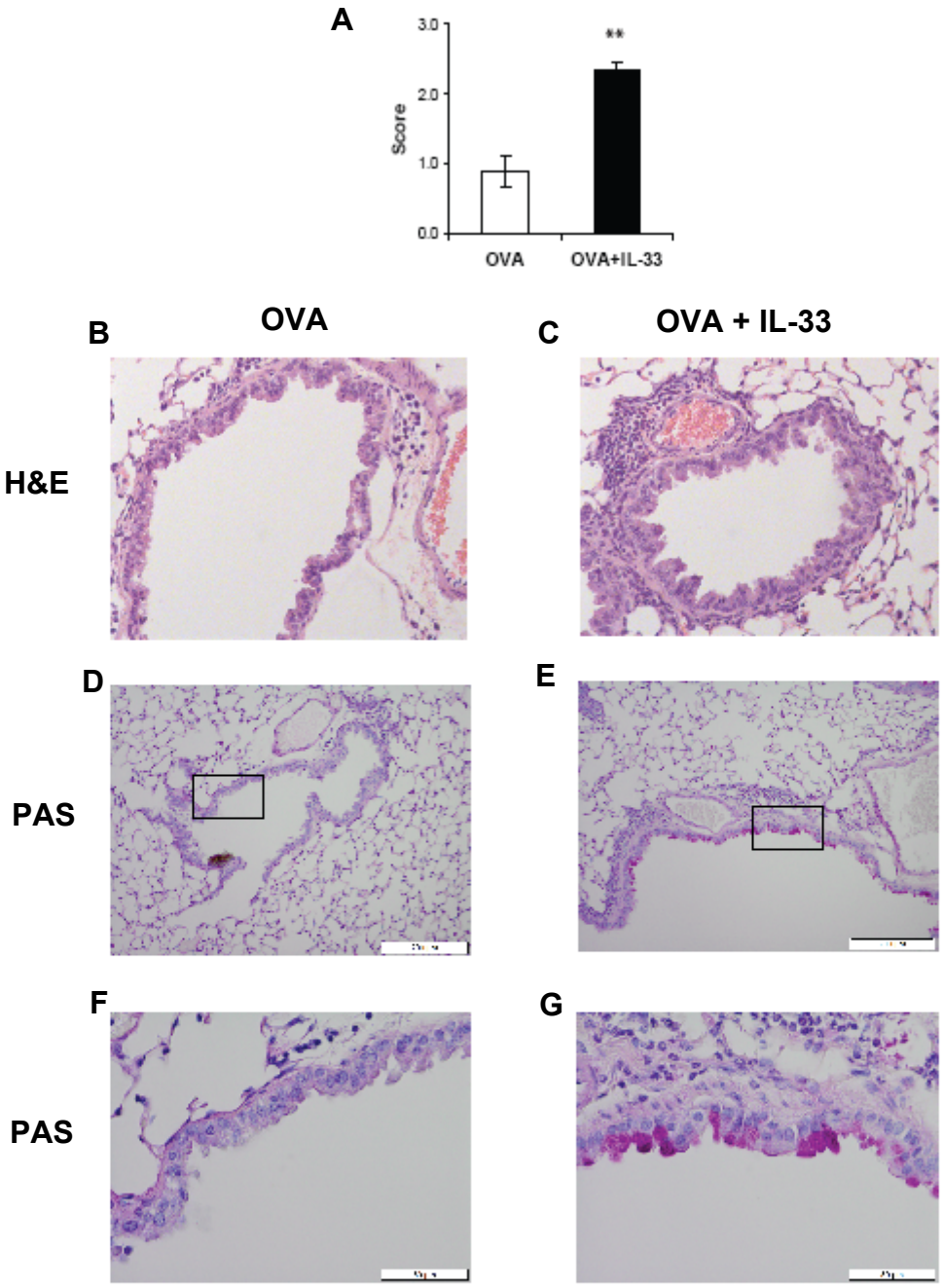


Figure 6.9: IL-33 at sensitisation increases the inflammatory infiltrate and mucus production in the lung
Mice treated as per protocol in Figure 6.8A had lungs removed and processed. H&E stained lung sections were examined by light microscopy and inflammatory cell infiltration and architecture assessed. Inflammation was quantitatively assessed using an arbitrary scoring system as described in chapter 2 A). Representative sections are shown in OVA only sensitised mice (OVA) B) and OVA and IL-33 sensitised mice (OVA+IL-33) C) original magnification x 20. Sections were also stained with PAS for mucus assessment in OVA D) and F) OVA+IL-33 E) and G). D) and E) x10 magnification and F) and G) x40. n=5 per group. Results are mean ± SEM. **p<0.01 when compared to OVA group.

6.2.3.3 IL-33 at sensitisation exacerbates chemokine production in the airway

IL-33 can directly influence cellular cytokine and chemokine production when administered into the airway. Therefore the effect IL-33, administered at the sensitisation phase, on these parameters in the BAL was assessed from experiments in Figure 6.8A. Levels of IL-5 and IL-4 were slightly elevated and reduced respectively in the BAL in mice treated with OVA and IL-33 at sensitisation compared to those treated only with OVA, but levels were low (Figure 6.10 A and B). The following were also detected at low levels but no difference between the groups was seen: IL-2, IL-10, IL-6, GM-CSF, VEGF, and TNF-α. IL-13, IFNγ, IL-12, IL-1β or IL-17 were not detectable in the BAL of either group (data not shown).

The most marked finding in the BAL fluid was in the level of chemokines detected. Eotaxin/CCL11, eotaxin-2/CCL24 and TARC/CCL17 were measured at increased levels in mice treated with both IL-33 and OVA at sensitisation compared to mice treated with OVA alone (Figure 6.10 C-E).

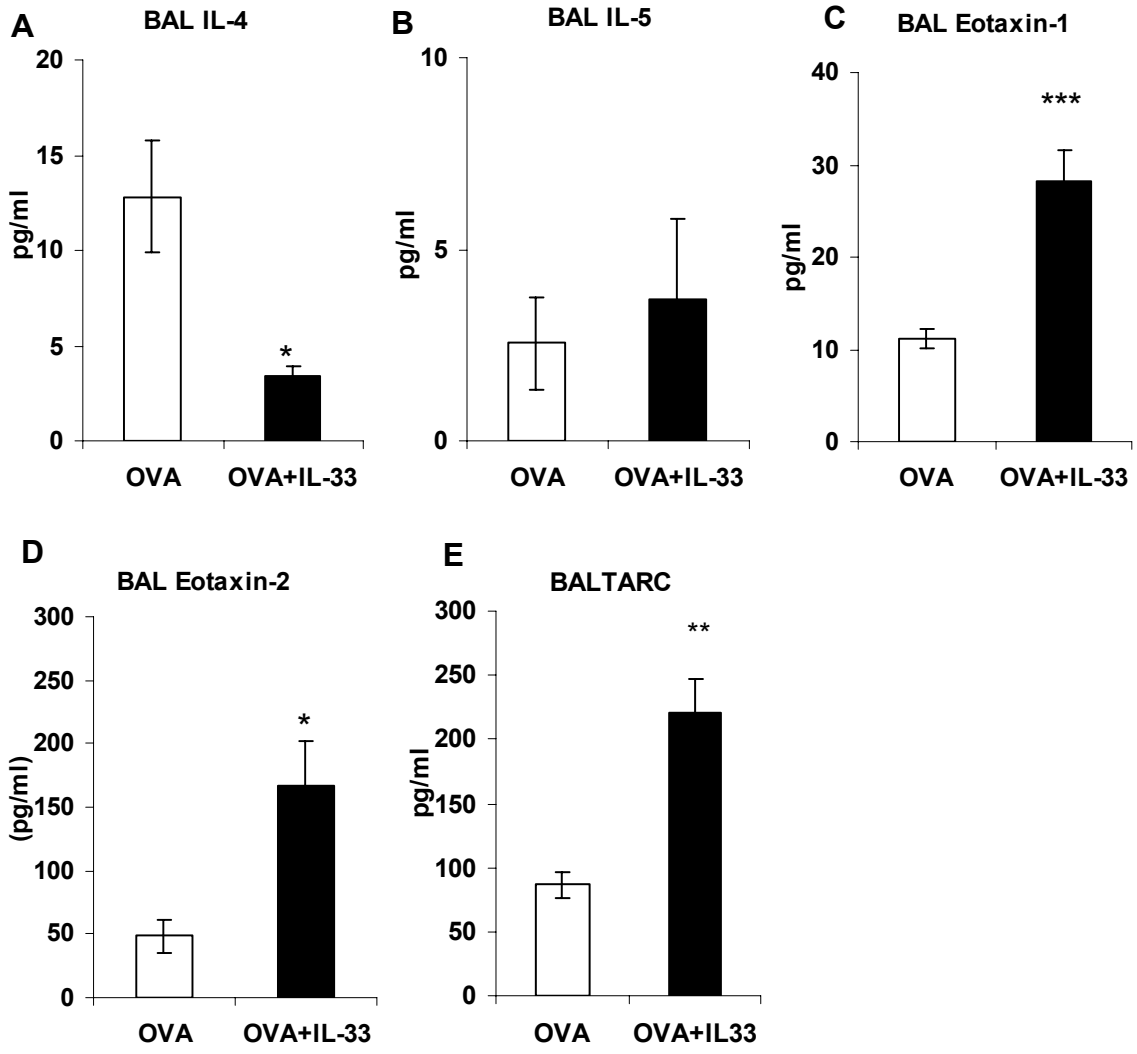


Figure 6.10: Increased levels of BAL eotaxin and eotaxin-2 following IL-33 treatment at sensitisation
24 hours following final i.n. OVA challenge, BAL from BALB/c mice treated with OVA or OVA+IL-33 at sensitisation were collected and processed. Levels of cytokines and chemokines were determined: IL-4 and IL-5 by multiplex immunoassay; eotaxins by ELISA. Data represent mean ± SEM of measurements from individual mice. n=10 per group.(*p<0.05, ***p<0.001). Data are representative of 3 experiments.

6.2.3.4 IL-33 at sensitisation increases levels of TARC in the serum

All cytokines and chemokines measured in the BAL fluid were also assessed in the serum. Levels of TARC, IL-13 and IP-10 were elevated, but only TARC levels reached statistical significance in the OVA plus IL-33 treated compared to the OVA treated group (Figure 6.11 A-C). No difference in the other analytes was demonstrated between the groups, including IL-5, eotaxin-1, eotaxin-2, GM-CSF and VEGF (data not shown). As the antibody memory response is initiated at sensitisation, levels of total IgE were measured to ascertain any affect of IL-33 but no differences were detected between the groups (Figure 6.11 D).

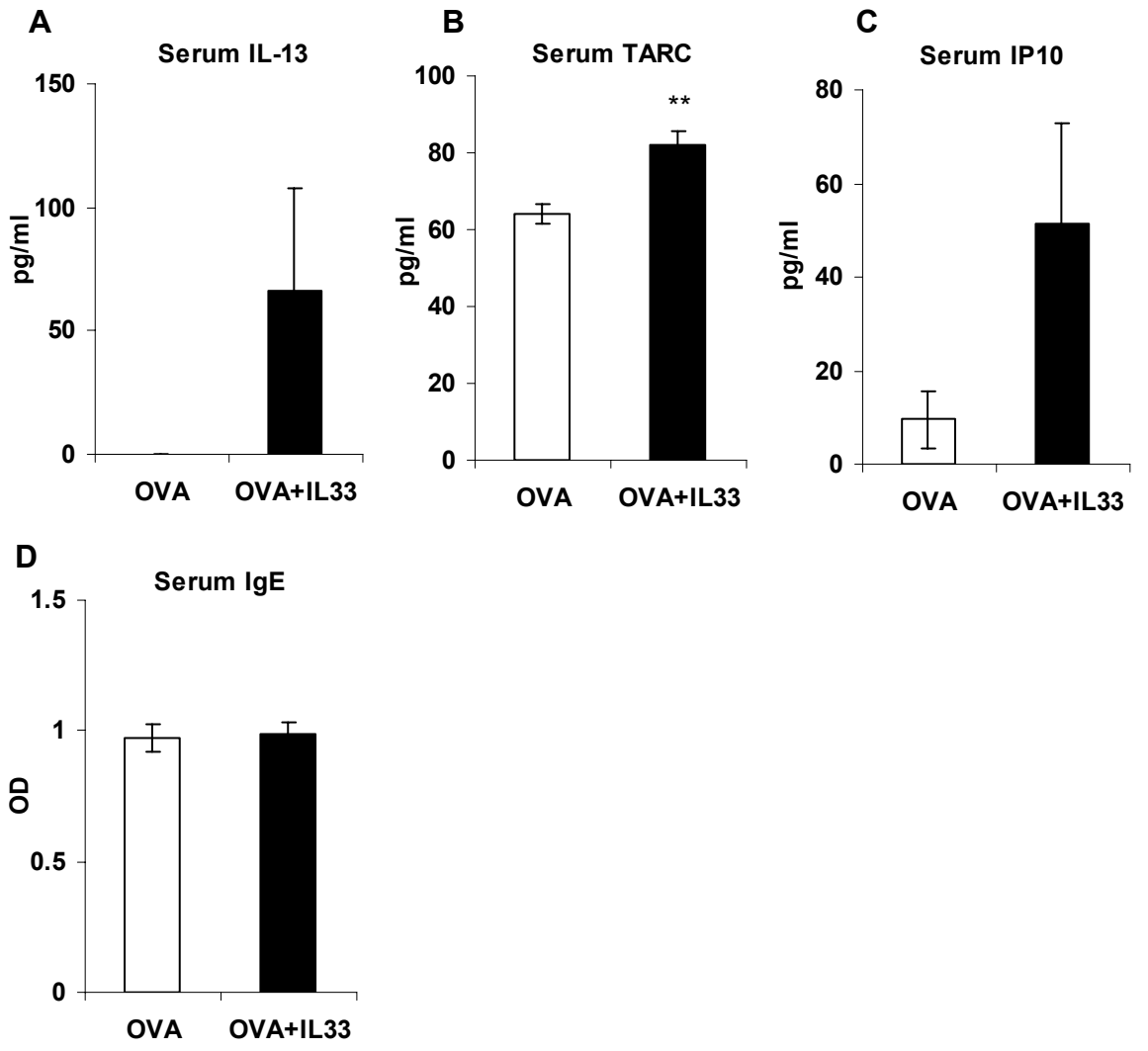


Figure 6.11: Increased levels of serum TARC following IL-33 treatment at sensitisation
24 hours following final i.n. OVA challenge, serum from WT mice treated with OVA or OVA+IL-33 were collected and processed. Levels of cytokines and chemokines were determined: by ELISA or multiplex immunoassay. Date represent means ±SEM of measurements from individual mice. n=10 per group. (**p<0.01). Data are representative of 3 experiments.

6.2.3.5 IL-33 at sensitisation increases the proportion of IL5$^+$ T cells in DLN

In vitro culture of CD4$^+$ cells with IL-33 enhances IL-5 production. Therefore to further explore the differences demonstrated by addition of IL-33 at sensitisation to a model of AAI, the T cell phenotype and cytokine responses in the DLN were examined. DLN were removed following antigen challenge and following processing, total cell counts performed and ST2 expression and cytokine profile assessed by FACS. Mice co-sensitised with IL-33 had an increase in cell numbers compared to OVA-only sensitised mice (Figure 6.12).

