IL-33-induced innate lymphoid cells and airway inflammation

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Abstract

Background: IL-33 is an innate cytokine and a member of the IL-1 superfamily. It is the ligand for ST2 and since it was first described, it has been shown to be involved in an increasing number of immune responses, both in health and disease. Greater understanding of this field has demonstrated that IL-33 is a dichotomous cytokine, able to function as both a classical cytokine and as a nuclear factor. Whilst numerous signalling pathways for IL-33 have been described, a role for the serine threonine kinase, mammalian target of rapamycin (mTOR), has not been assessed. mTOR activation can be inhibited by rapamycin, an immunospressant that is widely used in clinical practice.

One of the most recent advances in the field of IL-33 is its ability to induce a novel group of innate lymphocytes named type-2 innate lymphoid cells (ILC). These cells respond to IL-33 and other innate cytokines to produce the type-2 cytokines, IL-5 and IL-13. Much is yet to be discovered about these novel cells and their homeostatic and pathological roles. More so, very little is known about the signalling pathways involved in IL-33-driven ILC functions and their interactions with the adaptive immune system.

Aim: To determine the role of mTOR in IL-33-driven airway inflammation and in IL-33-induced ILC functions. To ascertain the interactions of ILC with CD4 T cells *in vitro* and *in vivo*.

Methods and Results: BALB/c mice were treated with intranasal IL-33 to induce airway inflammation in the presence or the absence of rapamycin. IL-33-induced ILC were sorted using fluorescence activated cell sorting (FACS) techniques to perform *in vitro* experiments to assess the role for mTOR in IL-33 signalling and the interactions with FACS sorted naïve CD4 T cells. Additionally, adoptive transfer experiments were performed to determine the roles of ILC in IL-33-driven airway inflammation as well as their effects on CD4 T cells *in vivo*.

IL-33-driven airway inflammation and ILC cytokine production *in vitro* is significantly inhibited by rapamycin. Additionally, ILC are the main source of type-2 cytokines in IL-33-treated lungs and they are sufficient to drive IL-33-

induced airway inflammation in ST2-deficient mice in an mTOR-dependent manner.

ILC respond to CD4 T cell-derived IL-2 by proliferating and expressing IL-4. In turn, ILC induce type-2 cytokine expression in naïve CD4 T cells *in vitro*, whilst inhibiting their expression of the type-1 cytokine, interferon-γ, in a contact-dependent manner. This effect is partially dependent on the adhesion molecule, intercellular adhesion molecule (ICAM) 1. More so, ILC are able to increase the proportion of activated CD4 T cells *in vivo* whilst enhancing their recruitment to the lung.

Conclusions: mTOR activation is required for optimum IL-33-induced airway inflammation and IL-33 signalling in ILC. Additionally, ILC interact with CD4 T cells *in vitro*, enhancing a Th2 phenotype in these cells. Furthermore, ILC augment CD4 T cell activation *in vivo,* demonstrating that ILC are able to influence adaptive immune responses and enhance type-2 immunity. Taken together, these results further the current understating of IL-33 and ILC biology. More so, the results included in this thesis offer future potential avenues to be explored in the development of better therapies for conditions in which IL-33 has been shown to be deleterious, such 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, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature……..

Printed name……

Definitions/Abbreviations

$^{-/-}$	knockout
γc	γ chain
AAM	alternatively activated macrophage
Ab	antibody
AFO	airflow obstruction
Ag	antigen
AHR	airway hyperresponsiveness
Alum	aluminium hydroxide
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP	activator protein
APC	antigen-presenting cells
ATP	adenosine triphosphate
ATS	American Thoracic Society
BAL	bronchoalveolar lavage
BALB	Bagg Albino (inbred research mouse strain)
BSA	bovine serum albumin

BTS	British Thoracic Society
CCL	C-C motif ligand
CCR	C-C motif receptor
CD	cluster of differentiation
cDNA	complementary Deoxyribonucleic acid
CFSE	carboxyfluorescein diacetate succinimidyl ester
c-kit	mast/stem cell growth factor receptor Kit
CLP	common-lymphoid progenitor
CM	complete medium
CS	corticosteroids
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
Deptor	DEP-domain-containing mTOR-interacting protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Di-n-butylPhthalate in Xylene
EAE	experimental allergic encephalitis

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eIF4	eukaryotic initiator factor 4
ELISA	enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
eYFP	enhanced yellow fluorescence protein
FACS	fluorescence-activated cell sorting
FALC	fat-associated lymphoid clusters
FBS	fetal bovine serum
FCεRI	high-affinity IgE receptor
FDA	Food and Drug Agency
FITC	Fluorescein isothiocyanate
FKBP12	FK506-binding protein of 12kDa
Flt3	fms-like tyrosine kinase-3
Foxp3	Forkhead box p3
FSC	Forward scatter
GATA	Trans-acting T-cell-specific transcription factor GATA
GFP	green fluorescent protein

GINA	Global Initiative for Asthma
GM-CSF	granulocyte monocyte-colony stimulating factor
GS	Golgi-stop
GVHD	Graft-versus-host disease
GWAS	genome-wide association study
HDM	house dust mite
HE	haematoxylin and eosin (stain)
HEV	high endothelial venules
HF	heart failure
HIF	hypoxia-inducible factor
HMGB1	high motility group box-1
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
ICOS	Inducible T-cell costimulator
ICS	inhaled corticosteroids
ID	inner diameter
Id2	inhibitor of differentiation 2
IFN	interferon
Ig	immunoglobulin

Ih2	Innate helper cell 2
IHC	immunohistochemistry
IκB-α	inhibitor of kappa B-α
IL	interleukin
ILC	innate lymphoid cells
i.p.	intraperitoneal
IRAK	IL-1 receptor-associated kinase
i.v.	intravenous
JAK	Janus kinase
HPRT	hypoxanthine phophoribosyltransferase
kb	kilobase
kDa	kiloDalton
KSHV	Kaposi sarcoma-associated herpes virus
LANA	latency-associated nuclear antigen
LFA	lymphocyte function-associated antigen
LMMP	lymphoid-primed multipotent lymphoid progenitors
LN	lymph node
LPS	lipopolysaccharide

MAP	mitogen-activated protein
Mac	Macrophage antigen
MC	Mast cell
MCP	Monocyte chemotactic protein
M-CSF	Macrophage-colony stimulating factor
MEK	Mitogen-activated protein kinase kinase
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex
MI	Myocardial infarction
MIP	Macrophage inflammatory protein
mLST8	mammalian lethal with Sec13 protein 8
mRNA	Messenger RNA
mSIN1	mammalian stress-activated protein kinase interacting protein
MTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NF	Nuclear factor
NFAT	Nuclear factor of activated T cells
NHC	Natural helper cell
NK	Natural killer

NKT	Natural killer T
OD	Optical density
OVA	Ovalbumin
PAS	Periodic-acid Schiff
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Penh	Enhanced pause
PI3K	phosphoinositide 3-kinase
PMA	phorbol 12-myristate 13-acetate
PRAS40	proline-rich AKT substrate 40kDa
Protor-1	protein observed with Rictor-1
PVDF	Polyvinlidene fluoride
RA	Rheumatoid arthritis
Rag	Recombination activating gene
Raptor	rapamycin-sensitive scaffolding protein regulatory-associated protein of mTOR
RELM	Resistin-like molecule
Rictor	rapamycin-insensitive companion of mTOR

RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic acid
ROR	RAR-related orphan receptor
RPMI	Roswell Park Memorial Institute
RSK	Ribosomal S6 kinase
RT	Reverse transcriptase
RTP801	HIF-1 activated RTP801
RV	Rhinovirus
Sca	Stem cell antigen
SCF	Stem cell factor
SD	Standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SIGN	Scottish Intercollegiate Guideline Network
SSC	Side scatter
ST2	Suppression of tumourgenicity 2
sST2	Soluble ST2
ST2L	Longer ST2
STAT	Signal transducer and activator of transcription

S6K	S6 kinase
TARC	Thymus and activation-regulated chemokine
T-bet	T-box expressed in T cells
Tc	Cytotoxic T cell
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSC	Tuberose sclerosis complex
TSG	Tumour suppressor gene
TSLP	Thymic stromal lymphopoeitin
TSLPR	Thymic stromal lymphopoeitin receptor
TW	transwell
UC	Ulcerative colitis
UK	United Kingdom

UVB	Ultraviolet band
WB	Western blot
WBP	Whole body plethysmography
WT	Wild type
4E-BP1	4E-binding protein 1

1 Introduction

1.1 Asthma

1.1.1 Prevalence and definition

Asthma is a common chronic inflammatory disorder of the lung characterised by airflow obstruction, inflammation and lung remodelling. The incidence and prevalence of asthma has been rising in the westernised world, although the rate of increase has slowed down in the past decade [1]. The most recent estimates however, suggest that up to 300 million people suffer from asthma worldwide [1]. A growing rate of urbanization has been suggested as a possible explanation for the increase in prevalence, since living in a city is a risk factor for asthma. Evidence has shown that children living in rural areas are less likely to develop asthma [2]. The reduction in asthma risk is associated with decreased levels of sensitisation to common allergens, therefore also linking the development of atopy and allergy to asthma [2]. Atopy and allergy have long been associated with asthma and up to 40% of the children and young adults in the westernised world have been shown to be atopic [3], yet of these, only a third develop asthma [3]. As such, the clinical burden of asthma remains a substantial one and in the United Kingdom (UK) alone, estimates suggest 1 in 7 children and 1 in 25 adults suffer from asthma symptoms requiring treatment [4].