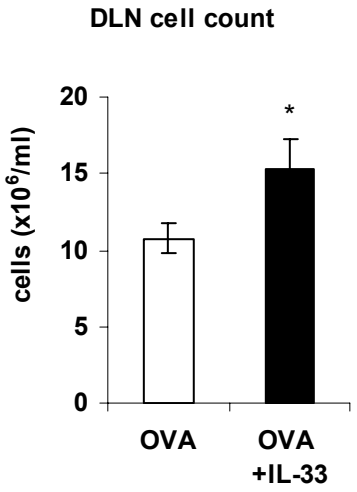


Figure 6.12: IL-33 at sensitisation increases cell number in DLN
Mice were treated and DLN removed 24 hours following final i.n. challenge. Full DLN counts were performed. Data are representative of mean of individual mice. n=10 per group (*p<0.05).

OVA-sensitised WT mice showed a substantial increase in the numbers of $CD4^+ST2^+$ cells compared to PBS-sensitised WT mice with 9.45% and 0.9% of DLN cells respectively. This was further substantially increased by co-administration of IL-33 with OVA to 16.7% $CD4^+ST2^+$ cells(Figure 6.13A). Intracellular staining of DLN $CD4^+$ T cells revealed that IL-33 alone had no effect on Th2 cytokine profile but increased the percentage of IFN-γ^+ cell population compared with the PBS group (Figure 6.13 B). In contrast, $CD4^+$ cells from OVA-sensitised mice showed an increase in proportion of IL-4^+ cells but not IL-5^+ cells compared to IL-33 alone of PBS-treated groups. The additional administration of IL-33 to OVA at sensitisation markedly enhanced the percentage of IL-5^+ cells but decreased the IL-4^+ cells compared to OVA group. The percentage of IFN-γ^+ cells did not change between these groups. There was no difference in cellular profile between OVA-sensitised and OVA+IL-33-sensitised groups in the $ST2^{-/-}$ mice. $ST2^{-/-}$ mice had an increased proportion of IFN-γ^+ cells compared to WT.

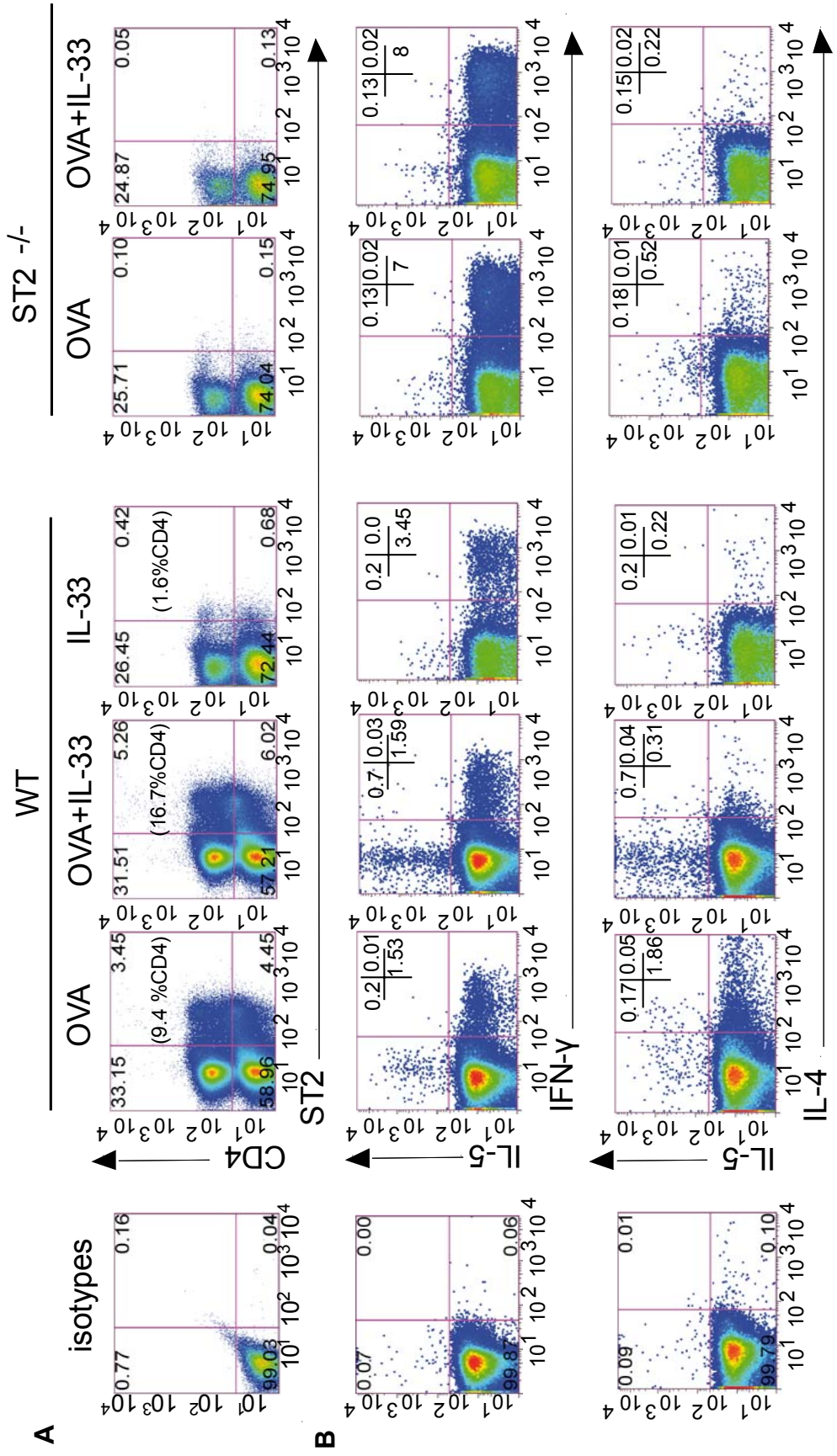


Figure 6.13: IL-33 increases the number of CD4$^+$IL-5$^+$ cells in the DLN
WT and ST2$^{-/-}$ mice were treated as described in Figure 6.8A. Draining mediastinal lymph nodes were removed and mechanically disrupted to form a single cell suspension when the total count was measured. Cells were stained with surface markers and using FACS, the percentage of CD4$^+$ cells and ST2$^+$ cells were determined A). CD4+ DLN cells were stimulated with PMA-I and golgi-stop before the presence intracellular cytokines IFNγ, IL-4 and IL-5 were assessed by FACS B). Data are representative of 2 experiments. n=5 per group.

6.2.3.6 IL-33 at sensitisation increases the antigen-specific recall response

The Ag-recall response was ascertained by culturing freshly isolated DLN mediastinal nodes with OVA *in vitro*. Levels of cytokine and chemokine production were low in the modified low dose OVA model. An increase in levels of TARC, IL-17 and non-significant increase in eotaxin/CCL11 and eotaxin-2/CCL24 was detected in the supernatants of cell cultures from mice treated with OVA in addition to IL-33 compared to those treated with OVA alone (Figure 6.14 A-D). A decreased IFN-γ and non-significant decreased IL-4 production was seen in the OVA only group (Figure 6.14E).

No significant differences in the levels of IL-5, IL-13, IL-10, IL-2, IL-6, TNF-α, VEGF, GM-CSF, IP-10, KC or MIP-1α between the groups and no IL-12 was detected in the culture supernatants (data not shown).

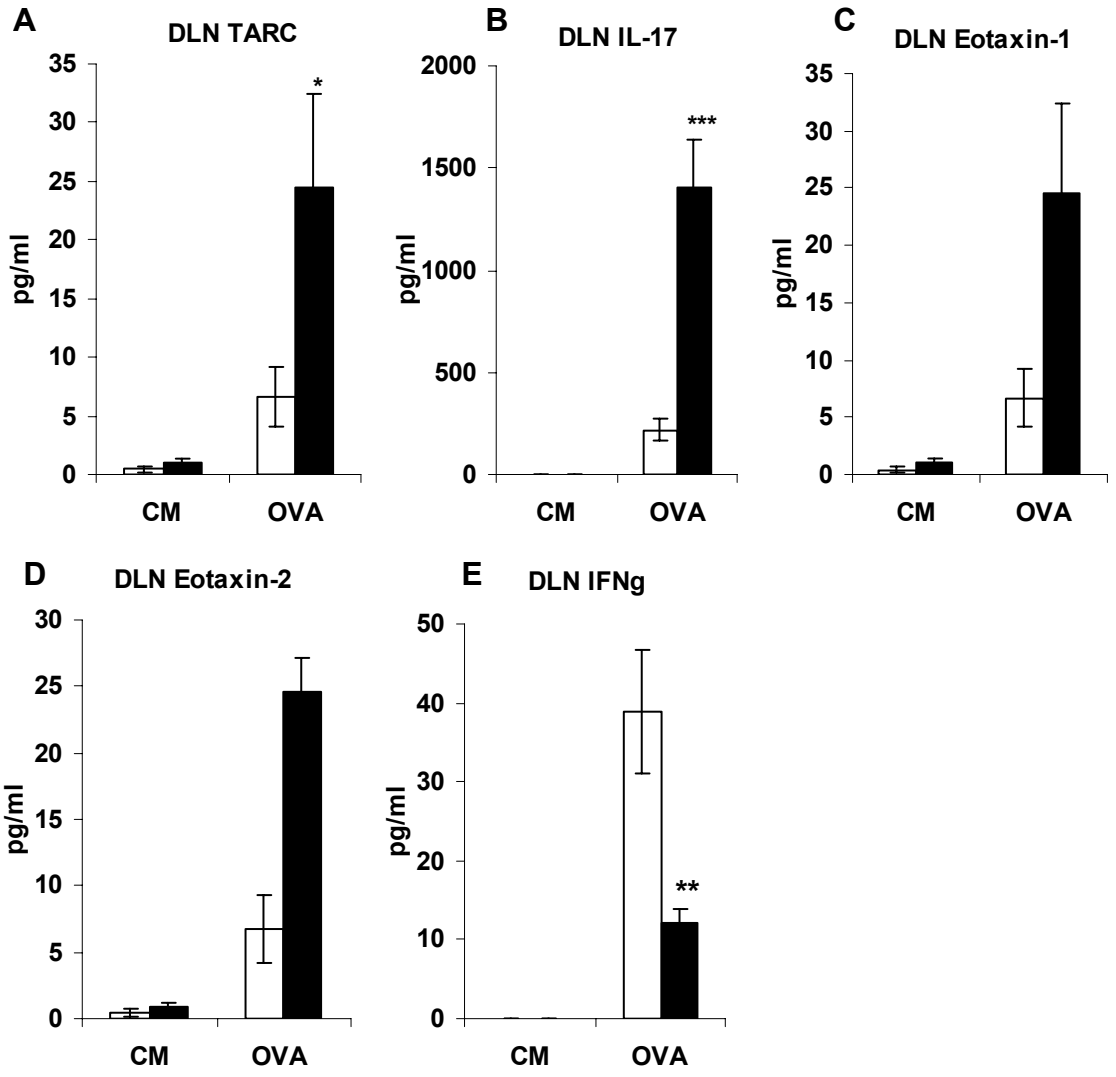


Figure 6.14: The effect of IL-33 at sensitisation on the Ag-recall response
WT mice were sensitised with OVA ± IL-33 and DLN removed following OVA allergen challenge. A single cell suspension was formed, counted and cultured with complete medium (cm) or with OVA 1mg/ml. Supernatants were collected after 72 hours and cytokine and chemokine levels were measured using multiplex immunoassay or ELISA for A) TARC, B) IL-17, C) eotaxin/CCL11, D) eotaxin-2/CCL24 and E)IFNγ. Unshaded bars OVA and shaded bars OVA plus IL-33. n=10 per group.(*$p<0.05$, **$p<0.01$, ***$p<0.001$). Data are representative of 3 experiments.

6.2.4 IL-33 at challenge induces allergic airways inflammation in ova sensitised mice

The application of i.n. IL-33 in addition to OVA at the challenge phase of AAI further exacerbates the ongoing Th2 inflammatory response (Kewin PK, 2007, PhD Thesis, University of Glasgow). This could occur via direct effects on ST2L expressing innate cells, Th2 cells or via a combination. Data in chapter 3 demonstrates i.n. IL-33 given directly into naïve lung induces an innate type-2 response, with at least 3 doses required to elicit an increase in BAL eosinophilia. In this non-sensitised environment however, IL-33 also has a direct effect on Th2 cells, increasing the expression of ST2L suggesting it has a role in modulating the adaptive immune response. This effect is non-essential in innate IL-33 driven inflammation as shown by the experiments in $RAG^{-/-}$ mice. Following on from the results in this chapter indicating IL-33 at sensitisation exacerbates AAI, it was interesting to consider whether pre-sensitisation to antigen would have a role in the innate response to IL-33, in the absence of further exposure to specific antigen.

Data shown in Figure 6.15 represents mice sensitised with OVA or PBS as in Figure 6.5 but these mice were given IL-33 at challenge only and not OVA. For comparison to the innate IL-33 experiments in chapter 4, C57BL/6 mice were used. The total number of cells in the BAL were low (Figure 6.15A) in keeping with experiments in chapter 4 when small number of eosinophils appear in BAL only following 3 days of i.n. IL-33. The differential cell count shows a higher proportion of eosinophils in mice sensitised with OVA compared to those sensitised with PBS (9% vs. 1.96%) (Figure 6.15B) and elevated serum IL-5 levels (Figure 6.15D). Low levels of IL-5 in BAL and IL-4 in BAL and serum of OVA sensitised mice were recorded but no significant differences were seen between the groups (data not shown). Corresponding experiments were also performed in BALB/c mice with similar results (data not shown).

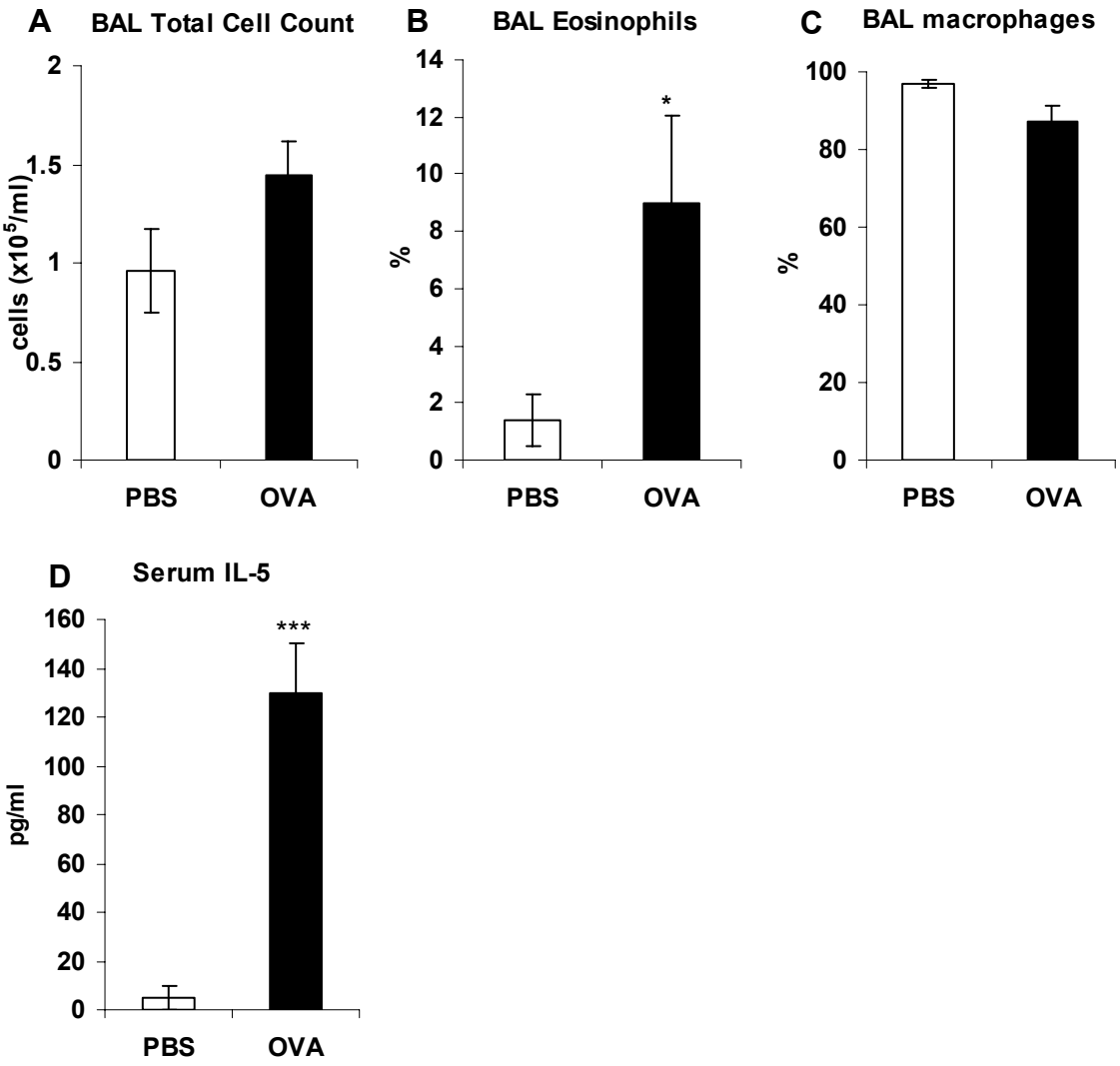


Figure 6.15: Sensitisation with OVA followed by IL-33 challenge exacerbates eosinophilic airway inflammation in C57BL/6 mice
WT mice were sensitized with 100 µg ovalbumin in 2% alum or PBS administered i.p. on day 1. On days 9-11, 2 µg of IL-33 was administered i.n. to all mice. Mice were sacrificed on day 12 and BAL and serum samples collected and processed. Cell counts performed on the BAL and serum IL-5 was measured by ELISA in supernatants. n=5. (*p<0.05, ***p<0.001). Data shown are representative of 2 independent experiments.

6.2.5 IL-33 at sensitisation exacerbates allergic airways inflammation in IL-4$^{-/-}$ mice

IL-4 is an important factor in Th2 cell differentiation and IgE production but its non-exclusive role in murine models of Th2 responses has been demonstrated [46,587,588]. To further investigate the relationship between IL-33 and IL-4 in polarisation and proliferation of IL-5 producing T cells and AAI, the experiments in section 6.2.3 were repeated in IL-4$^{-/-}$ mice. It was hypothesised that IL-33

given at sensitisation would exacerbate AAI via the IL-4 independent $CD4^+ST2^+IL\text{-}5^+$ pathway.

Mice were treated as per protocol in Figure 6.8A. There was no significant difference between BAL total cell counts between the groups of $IL\text{-}4^{-/-}$ mice (Figure 6.16A). There was a trend to increased eosinophils numbers in mice treated with IL-33 in addition to OVA at sensitisation compared to mice treated with OVA alone when the BAL is analysed by differential cell count (Figure 6.16B) or by FACS (Figure 6.16C) (FACS acquisition performed by Dr M Kurowska-Stolarska). Overall, the low counts in the BAL in this low dose OVA model meant further differences could not be easily elicited.

As expected, no IL-4 was measured in any sample. No statistically significant differences were measured in levels of IL-13 in the BAL (Figure 6.16E) and levels in the serum and DLN culture supernatants were low. Levels of IL-5 in culture supernatants from DLN stimulated with OVA antigen were higher in $IL\text{-}4^{-/-}$ mice sensitised with OVA plus IL-33 compared to groups sensitised with OVA plus PBS or PBS alone (Figure 6.16F). Levels of IL-5 in serum and BAL were low. No differences were seen in eotaxin/CCL11 levels in BAL or serum between groups.

An increased susceptibility to morbidity and mortality was observed in the $IL\text{-}4^{-/-}$ mice in experimental repeats with neutrophils comprising a higher proportion of the BAL in these animals compared to WT groups. This gave rise to limited numbers of mice and concern about pathogen contamination. These experimental protocols were then performed by collaborators in another laboratory, where a reduction in inflammation in $IL\text{-}4^{-/-}$ mice compared to WT mice was seen. This deficiency was overcome by the application of IL-33 at sensitisation [496].

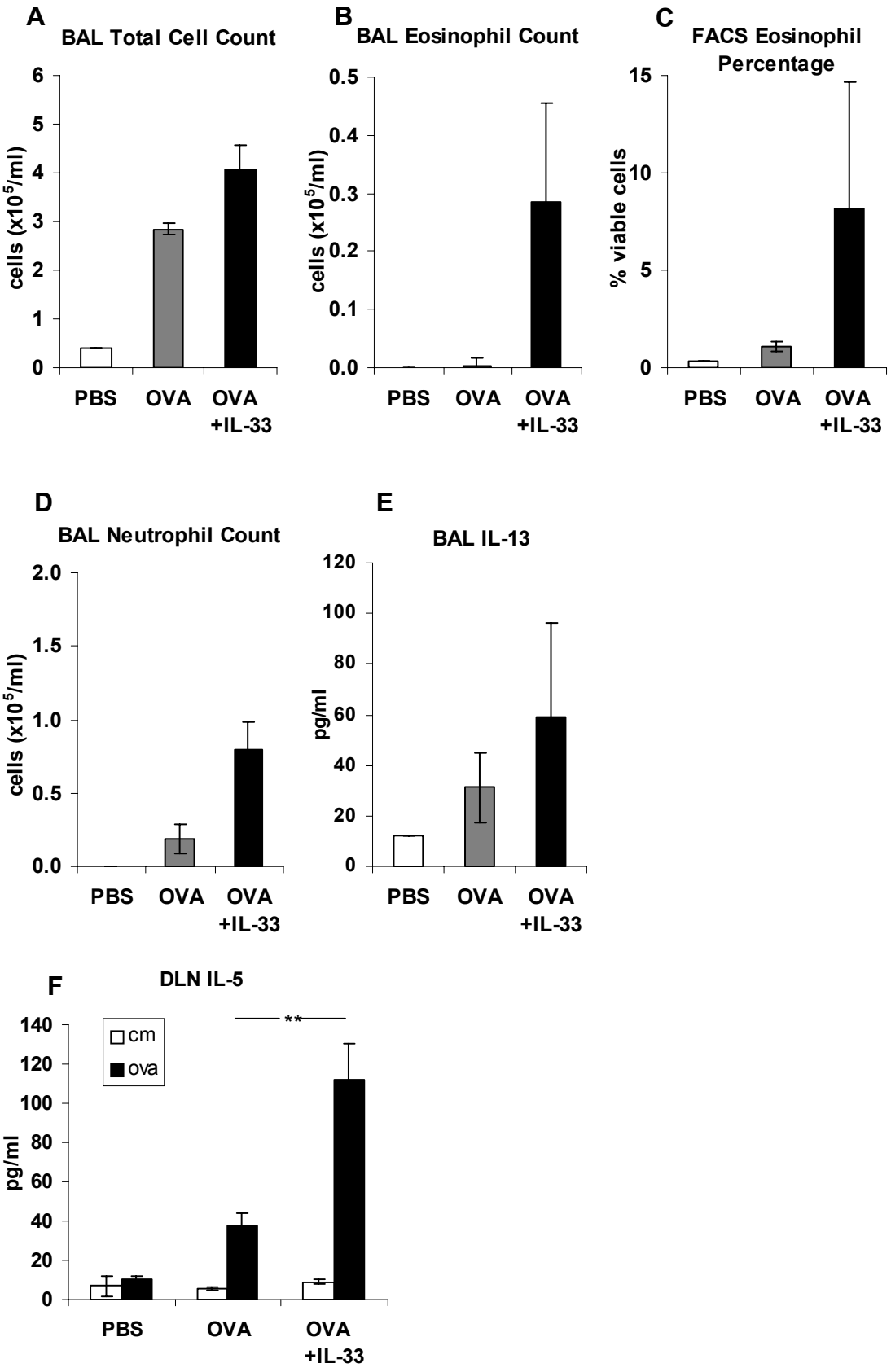


Figure 6.16: The effect of IL-33 at sensitisation in IL-4-/-mice
AAI was induced in IL-4$^{-/-}$ mice as per protocol in Figure 6.8A. BAL, serum, draining lymph nodes and lungs were obtained for analysis. A) Total cell and B) and D) differential cells counts in the BAL were analysed by haemocytometer and by C) cell surface marker analysis by FACS. Eosinophils population were identified as CD45^{+}Siglec-F^{hi}. E) BAL IL-13 measured by ELISA. F) DLN cells were cultured CM and OVA (1mg/ml). Results shown are mean±SEM of individual mice. Data shown are representative of 2 experiments. n=3-6 per group.**p<0.001.

6.3 The role of IL-4 and ST2 in an alum free sensitisation ovalbumin model in mice

Experiments in chapter 4 and presented in this chapter indicate a IL-33/ST2 mediated pathway can occur independently of IL-4. To further investigate this, the effect of loss of endogenous IL-33 in AAI was assessed. Problems in generating the anticipated IL-$33^{-/-}$ mice meant the decision was taken to assess receptor-ST2 deficiency and to consider how the absence of IL-4 might affect the dominance of these apparent pathways in the asthma model.

IL-4 is a mediator of Th2 biased sensitisation [42]. However, there is evidence that IL-4 free sensitisation can occur: for example, by using an epicutaneous (e.c.) rather than i.p. method of sensitisation, the need for IL-4 is bypassed [587]. Importantly, in these published experiments, no additional adjuvant was used in the sensitisation. Traditional allergen models use alum at time of sensitisation to boost response. The mechanism of this boosting action is unclear but a postulated mechanism is that IL- 4 induced by aluminium salts promote Th2 responses (and by enhancing Th2 mediated antibody production and inhibition of Th1 responses) [277] [588].

The role of ST2 in conventional AAI models have had conflicting reports [304,338,341,344]. In our laboratory using the short 12 day OVA model, ST2 gene deletion results in attenuated AI [496]. This is in keeping with the finding that soluble ST2 has a beneficial effect in airway inflammation [341].

Preliminary data in our laboratory (Pitman NI, 2012, PhD Thesis, University of Glasgow) has demonstrated a possible role for ST2 in an adjuvant free allergen long model of asthma. In this model, eosinophilia and IgE production but not AHR were partially ST2 dependent. This suggests the alternate or additional ST2/IL-33 dependent sensitisation pathway is overcome or overwhelmed by the addition of an adjuvant; this is relevant as adjuvant free models are clearly more physiological and thus considered be more representative of clinical disease.

The hypothesis tested is that ST2 and IL-4 are both independently necessary for sensitisation and AAI, and in the adjuvant model, alum overcomes ST2

deficiency. Furthermore, that a synergistic effect of IL-4 and IL-33 exists whereby IL-4 and ST2 double KO mice develop more attenuated AI compared to single KO mice. Experiments were designed to assess this hypothesis by the removal of cytokine function by receptor or cytokine knockout mice in alum and alum free models.

6.3.1 Generating IL-4$^{-/-}$ST2$^{-/-}$ mice

To investigate hierarchy of IL-4 and ST2 at sensitisation in allergic airways disease, double knockout mice were generated. The genes of IL-4, IL-13 and IL-5 are linked in an area on murine chromosome 11[589]. IL-4$^{-/-}$ mice were previously generated by directing targeting of IL-4 gene in BALB/c embryonic stem cells [450] and provided by Dr J Alexander (University of Strathclyde, Glasgow, UK). ST2 gene knockout mice are described in chapter 2. A schematic of the breeding programme is illustrated in Figure 6.17. Following cross of IL-4$^{-/-}$ and ST2$^{-/-}$ mice, F1 heterozygotes are re-crossed with a 1 in 16 chance of a double homozygote.

Genotype each parent F1	IL4^{+}ST2^{+}	IL4^{+}ST2^{-}	IL4^{-}ST2^{+}	IL4^{-}ST2^{-}
IL4^{+}ST2^{+}	IL4^{++}ST2^{++}	IL4^{++}ST2^{+-}	IL4^{+-}ST2^{++}	IL4^{+-}ST2^{+-}
IL4^{+}ST2^{-}	IL4^{++}ST2^{+-}	IL4^{++}ST2^{--}	IL4^{+-}ST2^{+-}	IL4^{+-}ST2^{--}
IL4^{-}ST2^{+}	IL4^{-+}ST2^{++}	IL4^{+-}ST2^{+-}	IL4^{--}ST2^{++}	IL4^{--}ST2^{+-}
IL4^{-}ST2^{-}	IL4^{-+}ST2^{+-}	IL4^{+-}ST2^{--}	IL4^{--}ST2^{+-}	**IL4^{--}ST2^{--}**

Figure 6.17: Generation of IL-4$^{-/-}$ST2$^{-/-}$ double knockout mice

Table of potential F2 genotype. IL-4$^{-/-}$ were crossed with ST2$^{-/-}$ mice (F0) and the mice generated (F1) were self crossed to generated the double KO mice (F2). A 1:16 chance exists of generating an IL4$^{-/-}$ST2$^{-/-}$mouse.

PCR screening of tail genomic DNA was used to detect heterozygotes in F2 (Figure 6.18 A and B). Double knockout mice in F3 were confirmed by PCR (Figure 6.18 C and D) before breeding pairs established.