One of the greatest challenges in the management of asthma at a global level is the lack of a distinct definition for the disease. The Global Initiative of Asthma (GINA) defined asthma as "… *chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness (AHR) that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable, airflow obstruction (AFO) within the lung that is often reversible either spontaneously or with treatment*" [1]. The lack of a precise pathologically-orientated definition makes the diagnosis of asthma difficult. In addition, as the understanding of allergy, airway inflammation and the causes of AHR have developed, more emphasis and interest has been placed on phenotyping the different "types" of asthma. This movement has increasingly gained support, as it appears that the immune characteristics of different

asthma sufferers vary significantly and treatments are not universally effective. It is therefore imperative to continue investigating the immunopathological mechanisms of this condition.

1.1.2 Clinical asthma management

The above-mentioned clinical definition of asthma encompasses several key clinical characteristics including AFO and AHR. In addition to these, airway inflammation and remodelling are now also considered important features of the disease [5, 6].

The management of asthma involves two main strategies to treat the condition: bronchodilators to improve AFO and AHR symptoms, and anti-inflammatory medication to treat the airway inflammation.

The British Thoracic Society (BTS) and Scottish Intercollegiate Guideline Network (SIGN) have developed national guidelines to aid the management of this heterogeneous condition (www.brit-thoracic.org.uk/guidelines/asthma-guidelines.aspx). The mainstay of asthma therapy includes the use of β2-adrenergic agonists as bronchodilators and inhaled corticosteroids (ICS) to control airway inflammation [7-9]. There are other drugs that are also used to regulate airway inflammation in asthma, including leukotriene antagonists [10], which interfere with the effects of leukotrienes, lipid mediators of the arachidonic acid pathway that are produced by various cell types including mast cells and eosinophils [11]. Importantly, smoking has been associated with reduced drug effectiveness in asthma patients [12, 13] and hence smoking cessation is also an important management strategy in this patient group. The majority of patients will have well-controlled asthma symptoms on combinations of the above medications. Unfortunately however, a proportion of patients continue to have debilitating symptoms despite these therapies, with estimates suggesting up to 5% of the asthma population fall into this category [5]. These patients often require regular oral corticosteroids, the use of which is associated with substantial longterm side effects [14]. One of the most recent additions to the asthma management treatment regime is a monoclonal antibody against immunoglobulin E (IgE) [15, 16]. This treatment is only available for a small minority of patients with severe asthma who remain uncontrolled despite

receiving oral corticosteroids (CS) (www.brit-thoracic.org.uk/guidelines/asthma-guidelines.aspx). Whilst it is extremely effective in some, it is certainly not associated with improvement in symptoms in all patients [17]. Patients with uncontrolled asthma despite maximal therapy remain an important unmet clinical need, incurring substantial healthcare and socioeconomic burdens. In fact, estimates suggest that whilst only representing a small proportion of all asthma patients, healthcare costs for these individuals amount to two thirds of total asthma expenditure in the UK [6]. Whilst the enhanced understanding of asthma pathogenesis has led to the development of numerous biological drugs (reviewed in [18]), most severe asthmatics still rely on systemic corticosteroids as their mainstay treatment [18]. Attempts to use steroid-sparing immunosuppressants have failed in the past due to unacceptable side effects [19]. For all these reasons, better understanding of the immunopathology of asthma is still required for many sufferers of this condition in order to allow the development of improved therapies with fewer side effects.

1.1.3 Immune responses in asthma

As previously discussed, airway inflammation has been implicated in asthma pathogenesis and increasingly sub-classification of the different forms of inflammation observed in patients diagnosed with asthma has been insightful into the potential mechanisms driving the disease. As such, many biased methods have been used to delineate different phenotypes of asthma, based around clinically defined, often subjective, asthma symptoms and signs.

Historically asthma was classified as “extrinsic” when associated with allergy and allergenic triggers, and “intrinsic” when there was no evidence of allergy driving the disease. These are also known as “atopic” or “non-atopic”, respectively. Further classifications based on the bronchoalveolar lavage (BAL) or induced sputum inflammatory cell compositions have also been proposed. As such, “eosinophilic”, “mixed granulocytic”, “neutrophilic” or “paucigranulocytic” forms have been described [20]. These classifications have broadened the understanding of asthma, whereby different phenotypes appear to have differing underlying disease processes.

Since the dichotomous classification of adaptive T helper (Th) cell responses as Th1 (mainly interferon (IFN)-γ producing) or Th2 (mainly type-2 cytokine-producing i.e. IL-4, IL-5, IL-13) [21], allergy and asthma have been classified as predominantly Th2-driven diseases [22]. This evidence is supported by the clinical finding that cells from BAL of asthma-suffers expressed higher levels of type-2 cytokines, compared to healthy controls [23], as well as data from animal models supporting the role of Th2 cells in mouse models of airway inflammation [24, 25]. Interestingly, in a cohort of mild ICS-naïve asthmatics, bronchial epithelial cell gene expression analysis demonstrated that up to 50% of that cohort expressed a "Th2-high" phenotype, denoted by increased expression of IL-13-induced genes. However, asthmatics lacking this phenotype had gene profiles similar to that of healthy individuals. Importantly, those with a "Th2-high" phenotype responded to ICS better than those with a "Th2-low" profile, suggesting different underlying immune mechanisms were present in this cohort of asthma-suffers [26], which in turn, had an impact on their response to treatment.