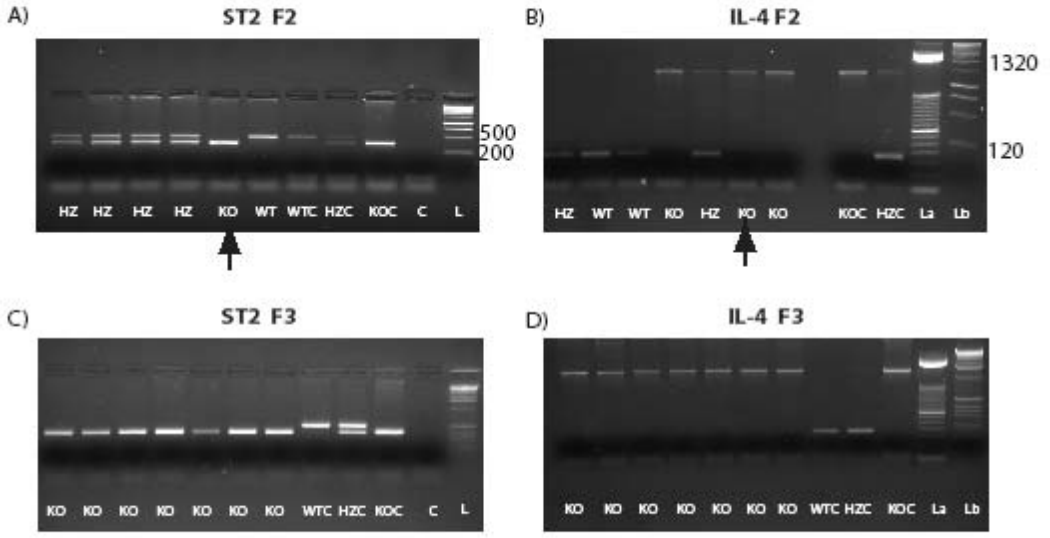


Figure 6.18: DNA detection gels viewed under UV light showing tail tip extracted genomic DNA following polymerase chain reaction for ST2 or IL-4

A mouse demonstrating both ST2 and IL-4 gene knockout in generation F2 (A and B). Male and female knockouts when bred produced generation F3, all of which were confirmed as double knock out (C and D).
Band sizes ST2 +/+(WT) 500bp, ST2 -/-(KO) 200bp, IL-4 +/+ (WT) 120bp and IL-4-/-(KO) 1320bp.
HZ= heterzygote, HZC=heterzygote control, KO=knockout (homozygous),
KOC=knockout control, WT=wildtype, WTC=wildype control, L=DNA ladder (1kb),
La=DNA ladder (1kb), Lb=DNA ladder (50-800bp), C=control H20.

6.3.2 The role of ST2 at sensitisation is less important than IL-4

AAI was induced in WT, ST2$^{-/-}$, IL-4$^{-/-}$ and ST2$^{-/-}$IL-4$^{-/-}$ mice. The protocol was based on a published adjuvant-free protocol and the same as that used in the laboratory pilot study [590]. These mice also underwent the protocol with the addition of alum in line with the standard 28 day AAI model. The protocol is shown in Figure 6.19.

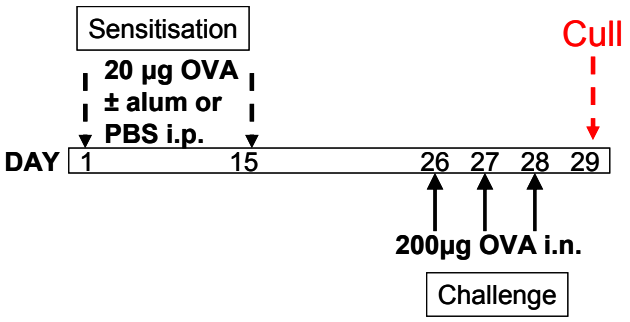


Figure 6.19: The adjuvant-free allergic airways inflammation model
BALB/c WT ST2$^{-/-}$, IL-4$^{-/-}$ and ST2$^{-/-}$IL-4$^{-/-}$ were administered OVA or OVA-alum or PBS on days 1 and 15, followed by i.n. OVA challenge on days 26-29. Mice were sacrificed 24 hours following the final challenge when samples were collected.

In keeping with published results, in the 28 day adjuvant model, no difference was seen in BAL cell counts between WT and ST2$^{-/-}$ mice (Figure 6.20A). In the IL-4$^{-/-}$ mice, alum boosts the ST2 pathway and generates airway eosinophilia but does not overcome IL-4 deficiency (Figure 6.20B). In double knockout mice, a small amount of eosinophilia persists, suggesting a further additional non-ST2, non-IL-4 alum driven pathway.

In the alum-free model, in contrast to pilot data, no difference was seen in eosinophilic inflammation in the BAL between WT and ST2$^{-/-}$ mice (Figure 6.20B). This suggests a ST2 dependent sensitisation pathway is not crucial in an adjuvant free environment as hypothesised. IL-4$^{-/-}$ mice exhibited very low levels of eosinophilia indicating that the ST2 pathway cannot overcome the importance of IL-4 in an alum-free model. Virtually no eosinophils were seen in the IL-4$^{-/-}$ST2$^{-/-}$ mice. However, given the already low levels in the IL-4$^{-/-}$ mice, the further

reduction of BAL eosinophils that could be considered dependent on the ST2 pathway was difficult to assess, was not statistically significant and unlikely to be of phenotypic significance.

Comparing conditions within mouse genotypes, in the adjuvant model as expected, the addition of alum exacerbated or boosts the immune response in WT mice. Similarly in $ST2^{-/-}$mice, alum boosts the sensitisation response. In $IL\text{-}4^{-/-}$ mice, the group given an adjuvant had a higher level of eosinophilia in BAL than in alum-free model mice, suggesting mechanism of alum is partially but not solely through IL-4. In $IL\text{-}4^{-/-}ST2^{-/}$ mice the loss of both pathways results in complete abrogation of AAI in the alum free group..

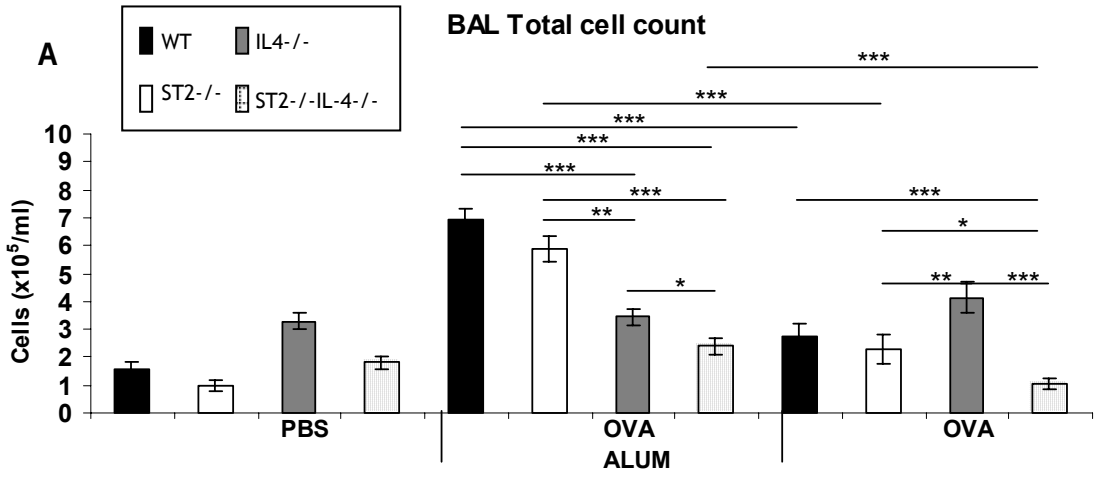


Figure 6.20: A. See over for legend.

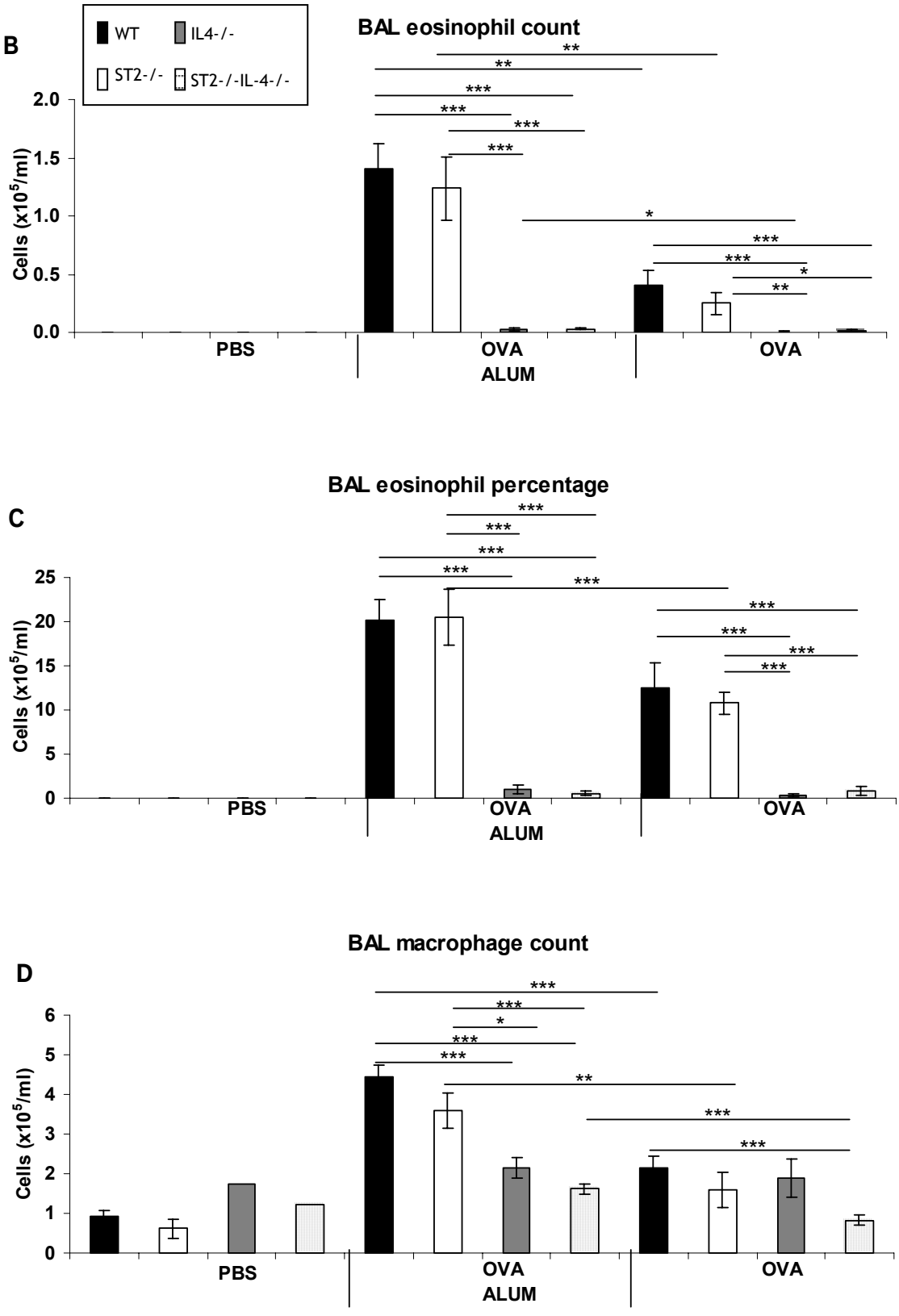


Figure 6.20: BAL cell counts in an adjuvant and adjuvant-free model of asthma
BALB/c WT ST2$^{-/-}$, IL-4$^{-/-}$ and ST2$^{-/-}$IL-4$^{-/-}$ were treated as per protocol in Figure 6.19. BAL was performed 24 hours following the final i.n. challenge. A) Total and B)-D) differential cell counts were performed on the BAL. (*p<0.05, **p<0.01 ***p<0.001). Data shown is mean ± SEM. Representative of 2 independent experiments.

The concentration of mediators measured in the BAL were at low levels. IL-4 appeared to be lower in $ST2^{-/-}$ compared to WT mice in the OVA-alum sensitised group despite similar level of inflammation but this was not statistically significant (Figure 6.21A) .

Production of MIG/CXCL9, appears dependent on ST2 as none is detected in the $ST2^{-/-}$ mice compared to WT or IL-$4^{-/-}$ in OVA-alum or OVA-only sensitisation groups (Figure 6.22B). Concentrations of IL-2, IL-13, IL-10, IL-17, KC, IL-12, MIP1α and FGF were very low or absent; and of IL-5, IFNγ, IL-1β, IL-6, GMCSF, TNFα, VEGF, MCP-1, IP-10, IL-1α low with no differences between the groups detected (data not shown).

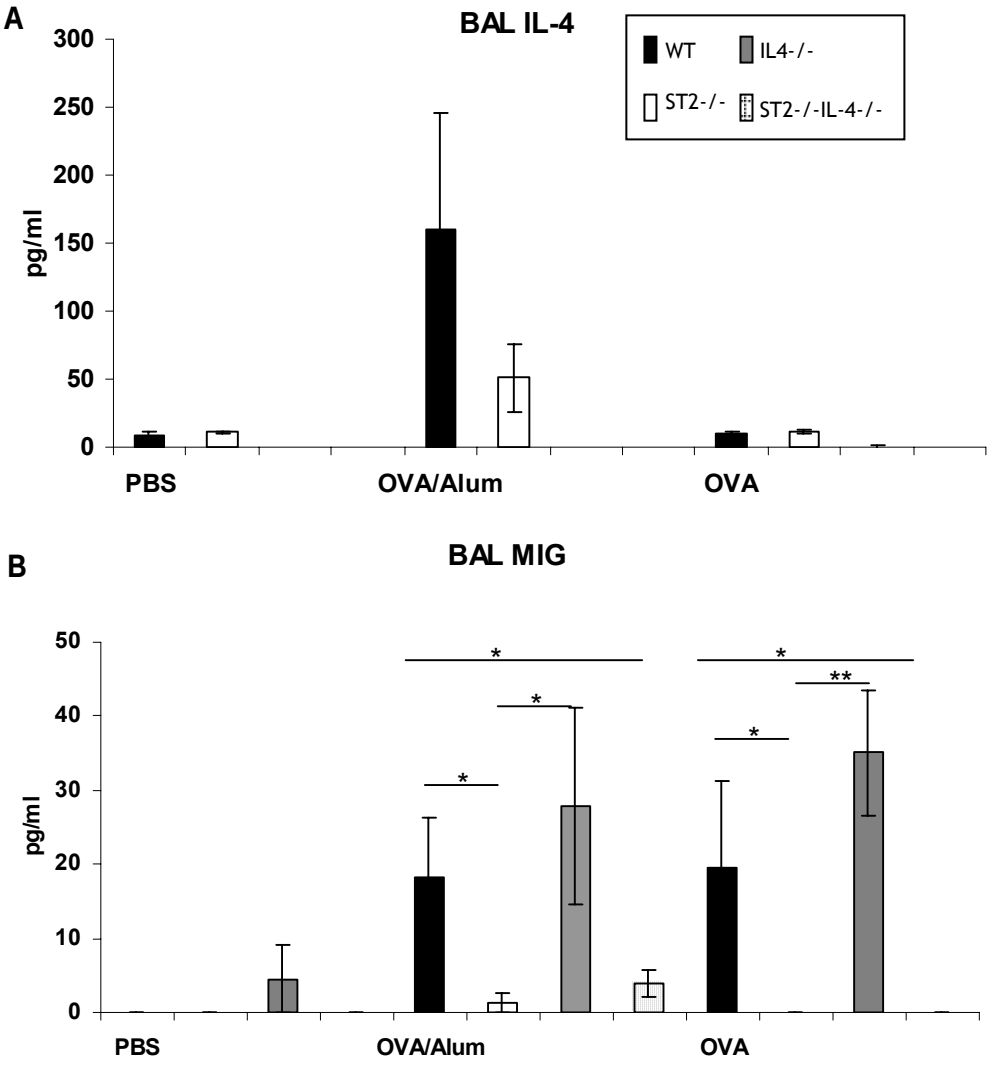


Figure 6.21: BAL cytokines in an adjuvant and adjuvant-free model of asthma
BALB/c WT $ST2^{-/-}$, IL-$4^{-/-}$ and $ST2^{-/-}$IL-$4^{-/-}$ Were treated as per protocol in Figure 6.19. BAL was performed 24 hours following the final i.n. challenge. Cytokine analysis was performed by ELISA or multiplex immunoassay. A) IL-4 and B) MIG (*$p<0.05$). Data shown is mean ± SEM. Representative of 1 experiment.