1.1.3.1 Immune cells in asthma

Numerous cells have been implicated in the development in asthma, and whilst it is likely that each cell type contributes to asthma pathogenesis and cross-talk undoubtedly is important, for clarity they shall be discussed individually. Furthermore, whilst innate lymphoid cells (ILC) have recently been implicated in asthma pathogenesis, since their description is inextricably linked to IL-33, they shall be discussed in section 1.3.5.2.1.

Atopic asthma has often been described as a Th2-mediated disease. In this regard, Th2 cells produce many of the cytokines implicated in asthma pathology including IL-4, IL-5 and IL-13 [3]. More so, BAL of asthma patients showed a Th2-like lymphocytosis [23]. Novel sub-types of Th cells have been described since the initial paradigm of Mossman and Coffman was proposed [21]. These include Th9 cells that produce IL-9, and Th17 cells, that secrete IL-17. Both these subgroups have been implicated in asthma [27, 28] and Th17 cells, due to their ability to recruit neutrophils, have been suggested to be pathological in non-eosinophilic, neutrophilic asthma [27, 29]. Interestingly, the lung has been shown to have large numbers of resident memory T cells at rest, many of which

are antigen-specific [30], suggesting that these cells are poised and ready to respond to re-encountering their cognate antigen in the lungs. As a result, much work has focused on the interaction of T cells with other innate cells, in particular, antigen-presenting cells (APC).

Dendritic cells (DC) are professional APC and their function in the lung has been studied extensively. DC transport antigen from sites of delivery to the draining lymph node (LN) [31]. More recently, they have been shown to sample antigen in the alveolar space, through epithelial tight junctions [31], placing DC in a privileged position to obtain and present inhaled antigen. In fact, DC have been shown to be present in induced sputa of patients with asthma and additionally, antigen-challenge further enhances their number within a few hours of antigen exposure [32], demonstrating their relevance in human asthma.

B cells are responsible for the production of IgE via a process of class-switch, which allows them to shift their production of IgM to IgE [33]. In order to develop into IgE-producing plasma cells, B cells require two signals, one provided by type-2 cytokines such as IL-4 and another via cell contact and CD40 engagement [34]. As aforementioned, IgE is a common marker of atopic asthma and anti-IgE therapy has been shown to be effective in some CS-resistant severe asthma patients [15, 16]. Whilst it was believed that B cell class switch only occurred in peripheral lymphoid tissue, evidence is emerging that this process may occur within the airways themselves [35], placing B cells at the forefront of the allergic asthma response. In addition to their role as IgE-producing cells, antigen-activated B cells have also been shown to be able to present antigen to cognate T cells [36], further supporting their pathological role in asthma.

Eosinophils are granulocytes that appear red when stained with eosin by the Romanovsky method due to their content of acidic granules. They develop and proliferate in response to IL-5 and when activated, they degranulate, releasing a variety of pro-inflammatory mediators, including histamines and peroxidases [37]. These cells were first associated with asthma with the discovery of eosinophil-derived granules in the airways of asthma patients at *post mortem* [38]. Eosinophils can be found in the airways and peripheral blood of patients with asthma and their numbers have been shown to correlate with disease severity [39]. Additionally, the presence of sputum eosinophilia in patients with

asthma predicts their likely response to CS treatment [40]. More so, the numbers of eosinophils in induced sputa of asthma patients have been used to guide asthma treatment successfully [41]. All this evidence places eosinophils at the forefront of asthma inflammation. However, some debate remains regarding their pathogenic role in the lung. Evidence from animal models, using a variety of transgenic mice and adoptive transfers, support their role as drivers of disease [42-44]. However, data from human studies are less clear. IL-5 is an important eosinophil chemoattractant and growth factor [37]. An anti-IL-5 study showed, whilst eosinophilia was decreased by the treatment in an asthmatic population, symptoms and exacerbations were not significantly reduced in the studied cohort [45], suggesting that eosinophil reduction did not alter the disease process. As discussed above, asthma is a heterogeneous disease in humans and subsequent studies assessing the effect of anti-IL-5 treatment in CS-resistant patients with ongoing pulmonary eosinophilia did show improved clinical outcomes with this therapy [46, 47], stressing the importance of phenotyping asthma patients.