The compartments most likely to reflect significant mechanisms of differences in sensitisation are serum and the draining mediastinal lymph nodes. In the serum, cytokine and chemokine concentrations were lower in OVA than OVA-alum treated mice reflecting lower levels of inflammation seen in the BAL. As such, any differences between groups sensitised with OVA were difficult to elucidate. As expected, no difference was seen in IL-5 levels between WT and $ST2^{-/-}$ in OVA-Alum group as no difference was seen in BAL eosinophilia. Lower IL-5 levels were seen in $IL\text{-}4^{-/-}$and $ST2^{-/-}IL\text{-}4^{-/-}$ compared to WT mice and in $ST2^{-/-}IL\text{-}4^{-/-}$ compared to $ST2^{-/-}$. Lower concentrations of serum IL-5 were seen in OVA sensitised WT and $ST2^{-/-}$ in comparison to OVA-alum sensitised mice (Figure 6.22A). $ST2^{-/-}$ mice had reduced serum levels compared to IL-4 and a trend towards reduced levels compared to WT but this was not statistically significant (Figure 6.22B).

Levels of IL-6 were lower in $IL\text{-}4^{-/-}$ and $ST2^{-/-}IL\text{-}4^{-/-}$ compared to WT and $ST2^{-/-}$ mice in OVA-alum group (Figure 6.22C). Like in the BAL, MIG concentrations appeared to be partially dependent on ST2 (Figure 6.22D). Levels of IL-12 were higher in all the adjuvant treated groups compared to OVA-only treated mice (Figure 6.22 E). No differences were seen in concentrations of IL-2, GM-CSF, TNFα, VEGF, IL-17, KC, IP-10, FGF or IL-1β. Very low or absent levels of IL-13, IL-10, MIP1α or IL-1α were detected (data not shown).

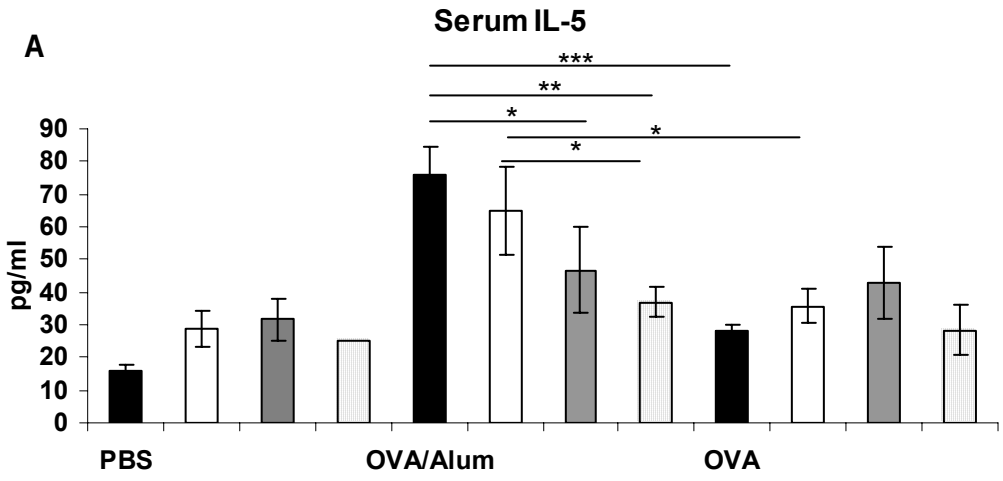


Figure 6.22: A. See over for legend.

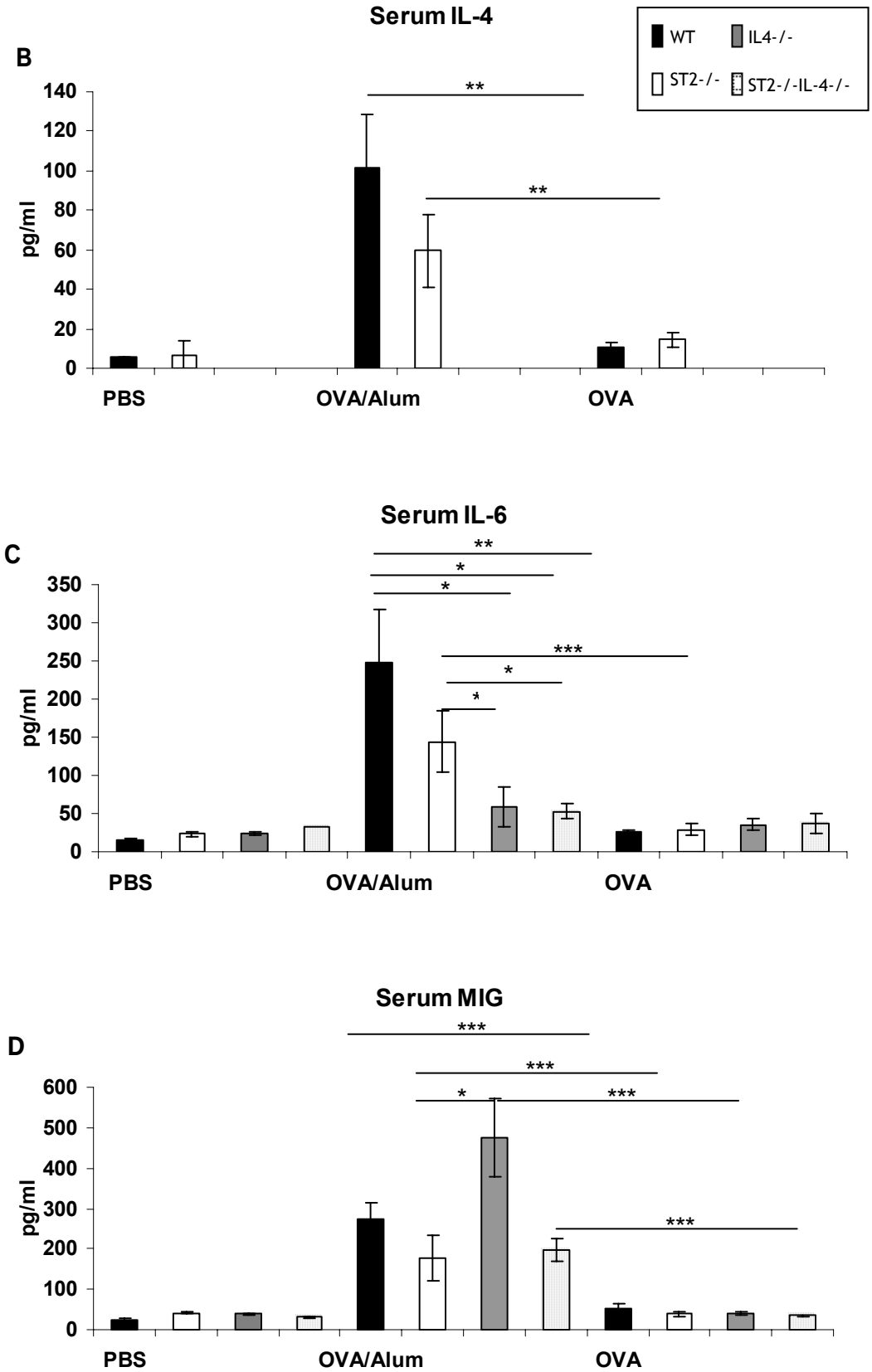


Figure 6.22: B-D. See over for legend.

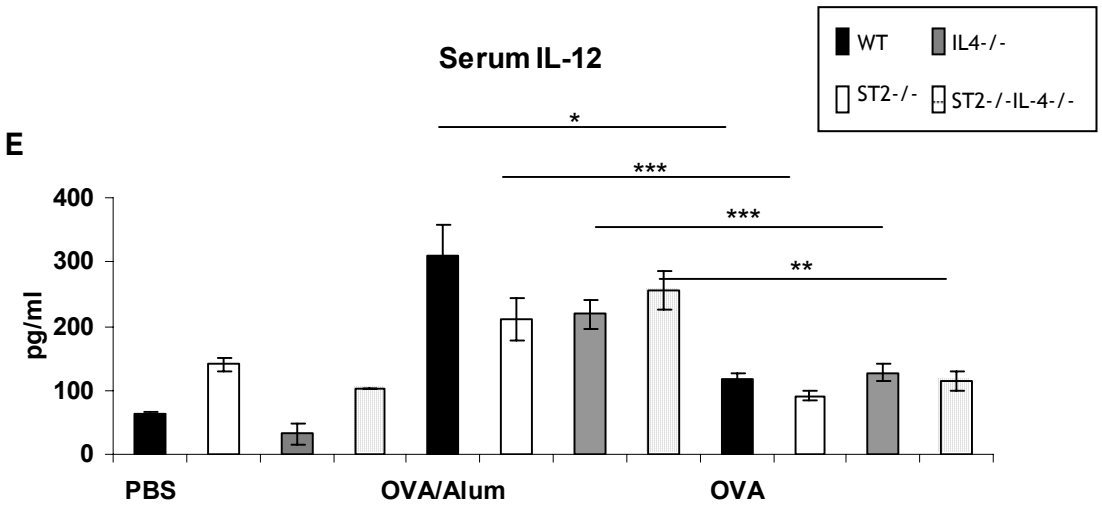


Figure 6.22: Serum cytokines in an adjuvant and adjuvant-free model of asthma
BALB/c WT ST2$^{-/-}$, IL-4$^{-/-}$ and ST2$^{-/-}$IL-4$^{-/-}$ were treated as per protocol in Figure 6.19. Serum was collected and processed 24 hours following the final i.n. challenge. Serum cytokine and chemokine levels were measured by ELISA or multiplex immunoassay. A) IL-5, B) IL-4, C) IL-6, D) MIG and E) IL-12. (*p<0.05, **p<0.01 ***p<0.001). Data shown is mean ± SEM. Representative of 1 experiment.

IgE production is a critical aspect in animal models of asthma and so total and Ag-specific levels were measured in the serum. There were no differences seen in levels of total IgE or OVA-specific IgE between WT and ST2$^{-/-}$ mice in OVA-alum or OVA alone groups indicating ST2 deficiency in the absence of an adjuvant has no effect on IgE production; this is in disagreement with previous pilot results (Figure 6.23 A and B). As expected, IL-4 deficiency resulted in significantly reduced levels of total and OVA-specific IgE in the IL-4$^{-/-}$ and ST2$^{-/-}$ IL-4$^{-/-}$ mice in comparison to WT or ST2$^{-/-}$ mice in the OVA/alum or OVA alone groups. Interestingly, the levels of OVA-specific IgE were enhanced in both IL-4 knockout groups by the addition of alum at sensitisation (Figure 6.23B).

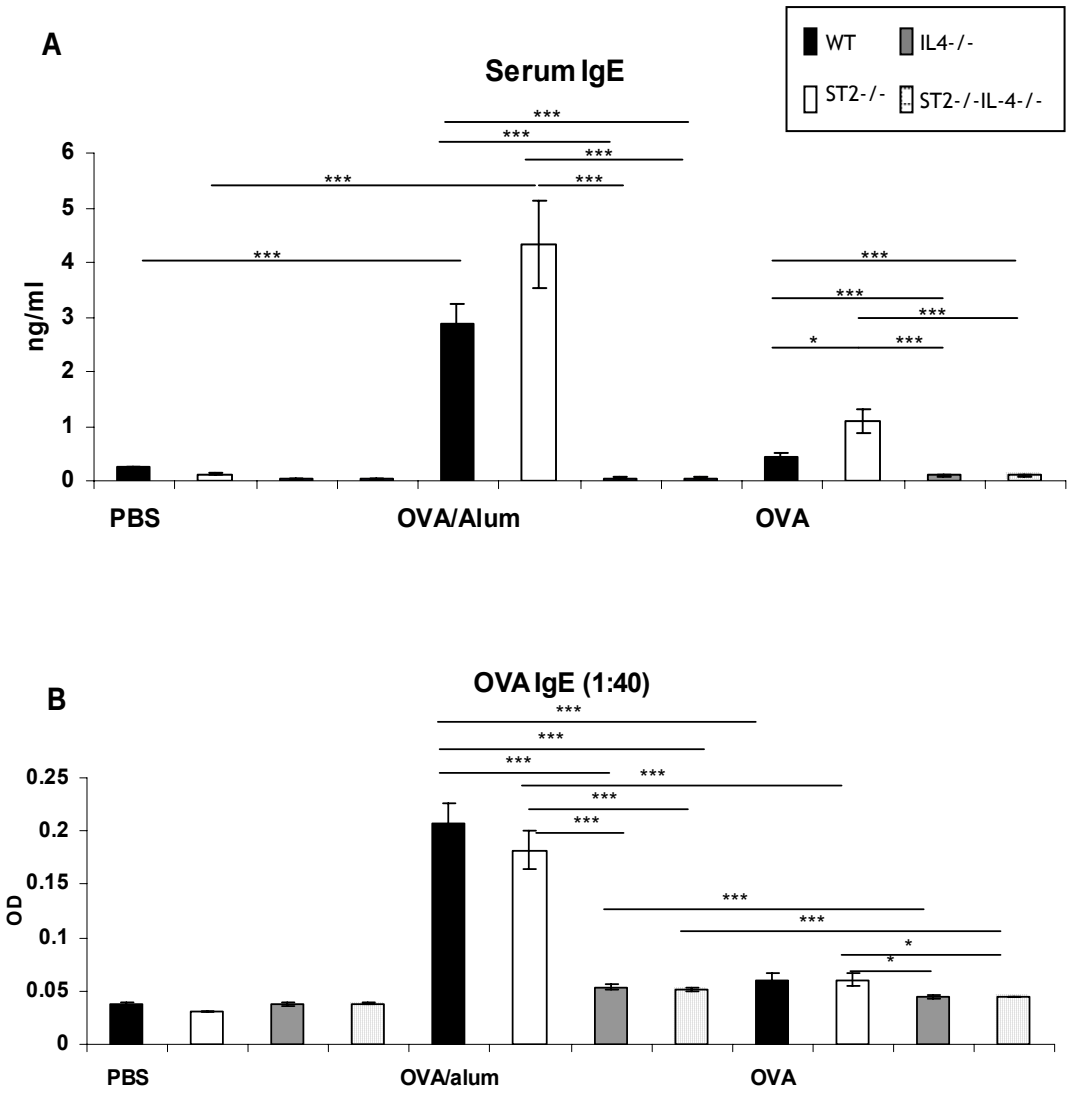


Figure 6.23: Serum IgE in an adjuvant and adjuvant-free model of asthma
BALB/c WT ST2$^{-/-}$, IL-4$^{-/-}$ and ST2$^{-/-}$IL-4$^{-/-}$ were treated as per protocol in Figure 6.19. Serum was collected and processed 24 hours following the final i.n. challenge. A) Total and B) OVA-specific IgE levels were performed by ELISA. (*p<0.05, ***p<0.001). Data shown is mean ± SEM. Representative of 1 experiment.