Macrophages are the most abundant immune cell in the lung and they are divided by their location to alveolar, interstitial or intravascular macrophages [48]. Much work on macrophages has concentrated on the role of the alveolar macrophage, in particular, assessing their function under the influence of type-2 cytokines, which skew them away from classically-activated (or M1) macrophages to an alternatively activated macrophage (AAM) (or M2) phenotype [49]. This dichotomy may be an oversimplification, in a similar way to the Th1/Th2 division. However these two types of macrophage phenotypes have distinct roles in the airway [49]. In human asthma patients, the number of IL-13-expressing alveolar macrophages is greatly increased compared to healthy controls [50]. More so, in a post-viral chronic inflammatory lung model, AAM appear responsible for persistent lung inflammation [50]. Interestingly, Tang and colleagues demonstrated that alveolar macrophage depletion exacerbates ova-induced allergic airway inflammation through enhanced Th2 responses, suggesting that alveolar macrophages may also be important regulators of inflammation [51]. Therefore the precise role of alveolar macrophages in asthma is yet to be fully elucidated.

Neutrophils are granulocytes most commonly associated with acute inflammatory responses and anti-bacterial functions. In the context of asthma, however, the neutrophilic phenotype is most commonly found in patients who do not appear to have allergy-driven disease [52]. As such, these patients have increased neutrophils in their BAL, rather than eosinophils, and they have been shown to be less responsive to ICS-treatment [53]. Additionally, patients with severe CS-resistant asthma have increased airway neutrophilia, compared to normal controls or moderate asthma patients [54], and patients requiring mechanical ventilation for non-infectious asthma exacerbations have increased airway neutrophils compared to controls [55]. More recently, a role for Th17 cells has been suggested in patients with neutrophilic asthma, since Th17 cells are able to recruit neutrophils to the airway, driving non-Th2 inflammation (reviewed in [27]). Whilst taken together these data demonstrate the presence of neutrophils in asthma inflammation, their role as pathogenic cells is less clear. This issue is further confounded by the fact that many of the patients with non-eosinophilic asthma are on higher CS doses and in turn, CS have been shown to enhance neutrophil survival by preventing apoptosis [56, 57], hence suggesting that the increased neutrophil presence in the airway may simply reflect the use of CS in these patients. Further research in this field is required to confirm whether the neutrophilic phenotype is a distinct inflammatory condition compared to eosinophilic asthma, and whether neutrophils drive this inflammatory response.

Mast cells and basophils have also been implicated in allergy and asthma. Both cell-types express the high affinity IgE receptor (FcεRI) and can bind IgE, leading to their degranulation, releasing numerous mediators such as histamine and serine proteases [58]. Additionally, both cells are sources of type-2 cytokines [58, 59] and have been shown to influence Th cell differentiation [60, 61]. Interestingly, basophils have recently been suggested to have key APC-functions *in vivo* [61-63]. Furthermore, the presence of mast cells in the smooth muscle layer of bronchi in patients with asthma appears to correlate with airway hyperresponsiveness [64] and can influence smooth muscle cell functions [65, 66] suggesting that cross-talk mechanisms between all the cell types in the lung may orchestrate the symptoms of asthma. The interactions between these cell types are briefly summarised in Figure 1-1.