As isotype switching is likely to occur in the draining lymph nodes under influence of IL-4, experimental plans included analysis of the production of cytokines from the lymph node cells on re-stimulation with OVA. There was no significant change in lymph node cell counts between groups in OVA-alum protocol (data not shown). Unfortunately, cell culture supernatants were not available for the OVA treated mice so further analysis of cytokine profile could not be undertaken. Spleen weights were higher in WT mice compared to other groups in the OVA-alum sensitisation group (data not shown).

6.4 Conclusions

Experiments in this chapter assessed the role of IL-33/ST2 in sensitisation of AAI. Firstly, the effect of adding IL-33 at the sensitisation phase of the asthma model was studied. This led to the exacerbation of airway inflammation by induction of Ag-specific $CD4^+IL\text{-}5^+$ cells. Secondly, a synergistic effect of IL-4 and IL-33 at sensitisation using single and double receptor knockout mice was investigated.

Initial experiments in this chapter served to develop a model where the effects of IL-33 at sensitisation could be assessed. Despite generating an airway eosinophilia using an *in vitro* generated transgenic T cell adoptive transfer model, this was at modest levels and this was dependent on the availability of a large number of $CD4^+IL\text{-}5^+$ cells for transfer. Thus, this increased requirement for animal numbers and effort to generate a sample size that would be useful for experiments favoured an alternative approach.

Adoptive models are advantageous as T cells can be tracked but there are concerns about their artificial nature; the number of Ag-specific T-cells required to induce eosinophilic inflammation can be 5000 fold less than *in vivo* generated T-cells [591]. Antigen sensitisation models are the classical and most widely model to study asthma but with a significant degree of variety in protocol [500]. For the experiments described here, a short 12 day protocol adapted from Stock *et al* was used and this has been validated in our laboratory with respect to airway eosinophilia, IgE production and AHR [452] and used by others [526]. This short model may reflect a different phenotype compared to better established 28 day models. Indeed, models reflective of remodelling changes associated with asthma have longer protocols with multiple challenges. Few studies to date have been performed using this short model but it seems likely to reflect the early and late asthmatic responses rather than sustained inflammation or remodelling features. We and others have demonstrated dependence for ST2 in this short model, which has not been shown in 28 day model, supporting the suggestion of an alternative phenotype [421,496]. One hypothesis is that this model may be somewhat dependent on mast cells; an alternative 12 day model showed dependence on mast cells [592] in contrast to the more established 28 day model showing no requirement for these cells in the development of AI [593].

The data presented here demonstrating that IL-33 given to WT mice during sensitisation exacerbates AAI is a novel finding. The finding is specific as IL-33-driven inflammation was not evident in $ST2^{-/-}$ mice. Furthermore, non-OVA sensitised mice given IL-33 and challenged with OVA did not show any significant eosinophilia in the BAL indicating an Ag-specific mechanism. DLN analysis demonstrated an increased number percentage of $CD4^{+}IL\text{-}5^{+}$ without increase $CD4^{+}IL\text{-}4^{+}$ population. Supportive parallel findings in our laboratory show IL-33 polarises naïve CD4 cells in the presence of TCR stimulation (anti-$CD3^{+}$ antibody or in presence of APC and OVA peptide antigen), to produce IL-5, IL-13 and not IL-4 [496]. This was found to be independent of IL-4, IL-4Rα as well as STAT6 and GATA3 signalling, which are essential for classical Th2 development [594]. Instead, differentiation was dependent on MyD88, an adaptor protein recruited by IL-33 activation of ST2 [293]. The ability to polarise T cells independently of IL-4 seems novel to IL-33. Polarised Th2 cells cultured with IL-33 and TCR stimulus produce IL-5 and IL-13 and is now well described [293,422]. As previously shown by our laboratory, IL-18, another member of the IL-1 cytokine family can similarly induce the production of IL-5 and IL-13 but also IL-4 production from T cells [337]. IL-25 induces Th2 pathologies via IL-4, IL-5 and IL-13 production though from innate immune cells *in vivo* [161]. Both classical and IL-33-dependent $IL\text{-}5^{+}$ T cells will co-exist following antigen sensitisation with IL-33 possibly indirectly stimulating the IL-4 dependent pathway with preference dependent on multiple local environmental factors. Taken together with these *in vitro* experiments, the *in vivo* results presented here suggest IL-33 drives Ag-specific airway inflammation by polarisation and proliferation of $CD4^{+}IL\text{-}5^{+}$ *in vivo*.

The dose of the antigen used in the 12 day model was important to detect a response to exogenous IL-33, with a reduction in the dose used. Differences in dose and route of antigen delivery are common in the asthma model [500]. Prolonged inhaled allergen exposure can induce tolerance [595]. Antigen dose can also influence the development of a specific antibody response: a low dose of antigen induces the optimum IgE response, whereas a high dose induces an IgG2a response *in vivo* [596]. Experiments performed here showed that a 100 µg OVA dose stimulates the classical pathway Th2-IL-4 dependent pathway with no difference in percentage of single positive $CD4^{+}IL\text{-}5^{+}$ T cells when IL-33 was added at sensitisation. The sensitisation dose of 10 µg used in data presented here is in

keeping with reports in the literature. Sakai *et al* demonstrated that increasing the sensitising dose above 10μg OVA actually attenuated rather than exacerbated all features of AAI [280].

The finding here that increased $CD4^+IFN\text{-}\gamma^+$ cells are produced in mice given only IL-33 at sensitisation indicates that under certain circumstances, IL-33 can induce Th1-type responses. This is in agreement with subsequent work by Smithgall *et al* showing IL-33 can amplify Th1- as well Th2-type responses from various cell types [326]. IL-33 enhances the production of IFNγ from iNKT, NK as well as polarised Th2 cells. IL-18, preferentially differentiates Th1 or Th2 cells depending on cytokine environment and genetic background but it is not clear if this could be the case for IL-33 [337]. Whilst IL-33 stimulates T cells to $CD4^+IFN\text{-}\gamma^+$ producing Th1 there is no resultant phenotype which is dominated by the disease state Th2 response.

IL-33 also enhances Ag-mediated Th2 inflammation in an Ag-independent manner. Mice sensitised with OVA, and subsequently challenged with IL-33 without antigen, go on to demonstrate eosinophilic inflammation in the BAL. *In vitro* work from Smithgall *et al* demonstrates this Ag-dependent and Ag-independent enhancement of polarised Th2 cell IL-5 production by IL-33 [326]. As well as by specific Ag-dependent and Ag-independent T cell responses, IL-33 enhances ongoing type-2 responses non-specifically and its role in many disease states by Th-2 mediated immune responses has been described (Chapter 1). As asthma becomes severe and chronic it adopts an altered inflammatory profile towards a Th1 type response with neutrophilia and steroid unresponsiveness. IFNγ and TNFα have roles in this severe asthma phenotype [211,597]. Thus, it would be interesting to examine the effects of IL-33 and its inhibition in chronic allergen models, considered reflective of chronic human asthma.

Although IL-33 enhanced the production of $CD4^+IL\text{-}5^+$ cells without the requirement for IL-4, it was important to investigate whether: these cells could develop in a completely IL-4 free environment and; are suffice to generate AAI in absence of classical $CD4^+IL\text{-}5^+IL\text{-}4^+$cells. This was investigated in $IL\text{-}4^{-/-}$ mice. Pilot findings presented showing non-statistically significant changes and experiments were repeated in a collaborative laboratory using these protocols.

IL-33 enhanced AAI in absence of IL-4 via the mechanism of increasing the population of IL-5 producing T cells [496]. Differences in my findings could be explained by incorrect sample size and altered microbial colonisation in different animal facilities. In particular, mice used in the data shown were not kept in barrier condition in local facilities and concomitant infection is possible. The IL-4 deficient mice used were generated by Noben-Trauth *et al* using targeted construct injection into embryonic cell lines [450]. IL-4 deficiency was confirmed here using tail genomic DNA PCR. Naïve IL-$4^{-/-}$ mice do not display an abnormal phenotype with normal numbers of CD3, CD4 and CD8 cells [451]. The effect of IL-4 deficiency is apparent in the disease state with susceptibility to Th2-mediated disease, in particular helminth parasitic infections. Furthermore, Th1-mediated conditions lose a level of cross-regulation whereby Th2 helper cells regulate the development of the Th1 cell subset by the secretion of IL-4 [42]. IL-4 in parasitic infection is complex and this is demonstrated by its role in *Leishmaniasis.* In WT mice, IL-4 blockade reduced parasite burden and illness however IL-$4^{-/-}$ mice remained susceptible to infection and did not improve with anti-IL-4 therapy suggesting compensatory mechanisms [451]. However, the opposite conclusion of IL-4 mediated resistance was reached by separate investigators using the same mice [598]. The timing of the presence of the presence of IL-4 is important [599] and it can in some circumstances promote Th1 responses [600].

To confirm that the $CD4^{+}$IL-5^{+}IL-4^{-} cells specifically mediated AAI in absence of IL-4, the cells were adoptively transferred by collaborators into IL-$4^{-/-}$ mice which developed more BAL and tissue eosinophilia. Published data show $CD4^{+}$Th2 cells producing type 2 cytokines can develop from IL-4-dependent or IL-4-independent pathways. In IL-4 deficient mice, eosinophilic airway inflammation was impaired but CD4 T cells from the IL-$4^{-/-}$mice behaved in a similar fashion to wildtype, in a model of AAI[46]. CD4 IL-4 independent responses are also seen in other airway or vaccination models as well in parasitic infections described [587,588]. Overall these findings demonstrate IL-33 drives the development of IL-4 independent $CD4^{+}$IL-5^{+} T cells in AAI and raises the possibility IL-33 could be an important mediator in other IL-4 independent responses. However, it must be considered how IL-33 might be available in local environment to polarise Th2 cells in an IL-4 independent fashion.

In agreement with a role for IL-33 at sensitisation, in published studies, blockade of endogenous IL-33 by anti-IL-33 antibody delivered at sensitisation and challenge results in abrogated AI, reduced cytokine and antibody production [601]. Others have now found that IL-33 exacerbates AAI via effects on DCs suggesting this is the mechanism of action for IL-33 at sensitisation. IL-33 enhanced DC activation, maturation, cytokine production and initiation of Th2 responses. The transfer of IL-33 treated OVA-pulsed DCs worsened AAI following adoptive transfer and OVA i.n. challenge compared to mice receiving OVA-pulsed DCs without IL-33 pre-treatment[421]. These authors did not find IL-33 could polarise CD4 T cells without DC co-culture but the resultant cells were consistent with non-classical T cell producing IL-5 and IL-13 but not IL-4.

Secondly in this chapter, the effect of ST2 deletion at sensitisation in AAI was assessed. The role of ST2 in the asthma model has been controversial. Our laboratory has found overall loss of ST2 in a 12 day model results in attenuated OVA-induced inflammation with reduced BAL eosinophilia, IL-5 (but not IL-4 or IL-13) production as well as lower lung inflammation [496]; however, using 28 day models, others have disagreed [338,344]. These differences could to some degree be explained by model protocol and mouse strain differences. In the 12 day model we used, ST2 dependency could indicate loss of ST2 cells required at sensitisation, namely Th2 cells or commitment to a pathway that later requires ST2 at antigen challenge such as on Th2 cells or mast cells. In view of the effect of IL-33 at sensitisation demonstrated above and given that IL-4 was not required in these responses, the presence of ST2 mediated pathway at sensitisation which is not dependent on IL-4 was determined. Given the IL-4 independence, this pathway was hypothesised to be an adjuvant-free. Adjuvant use in vaccination and animal models is commonplace but the exact mechanism of the role of aluminium salts is not clear and it has been thought to enhance sensitisation via IL-4 [602,603]. As adjuvant-free models, thought to be more representative of clinical disease, have longer AAI protocols, a model based on this was chosen. Pilot data obtained in the laboratory indicate ST2 is critical in this 28 day adjuvant free model.

No difference was seen in levels of AAI between WT and $ST2^{-/-}$ mice in the adjuvant model consistent with the 28 day model literature and our previous experience [338,344]. This suggests any ST2 dependent pathway that does exist can

be overcome by alum, via IL-4 or non-specific enhancement. However, in the adjuvant free model, pilot data showing a clear requirement for ST2 at sensitisation in the development of eosinophilic airway inflammation was not confirmed. Despite this, some conclusions about the role of IL-4, ST2 and alum in these models can be drawn.

In comparison to the effects of ST2 at sensitisation in adjuvant or adjuvant free conditions; the IL-4 mediated pathway is dominant. IL-4 induces isotype switching, secretion of IgE by B lymphocytes along with the differentiation of Th2 lymphocytes which in turn leads to cytokine release [604]. Whilst most animal models of asthma show a partial dependence on IL-4, this has not translated in major clinical advances from IL-4 blockade. In agreement with the results here, IL-$4^{-/-}$ mice have been shown to have impaired but not absent AAI without effect on AHR [46]. Although AHR was not assessed in experiments here it is therefore unlikely any differences would be seen. IL-4 independent pathways are known to exist [605] and as discussed, IL-33 can polarise Th2 cells to an IL-5^{+}IL-4^{-} producing cell independent of IL-4 [496]. IL-13 shares a cell-receptor IL-4R with IL-4 thus providing compensatory mechanism for some roles [606]. IgE production can occur via IL-13 [54] and of course non-atopic asthma can develop without IgE. Therefore, in the absence of IL-4 (in the adjuvant free model), a less dominant ST2 pathway may be able to be exert an effect. However, the loss of IL-4 in this model resulted in very low levels of inflammation, making further effects attributable to ST2 in the double knockout mice difficult to delineate.

IL-5^{+}IL-4^{-}Th2 cells that develop at sensitisation with IL-33 treatment are sufficient to cause AAI following adoptive transfer to IL-$4^{-/-}$ mice. The subsequent IL-4/ST2 experiments now demonstrate (at least in this particular model) that these cells are not are essential for the development of AAI as in the absence of ST2, mice successfully sensitise and develop AAI. This suggests IL-33, if present endogenously, can exacerbate AAI via this pathway over the classical IL-4 dependent route should environmental conditions be favourable. In BAL and serum of $ST2^{-/-}$ mice levels of IL-4 are lower, this in consistent with the notion that, IL-4 does not play a major role in IL-33/ST2 mediated AAI.