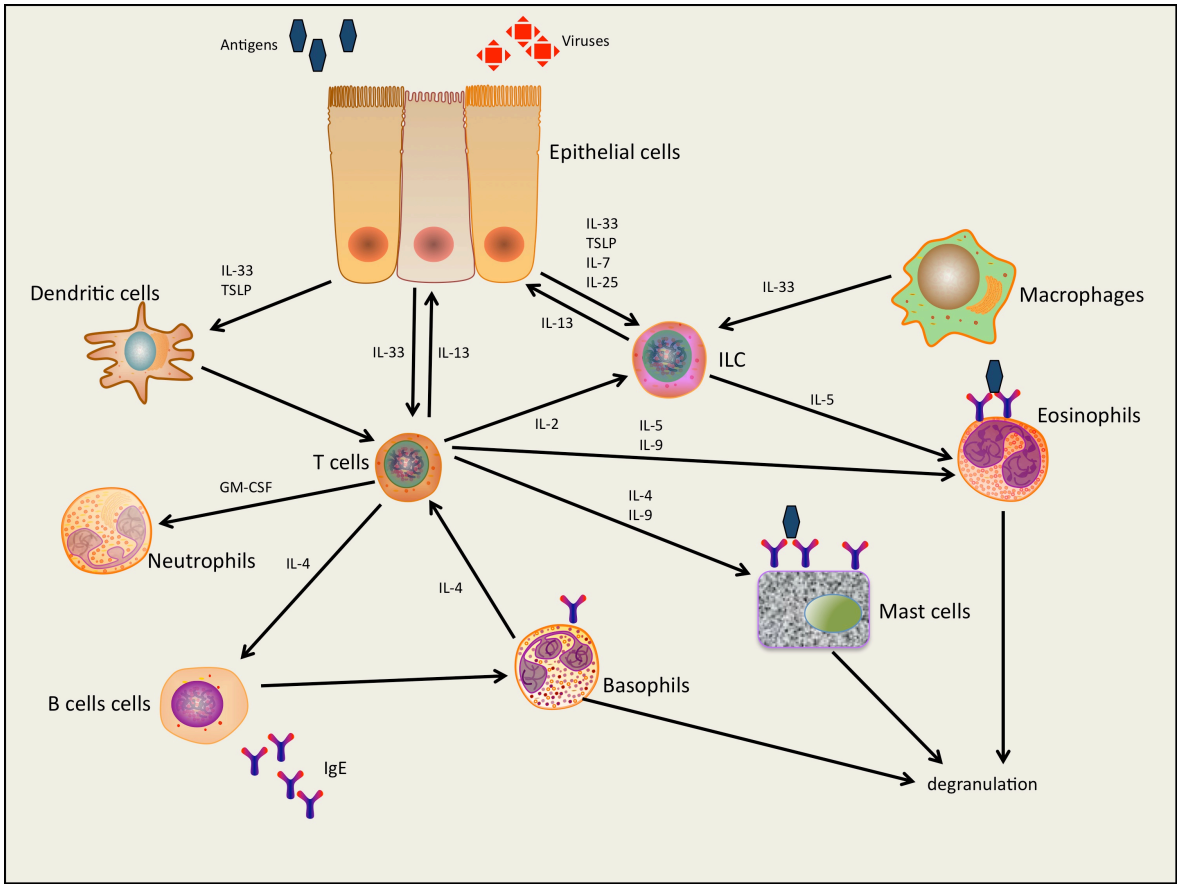

Figure 1-1 Summary of important cellular interactions in asthma

Diagram depicting the important cross-talk between immune cells and non-immune cells in asthma, driven by their cytokine production. Common triggers of asthma exacerbations such as viruses and antigens are shown as they can trigger this cytokine cascade in the lung.

The inflammatory milieu induced by the immune cells and the release of their inflammatory mediators affect the stromal cells in the lung itself, driving the clinical features of asthma. These are briefly described in Figure 1-2.

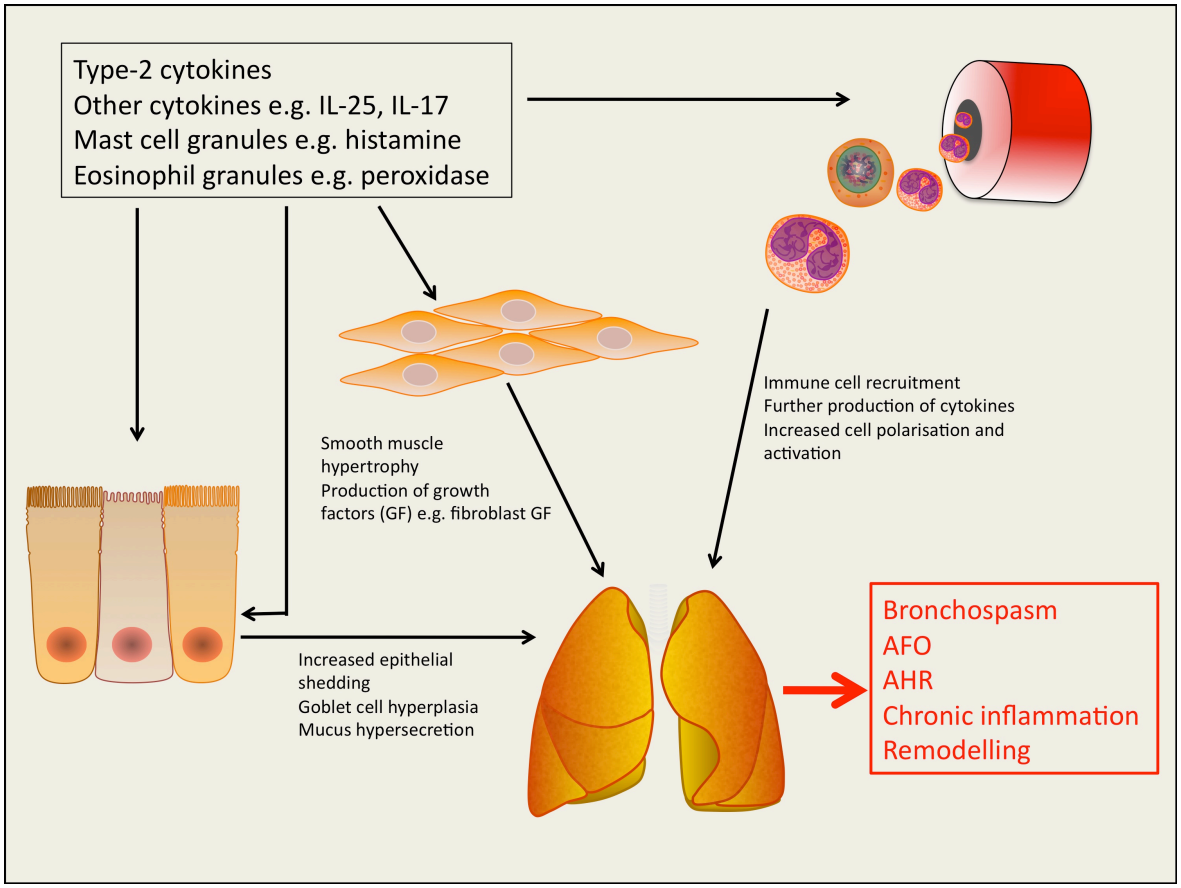

Figure 1-2 Summary of asthma pathogenesis and clinical features

Diagram summarising the important interactions of the different tissues and cells types in asthma, leading to the characteristic symptoms of asthma.

1.1.3.2 Novel cytokines in asthma

The key role of epithelial cells in asthma has been increasingly explored. In particular, disruption of the normal barrier function in asthmatic airways has been suggested as a self-perpetuating mechanism for chronic inflammation [67]. Epithelial cells can produce a variety of chemokines and cytokines, and recently much interest has been placed on three stromal cell-derived cytokines in asthma pathogenesis, thymic stromal lymphopoeitin (TSLP), IL-25 (also known as IL-17E) and IL-33.

TSLP was first described as a cytokine found in a murine thymic cell line and was described as a B cell and T cell growth factor [68]. However, its roles have extended to involve functions in basophil differentiation [69], DC activation driving Th2 polarisation [70] and mast cell activation [71]. Its levels are increased in patients with asthma, and levels of expression correlate with

disease severity [72]. More so, transgenic mice over-expressing TSLP in their airway epithelium develop spontaneous pulmonary inflammation with features resembling human asthma [73]. The evidence for an important role for TSLP is therefore substantial. In fact, data described in chapter 4 and by others [74, 75] have demonstrated a role for TSLP in the functions of novel type 2 ILC populations.

IL-25 is a recently described member of the IL-17 family of cytokines and it signals through both the IL-17RB and IL-17RA receptors [76]. Over-expression of this protein in mice *in vivo* leads to type-2 lung inflammation with eosinophilia, mucus hypersecretion and AHR as well as augmented IL-4, IL-5 and IL-13 levels [77, 78] suggesting a role for IL-25 in allergic disease. In fact, neutralising IL-25 in a mouse model of allergic airways inflammation blocked the onset of AHR and reduced pulmonary inflammation [79]. In addition to these findings, TSLP-treated DC enhanced memory Th2 cells' expression of IL-17RB, making them more responsive to IL-25. IL-25, in turn, was able to increase type-2 cytokine production from Th2 cells and enhance their proliferation *in vitro* [80]. More recently, IL-25 has been shown to be able to induce ILC (see below) and a population of cells in gut lymphoid tissue named multipotent progenitor cells type 2 [81]. These cells have progenitor capacity and can drive type-2 immune responses in the gut. Their role in the lung, if any, has not yet been determined.

Finally, IL-33 is one of the most recently described members of the IL-1 family of cytokines [82]. The role of IL-33 in airway inflammation is the main focus of this thesis and will be discussed at length below.

1.1.4 Animal models in asthma

Much of what is currently known of the immunobiology of asthma is derived from murine models of type 2 allergic airway inflammation. In an attempt to mirror human atopic asthma, these models often have the characteristic features of eosinophilic inflammation and AHR [83]. In order to induce these features, many models of allergic airway inflammation involve immune sensitisation with a protein, commonly chicken ovalbumin (ova), and subsequent challenges into the airway with the same protein [83]. The advantages of using ova are numerous,

most notably the fact that its epitope structure is well described and transgenic mice expressing ova-specific T cell receptor (TCR) are available. More recently, numerous groups have employed antigens more relevant to human disease [74, 84], including house-dust mite [85] and cockroach extracts [85], common allergens in human asthma, as well as chronic exposure of rodents to these in order to better replicate the disease [86, 87]. The sensitisation process is often via systemic administration of allergen. However, small variations within protocols appear to give different results and hence the route of sensitisation appears to be important [88, 89]. Moreover, the addition of adjuvants can alter the phenotype. The use of aluminium hydroxide (alum) enhances the sensitisation process via the activation of the inflammasome [90]. There are different methods for antigen delivery to the airway, but less invasive methods, such as intranasal or nebulised routes are favoured.

Whilst variations in protocols can alter the outcomes of the models, so can the genetic background of the mice [89]. This is of particular importance since transgenic mice are often developed to investigate the contribution of a particular molecule or pathway and the genome of certain genetic backgrounds are easier to target [91]. Whilst the use of transgenic mice is a very attractive option, the impact of the genetic background used must be taken into account [91, 92].

The mainstay method for measurement of airway inflammation has involved performing BAL and subsequent Romanovsky staining to provide differential cell counts. Measurements of cytokine levels in the lungs are also commonly used. More recently, fluorescence-assisted cell sorting (FACS) techniques have been used to assess cell types, allowing more in-depth characterisation of the different cells involved in the inflammatory process.