$ST2^{-/-}$ mice in this AAI model have lower levels of MIG/CXCL9, contrasted by increased levels in IL-$4^{-/-}$ mice, suggesting a ST2-dependent, IL-4-independent

mechanism for monocyte/macrophage recruitment. As discussed in chapter 4, a non-antigen colitis model which is exacerbated by IL-33 in an IL-4 dependent manner, levels of MIG were enhanced by IL-33 also in an IL-4 dependent fashion [447]. In humans, levels of elevated MIG/CXCL9 were associated with IL-33 in serum of patients with malaria but the mechanism could not be determined [607]. In innate IL-33 AI in chapter 4, MIG/CXCL9 was not detected indicating differential Ag- and non-Ag-dependent responses.

A similar pattern of enhanced immune response was seen in all groups co-treated with alum at sensitisation. Alum contributes to development of inflammation in IL-$4^{-/-}$ mice suggesting alum does not act not solely via IL-4. Alum could act via ST2 and furthermore drive a small non-ST2, non-IL-4 pathway. Aluminium adjuvants are the most common adjuvant in use and elicit strong humoral immune responses, but the mechanism of action is not well understood. As IL-4 is a key feature in the induction of Th2 responses it has been proposed to be important in mediating the boost in this response by alum [277,608,609]. Alum can preferentially induce $CD4^+$ cells which in turn mediate differentiation of B cells that produce IgG1 and IgE [608]. This is in part by the generation of IL-4 from $Gr1^+$ splenic eosinophils [603] but also by inhibiting Th1 responses [609]. The increase in IL-6 in IL-$4^{-/-}$ mice in data presented here is in keeping with loss of IL-4 inhibiting Th1 responses; however this difference was only seen in the OVA treated and not OVA-alum treated group. No changes however were seen in levels of IFNγ. It has also previously been shown that alum can enhance Th2 responses in the absence of IL-4 and results presented here are consistent with this finding as OVA-alum treated IL-$4^{-/-}$ mice had increased levels of BAL eosinophilia compared to OVA treated IL-$4^{-/-}$ mice[277,588]. Despite this, the overall the magnitude of reduction of the Th2 response in the absence of IL-4 is considerable.

More recently other mechanisms for the mode of action of alum have been postulated. Intraperitoneal OVA is taken up by DCs in DLN. In the presence of alum, inflammatory peritoneal monocytes can take up the OVA, mature to DCs which travel to DLN. Co-injection of alum is cytotoxic directly leading to (UA) uric acid production. UA activates DAMPs resulting in T cell effector rather than tolerance responses [610]. Also, after alum injection it appears DAMP host DNA is

released [611] but while this alum-induced DNA can be involved in DC-T cell interactions, the receptor pathway is uncertain [612]. The initial recognition of alum by the innate system has recently been suggested to occur via plasma membrane lipids on DCs, leading to activation of spleen tyrosine kinase (Syk-) and P13- kinase signalling. This pathway is implicated in PGE_2 secretion and IgE production along with inhibition of IL-12p70 and thus Th1 responses[613-615]. Furthermore, UA alone can induce and amplify Th2 immunity via this DC pathway without alum or NLRP3 in a HDM model [616]. Results shown here contrast with the IL-12 finding with increased IL-12 in all OVA-alum groups compared to OVA groups. It is unclear as to why Th1 responses are directly activated in all groups but could indirectly reflect a higher level of inflammation in these groups. However, the IL-$4^{-/-}$ mice had proportionately higher serum IL-12 in the OVA-alum group compared to $ST2^{-/-}$ mice and their respective control groups possibly indicating some loss of Th1 regulation by loss of IL-4.

One of the key findings in relation to the properties of alum is that it activates the NLRP3 inflammasome. This in turn activates caspase-1 which has the ability to cleave pro-IL-1β, pro-IL-18 and pro-IL-33 [383,610,617] although this mechanism appears dispensable for IL-33 release [618]. In studies of AAI in $NLRP3^{-/-}$ mice, a critical role for NLRP3 was shown in the presence of alum[617] but also in its absence in a low dose short model where DC migration and IL-33 levels were reduced[421]. The AAI models used were not identical but this suggests alum administration could actually be contributing to IL-33 (and IL-1α and IL-1β) generation but also in alums absence, NLRP3 could have an indirect or direct role in endogenous IL-33 production. Data presented here shows that as alum boosts the immune response in absence of ST2 therefore bypassing an IL-33 pathway, IL-33 is not critical in the response to alum. However, alum dependent or independent enhanced IL-33 production could potentially enhance the NLRP3 pathway at sensitisation. A further study examining the role of NLRP3 *in vivo* in several AAI models including alum-free, concluded there was no prominent role for the inflammasome regardless of model used. However, reduced IL-13 and IL-33 levels were seen in NLRP3 mice confirming a potential role for NLRP3 in endogenous IL-33 processing [619]. Overall, there are multiple simultaneously triggered responses, none of which are selectively responsible for the adjuvant

effect of alum, which reflects its role here in boosting non-ST2, non-IL-4 and non-ST2/IL-4 pathways.

In this model, the loss of ST2 and IL-4 occurs not only at sensitisation but also downstream by loss on effector cells. Thus considering the generated antibody profile is more likely to identify a specific role of at sensitisation. Whilst the data presented here did not demonstrate that ST2 was required in an adjuvant free model for IgE production, it confirmed the crucial role of IL-4 in type-2 antibody production. In the absence of IL-4, it appears alum can boost this response, possibly via the NLRP3 inflammasome that boosts the adaptive response [617]. IL-4 promotes adaptive immunity by class switching to IgE and IgG1 producing isotypes in activated B cells [43]. Loss of IL-4 results in slight reduction in IgG1, absent IgE and increased IgG2a without the inhibitory effects of IL-4 [277]. The IgE results presented in this thesis disagree but secondary mechanisms for IgE production in the absence of IL-4 have been described. The model used in these published studies involved an OVA-alum sensitisation followed by DLN OVA-stimulation *ex-vivo* without an airway challenge thus potentially different kinetics may exist generating IgE via a non-IL-4 pathway. As IL-13 is able to play a partially compensatory role in IgE production in OVA-alum immunisation [606,620] IL-4 receptor (IL-4R) knockout were also investigated [588]. IL-4R$^{-/-}$ mice had similar results to IL-4$^{-/-}$ mice with IgG_1 but not IgE produced, indicating independent function of IL-4 or IL-13. Indeed under certain circumstances, mice lacking IL-4 and IL-13 have capacity for IgE production [86]. Neither IgG2a and IgG1 subclass levels nor DLN recall were assessed in data presented here for comparison.

The route of allergen delivery can affect the resultant Ag-specific response in IL-4$^{-/-}$ mice. Such IL-4 independent pathways have been described particularly with sensitisation via the e.c. route. Th2 responses were equivalent to those which developed in WT mice when an e.c. sensitisation route was followed by i.n. challenge, with the exception of the antibody profile, as no IgE was produced [587]. In this study alum was not used to compare possible enhancement. Alternative sites for class switching and IgE production have been described. In allergic rhinitis, class switching and local IgE production occur in the presence of IL-4 and or IL-13 [77]thus intranasal sensitisation is a route to be considered and where in AAI, the role of IL-4 is unknown.

Data presented did not agree with preliminary work suggesting that ST2 and IL-4 are both independently necessary for sensitisation and AAI, and there are no comparable experiments in the literature to draw firm conclusions about the role of ST2/IL-4 synergistically at sensitisation. There are signals to suggest that a ST2 pathway at sensitisation can be elicited under certain circumstances. In the shorter model, ST2 has a role in alum and alum-free models [421,496]. In understanding possible reasons why these experiments did not come to the same conclusion regarding ST2 as the previous pilot data, many confounding experimental variables must be considered. Any requirement of IL-4 and IL-33/ST2 at sensitisation cannot be studied without accounting for loss IL-4 and IL-33/ST2 on effector cells at subsequent challenge. One approach would be to adoptively transfer T cells obtained post sensitisation from knock out mice and transfer into WT and knockout mice. Although AAI can develop in IL-$4^{-/-}$ mice, the model used here was not validated in these knockout mice and they did not generate sufficient inflammation to ascertain any additional information about the contribution of ST2. Because of the scale of these experiments and resources required, the number of mice per group was deemed sufficient by power calculations. However, statistical conclusions were limited by a decreased in final numbers in some groups. This particularly affected IL-$4^{-/-}$ and $ST2^{-/-}$IL-$4^{-/-}$ mice with susceptibility to illness in the IL-4 mice suspected as discussed in chapter 4. Sterile conditions should be used in further studies.

In summary, IL-4 plays a dominant role in this model but the role of the ST2 pathway still merits further investigation due to potential potent effects of IL-33. Studying alternative sensitisation pathways where IL-4 is not required along with using alternative allergens such as Der P1 could lead to discovery of critical IL-33/ST2 pathways reflecting different asthma phenotypes. Finally, as IL-4 shares a receptor with IL-13, it should be anticipated to perform experiments in IL-4 and IL-$4R^{-/-}$ mice.

The role of cytokine and a cytokine receptor should be considered separately due to potential cytokine redundancy. To further investigate the role of endogenous IL-33 in AAI, repeat experiments in IL-$33^{-/-}$ mice were intended. The mice were unable to be generated in the laboratory during the timeframe available. Following the completion of the works presented in this thesis; studies looking at his have been published. In OVA-alum 28 day AAI, IL-$33^{-/-}$ mice showed

attenuated AI and AHR responses without change in cytokine and OVA-IgE levels. $CD4^{+}ST2^{+}$ cell and OVA-specific spleen responses were similar between IL-$33^{-/-}$ and IL-$33^{+/+}$ mice. This indicates that at least in the presence of alum, IL-33 is not necessary for Ag-specific differentiation but in the Ag-dependent local response by a loss on effector cells[515]. With a similar protocol, another study in the same strain of mouse again showed lack of endogenous IL-33 affected cellular inflammation and not IgE but here this was associated with cytokine IL-5 and 13 reduction andimpaired DLN responses but AHR was not affected [621]. Overall IL-33 appears to have a role in sensitisation but an influence on the effector response predominates.

7 General discussion

7.1 Summary

At the outset of the experimental work presented in this thesis, the role of ST2 in asthma was unclear and the ligand for this receptor, IL-33, had only recently been described. IL-33 is a potent cytokine with innate type 2 effects but its role in AI and asthma was unknown. Since then, research has expanded massively into this field. In the following sections the data presented in this thesis will be summarised in the context of the current understanding of ST2/IL-33 immunobiology with specific consideration to its role in the airway.

7.2 ST2 in asthma

7.2.1 Soluble ST2

The studies in chapter 3 have demonstrated that ST2 and IL-33 are expressed in cells important in asthma pathophysiology. Measuring these determinants in induced sputum and plasma of asthmatics patients was found to be unrelated to clinical disease or smoking status, but interpretation was limited by available assays at the time. An unexpected finding was the increase in sST2 associated with CS treatment. A number have studies have now described elevated levels of sputum IL-33 and sST2, the latest in young asthmatic children [479].

A recent study investigating the effect of smoking on IL-33 expression shows a reduction in BAL IL-33 but not serum levels; the expression of IL-33 in the epithelium was increased. Accompanying *in vitro* studies indicate that expression, but not release of IL-33, is increased in EC exposed to cigarette smoke extract [622]. Thus the findings of raised sST2 presented in this thesis could not be explained as a result of elevated IL-33, present but undetected in the samples. Other than sequestration, a non-canonical role has been described where sST2 is internalised into DCs; although this would not explain raised sST2 levels seen in the CS treated patients, it suggests that other mechanisms for sST2 function exist [623].

Thus smoking influences both IL-33 and sST2 expression but the overall outcome is uncertain but this could suggest an intracellular/nuclear IL-33 pathway is activated by smoking.

7.3 IL-33 and ST2 in airways disease

The roles for IL-33/ST2 in airways disease are multifold; in sensitisation and challenge phases of AAI and asthma but also during exacerbations and in the resolution phase and remodelling.

7.3.1 Sensitisation

Despite compelling evidence regarding the role of Th2-mediated immune responses in asthma, the mechanisms underlying their initiation remain elusive. Results presented in this thesis in chapter 6 and supporting data show IL-33 can induce the differentiation of $CD4^{+}IL\text{-}5^{+}IL\text{-}4^{-}$ cells which exacerbate AAI suggesting a role for IL-33/ST2 at sensitisation [496]. Further data in this thesis shows absence of endogenous IL-33/ST2 at sensitisation does not significantly alter inflammation. Interpretation of these results is difficult to dissect given the loss of ST2 in this model will affect sensitisation and challenge phases, and there the differing roles of ST2L and sST2 both potentially affected. Further studies of loss endogenous IL-33 in AAI have been performed. Using $IL\text{-}33^{-/-}$ mice, attenuated responses in the AAI model was demonstrated with effects seen in the both phases; reduced numbers of DLN cells responsive to OVA-recall assay as well as inflammation in the BAL[621]. Whether the attenuated effector response is due to reduced CD4 cells is uncertain. Similarly, another study of endogenous IL-33 blockade resulted in attenuated AAI when anti-IL-33 antibody was administered at both sensitisation and challenge phases [624]. Blockage of endogenous IL-33 at sensitisation or challenge only may help to unravel these complexities.

IL-33 also has a role on other cells important at sensitisation, in particular on DCs [421]. DC-driven Th2 polarisation is ST2 dependent in an adjuvant-free cypress pollen model of AAI where increased levels of IL-33 were measured; this suggests co-presence of IL-33 could initiate the development of a Th2 response in

response to naturally occurring allergens [625]. Furthermore, IL-33 promotes DC/T cell interaction by enhancing the function of OX40/OX40L ligand [625,626].

Data presented in chapter 6, did not confirm a significant additional role for ST2 above IL-4 in the adjuvant-free pathway in AAI. The adjuvant-free ST2 pathway could well be important in alternate AAI sensitisation routes. ST2-dependence was found in an e.c. sensitisation model when mice were challenged with i.n. allergen, in contrast to findings in the skin where repeated challenge did not demonstrate a critical ST2/IL-33 pathway [627]. This also suggests important mechanisms in links between allergic skin and airways disease. In a HDM i.n. sensitisation model, where i.n. challenge was not performed, soluble ST2 (or anti-GM-CSF) given at sensitisation reduced the Th2 inflammation [469]. The source of IL-33 (and GM-CSF) in this model was the epithelial cell. In the HDM AAI model, inflammation was significantly attenuated in IL-1R$^{-/-}$ mice and by using neutralising IL-1 antibodies at sensitisation, these investigators showed IL-1α was required. IL-1α induced EC cytokine production and so it appeared IL-1α was upstream in HDM-derived cytokine cascade including IL-33 in this particular model [469].

Data presented in this thesis show that in the i.p. model of AAI sensitisation IL-4 was critical. The source of IL-4 at Th2 differentiation in asthma/AAI has been unclear. In the case of protease allergens, directly activated basophils produce cytokines including IL-4, and TSLP *in vivo* [95]. In other models and in asthma, the initial source for IL-4 is a mater of debate with other proposed sources including, γδ T cells, eosinophils, NKT cells, mast cells, ILC and naïve CD4 cells. This remains an outstanding unanswered question.

Beyond Th2 responses, a new role for IL-33 in differentiation of Th1 and CD8 T cell responses is now being appreciated. This has implications in tumour and viral immunity as well as in immunisation where the role of IL-33 as an adjuvant may be more effective [628]. IL-33 is now appears to be a pleiotropic cytokine with increasing functions likely to depend on the surrounding cytokine milleu.

7.3.2 Remodelling

The role of IL-33 in AAI and asthma has extended into chronic inflammation and remodelling. The importance of the bronchial epithelium in this disease process is becoming clearer. Not only does EC produce IL-33, but data presented in this thesis indicates that as ST2 expressing cells, they have an additional role responding to IL-33. IL-33 stimulated ECs produce a number of mediators and drive VEGF-mediated angiogenesis in IL-33 driven AI.

Less is known about the resolution of inflammation in AAI and asthma and how this is linked to clear development of remodelling. The IL-33/ST2 pathway was required for persistence of inflammation in a model of AAI which was mostly dependent on Th2 cells[282]. In a further study, the administration of anti-IL-33 or anti-ST2 antibody once AI was established, resulted in reduced AI and remodelling changes such as epithelial cell hyperplasia, goblet cell hyperplasia, collagen, smooth muscle hypertrophy and sub-epithelial fibrosis. Depletion of macrophages suppressed the remodelling changes, suggesting immune cells are also important contributors to IL-33-driven remodelling [629].

Clinically, an association of IL-33 with parameters of airway remodelling is becoming clear. An increased expression of IL-33 in endobronchial biopsies associated with BM thickness from paediatric patients with severe steroid-resistant asthma was observed [630]. In an accompanying mouse model, this study showed that the remodelling effect was not inhibited by IL-13 blockade despite reduction in AHR; furthermore remodelling was absent in $ST2^{-/-}$ mice [630]. Recently, another study in adult asthmatics demonstrated increased levels of serum IL-33 correlated with BM thickness [491]. *In vitro* observations in both these studies show IL-33 induced fibroblasts fibronectin and collagen production contribute to this pathology [491,630]. Importantly IL-33 mediated changes were steroid resistant, similar to the finding of VEGF-resistant production from EC demonstrated in this thesis in chapter 5.

Overall these findings indicate a wider role for IL-33 in mechanisms of remodelling; this aspect of IL-33 driven disease merits further work, in particular the role of IL-33 in angiogenesis in AAI and asthma.

7.4 Cellular release of IL-33

Whilst studying the effects of exogenous IL-33 is useful to determine biological actions, an important question in IL-33 immunobiology is what cell types are actually capable of releasing IL-33 in a biologically active form? Aside from nuclear IL-33, IL-33 is released from cells in various active forms although there is still some debate over bioactivity of these forms [631]. The dominant hypothesis is that IL-33 is released after necrosis or cell damage but it can also be secreted.

In relation to the airway, the epithelium is a primary source of released IL-33 [186,465,467 469,514 527]. Ideally positioned to respond to inhaled triggers, straightforward damage dependent IL-33 release acting as an alarmin is logical. However, the situation appears more complex and directly activated release and secretion are reported, the later which could perpetuate inflammation, an important aspect of asthma.

Direct release of IL-33 by TLR4 signalling is stimulated by HDM allergen or endogenous triggers [469]. Alternatively, ATP-mediated non-TLR dependent non-necrotic release in response to a fungal allergen is reported [467].

EC derived enzyme transglutaminase 2 (TG2) was found to be important in IL-33 expression and initiating IL-33 mediated Th2 (and Th17) responses in a OVA AAI model; this suggests IL-33 amplifies the OVA model via a common epithelial damage pathway for TG2 and IL-33 production [463]. Another EC damage mechanism is the production of trefoil factor family peptide-2, an epithelial derived repair molecule, which promoted IL-33 production from EC but also alveolar macrophages and DCs to drive Th2 differentiation *in vivo* [574].

Alveolar type 2 and mucus producing ECs have increased IL-33 gene expression in patients with severe Chronic Obstructive Pulmonary Diseases (COPD) and post-viral infected EC in mice. IL-33 expression in the COPD epithelium was localised to the nuclei in the basal cells which are linked to progenitor function and *ex-vivo*, IL-33 was found to be released in an ATP dependent fashion. This suggests a programmed excess IL-33 production in COPD [633]. In this study, IL-33 drove the innate immune response in an IL-13 dependent manner akin to the innate IL-33 model of AI data shown in chapter 4. If similar mechanisms existed in asthma,

this is intriguing as these cells could potentially be a source of chronic IL-33 production triggered by viral infection that perpetuates inflammation.

The EC is at forefront of response to IL-33 in the airway but the mechanisms that underlie release of IL-33 by differing allergens, microbes, virus, smoke as well as endogenous triggers and subsequent signals triggered need to be clarified further.

As well as being IL-33 responsive cells, data in chapter 3 demonstrates IL-33 expression was most likely seen within alveolar macrophages. IL-33 production from the macrophages has now been demonstrated [408,527,574]. A study by Polumuri *et al* used a range of TLR and non-TLR agonists and found distinct post transcriptional regulation dependent on the agonist, indicating multiple triggers for IL-33 production from macrophages [634]. In considering virus as a major trigger of exacerbations in asthma this could indicate a mechanism for viral triggered IL-33 release and amplification of the Th2 response. Indeed, influenza results in increased production of IL-33 by NLRP3 inflammasome activation in macrophages which results in IL-13 dependent AHR in mice [408].

7.5 IL-33 at challenge in AAI

Blockade of endogenous IL-33 at both sensitisation and the challenge phase of AAI attenuates the inflammatory response [621,624]. Thus the role of IL-33 in exacerbations of asthma requires further investigation.

Data presented in chapter 4 shows innate IL-33-driven AI replicates many of the features of asthma. Neither the adaptive immune response nor IL-4 contributed to this effect, with the eotaxin chemokines partially responsible for mediating IL-33 effects along with IL-5 and IL-13. AAM play a crucial role in innate IL-33 driven inflammation through production of eotaxin-2/CCL24. These innate responses may enhance the response to IL-33 when present at the challenge phase of AAI. A recent study looking at this important area of exacerbations in asthma, found IL-33 activated AAM to increase the inflammatory response [635]. The model used in this study was a low allergen dose chronic airway inflammation model generated over many weeks, with exacerbation modelled by a single higher challenge. Markers of AAM were elevated as well as eotaxin-

2/CCl24 levels, supporting the data presented in this thesis and extending the hypothesis that IL-33 innate response could exacerbate an ongoing allergic response.

Another key area in considering how IL-33 might interact with asthma or AAI in exacerbation is in a role related to infective triggers. As mentioned, viral induced IL-33 is released mediating IL-13 dependent AHR. In this manner, IL-33 is released from EC as well as alveolar macrophages and DC [408]. However, IL-33 appears to augment anti-viral Tc responses IFN-γ production [425 426]. Therefore the balance of these mechanisms will be important as surrounding mediators and timing of the viral stimulus seem to affect outcome [636].

Glycolipids present on the *Sphinogmonus* bacterium induce AHR in WT but not $ST2^{-/-}$mice as well as increasing IL-33 expression in ECs and DCs. These bacteria are present in the lungs of some patients with poorly controlled asthma and suggests a possible mechanism for bacteria-driven exacerbation [527]. It is interesting to speculate that IL-33 could be a common pathway for many exacerbating factors in asthma and this merits further investigation.

7.6 Cellular response to IL-33

The number of immune cell types responding to IL-33 and therefore possibly implicated in the pathogenesis of asthma have been well studied. These include mast cells, basophils, eosinophils, T cells and DCs. The recent discovery of a novel population of cells, theILC) responsive to IL-33 has led to intense investigation in their potential role in AAI and asthma.

Findings presented in chapter 4 demonstrated that the type-2 profile of IL-33 driven AI was not dependent on the adaptive immune system. Thus the search for a critical responsive innate source of IL-5 and IL-13 was undertaken. In now appears that pulmonary ILC are a major source of IL-5 and IL-13 seen in IL-33 induced AI [58,526,527]. IL-33 mediated ILC IL-13 production was sufficient for development of AHR in absence of adaptive immune response [527]. Furthermore, in allergen models, IL-33 induced ILC IL-5 and IL-13 production were critical in a protease allergen induced AAI [525,526]. In the ovalbumin AAI model, ILC were present in the lungs in increased numbers following challenge and were the

source of IL-13 mediated AHR [526]. ILC derived IL-5 and IL-13 mediated downstream type 2 effects in the absence of allergen as well as promoting an allergic response to a fungal allergen [528].

Together with CD4 T cells, ILC may contribute to the induction and maintenance of allergic inflammation and evidence shows ILC and CD4 cells actually cooperate in development of response to allergen. Allergen-induced Th2 differentiation was dependent on ILC-derived IL-13 with ILC activation further promoted by EC IL-33 production [637]. Further recent studies indicate these cells can modulate each others function, both contributing to the promotion of adaptive immunity and Th2 inflammation [535,638]. ILC are described in humans but further work is needed to confirm role of ILC in human disease [163].

Other IL-33 responsive cells include EC which clearly express ST2 and are capable of responding to IL-33 as shown in chapter 5. As discussed, IL-33-stimulated EC contributed to angiogenesis but other factors were released on IL-33 exposure. Some recent studies have extended this research into the role of IL-33 on differentiated EC [556]. Using improved models of primary AEC on native BMs and on established models of the EMTU and chronic AAI models should help to further elucidate some of these mechanisms more akin to an *in vivo* setting. As EC are now viewed as central in the pathogenesis of asthma, the elucidation of how IL-33 interacts with them is crucial [639].

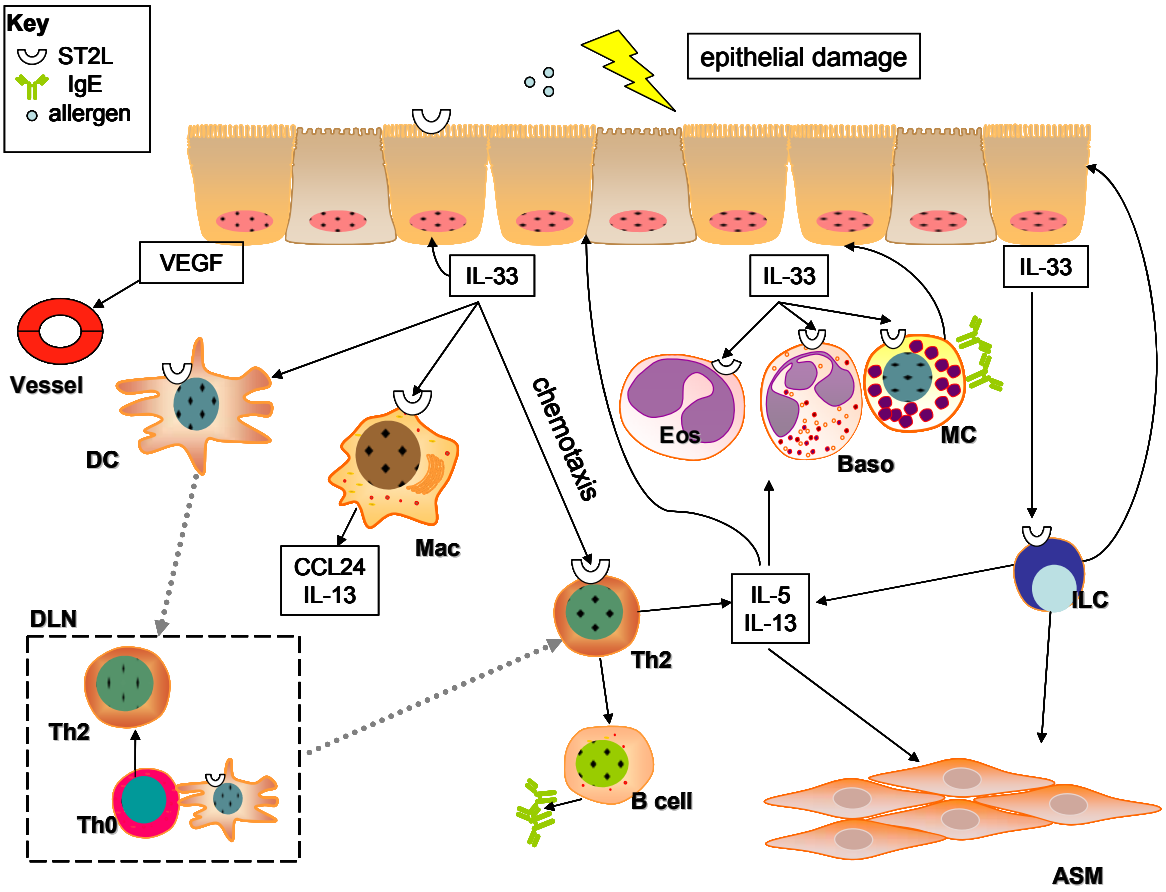


Figure 7.1: The role of ST2 and IL-33 in the airway
Illustrative summary of the main cellular response generated by epithelial cell released IL-33. Release of IL-33 as a consequence of allergen, virus or epithelial damage. DLN=draining lymph node, DC=dendritic cell, Mac=macrophage, Eos=eosinophil, Baso=basophil, MC=mast cell, ILC=innate lymphoid cell, ASM=airway smooth muscle

7.7 Clinical asthma

Along with areas discussed which mechanistically link IL-33 and AAI, genetic studies in humans suggest variations in the IL-33 gene are associated with increased susceptibility to asthma [640]. Asthma is heterogenous thus a better understating of clinical phenotypes in asthma and AAI models has led to clinical trials targeting cytokines with targeted patient selection and improved outcomes. Atopic asthma affects up to 50% those with the disease and severe asthma remains a major cause of illness. It could be postulated that greater benefit might be achieved by blocking an upstream target like IL-33. Although no studies in man have so far targeted IL-33, these are no doubt anticipated.

7.8 Concluding remarks

IL-33 binds to its receptor, ST2, and triggers AI and contributes to AAI by interacting with both the innate and adaptive arms of the immune system. Data presented in this thesis contribute to the increased understanding of IL-33 biology.

Exogenous and endogenous stimuli induce EC and other cells to produce IL-33 which acts on DCs, ILCs andCD4 cells initiating the release of Th2 cytokines, principally IL-5 and IL-13. These cytokines act in an autocrine and paracrine manner and on cells including macrophages and ECs.

This leads to the release of further mediators including chemokines and remodelling factors which drive the pathological response of AI, AHR and remodelling, akin to asthma and could contribute to symptomatic disease in *vivo*.

Many of the questions generated from the data presented in this thesis have been addressed in recent studies. Outstanding areas of knowledge in ST2/IL-33 biology include understanding: the role of ST2 in non-adjuvant sensitisation and IL-33 as an adjuvant; the exact role of sST2 and the possibility it may have a role independent of IL-33; defining the function of intranuclear IL-33 in expressing cells; further investigation of ILCs particularly in humans; and the role of IL-33 in asthma exacerbations requires further attention.

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