Numerous methods to measure AHR in mice have been developed. The gold-standard method remains invasive measurements of direct airway resistance using tracheal intubation of anaesthetised mice [93]. The description of a non-invasive method measuring enhanced pause (Penh) [94], using whole body plethysmography (WBP), allowed many groups to perform AHR without the need for the expensive equipment and highly technical skills required for the gold-

standard methods [95]. This technique has been shown to have limitations [96] and hence invasive methods remain the method of choice [93].

Whilst informative, mouse models of allergic inflammation have received criticism over the last decade. The main reason has been the variable effects of numerous drugs, developed using mouse models, in human trials (reviewed in [97]), leading to the suggestion that allergic airway inflammation induced in mice does not mimic asthma [97]. A well-described example of this is the anti-interleukin (IL)-5 monoclonal antibody drug, Mepolizumab [98]. Treating mice undergoing an allergic airways disease model with anti-IL-5 therapy improved their inflammatory scores as well as AHR [99]. Initial trials in humans, however, failed to show clinically-significant improvements in disease [98]. Importantly however, since then, anti-IL-5 therapy has been shown to be effective in a subgroup of CS-resistant asthma patients [46, 47]. These findings demonstrate the relevance of mouse models of allergic inflammation, yet they stress the need for better patient phenotyping in order to be able to target therapies more successfully.

Much effort has been put in creating better models of asthma that reflect other key aspects of the disease process, notably, the epithelial cell involvement, lung tissue remodelling and non-atopic inflammatory characteristics of human disease [83, 97]. These ongoing efforts, as well as knowledge of the important limitations of animal models, are ensuring that these methods of modelling disease remain a useful and key tool to enhancing the understanding of this heterogeneous disease.

1.2 ST2 and IL-33

1.2.1 ST2, in the beginning

ST2 (suppression of tumourgenicity 2) (*il1rl1*), the IL-33 receptor, was first described by two separate groups working independently on a mouse fibroblast cell line, 3T3. ST2 was induced in this cell line in the presence of serum, oncoproteins [100] and during proliferation [101]. Interestingly, whilst one group named the protein TI, they focused on its resemblance with human carcinoembryonic antigen [100]. Tominaga and colleagues named the protein

ST2, and described the similarity of the ST2 structure with that of the immunoglobulin (Ig) family [101]. More so, further assessment of the ST2 protein amino-acid sequence led to the conclusion that ST2 is analogous to the extracellular portion of the murine interleukin-1 (IL-1) receptor [101]. However, no intracellular portion was found in this protein. The discovery of ST2 was followed by the description of the human (IL1RL1) [102] and rat orthologs [103], both of which demonstrated the similarity of these proteins with the IL-1 receptors.

Proliferation in mouse fibroblasts was subsequently shown to produce two similar transcripts from the ST2/T1 gene, a short and more abundant form and a long, sparser form [104]. Furthermore, whilst the short form resembled the IL-1 receptor (IL-1R), the longer protein was shown to have both an intracellular and extracellular domain suggesting that it could possibly induce an intracellular signal. These have since been named soluble ST2 (sST2) and longer ST2 (ST2L), respectively. From here therein, the ST2L form will be described as ST2. The ligand for ST2 remained unknown until the discovery of IL-33 in 2005 by Schmitz *et al* [82]. In the intervening years, however, the understanding of the IL-33/ST2 axis progressed with studies focusing on ST2.

1.2.2 St2 gene

The *St2* gene is highly conserved amongst species, *from Drosophila* to humans. In mice, the *St2* gene is located on chromosome 1, in close proximity to the IL-1R-type 1 locus (IL1RI) [105]. In humans, the *St2* gene is located on chromosome 2 [106]. Similarly to the mouse gene, in humans, the *St2* gene is localised within the IL-1R gene cluster, suggesting their close relationship [106].

The product of the *St2* gene has been found to have several splice sites and it has been shown to produce up to four different isoforms, in different species. The first description of *St2* was shown to represent a 2.7kilobase (kb) messenger ribonucleic acid (mRNA) sequence [101], the product of which is now known as sST2. Subsequently, it was shown that fibroblasts made another, longer form of ST2, encoded by a 5kb sequence which interestingly could be enhanced in leukocytes more readily than in fibroblasts [104]. This is now known as ST2L. A further isoform, ST2V, was described in human cells as the third splice variant of

the *St2* human gene [107]. This variant has been shown to be located bound to the plasma membrane of cells in organs such as stomach and colon [108], yet the function of the translated protein remains elusive. Finally, during the cloning process in the search for the chicken sST2 and ST2L orthologs, a fourth variant was found. The mRNA encoded a protein similar to the ST2L form, but lacking the transmembrane portion of the receptor and which appeared to be secreted from the cell [109]. The functional relevance of this splice variant is unknown and has to date not been identified in other species.

1.2.3 ST2 protein and its expression

As aforementioned, ST2 is a member of the Toll-like/Interleukin-1 receptor superfamily. One of the key features of this family of receptors is their shared expression of a Toll-like/IL-1R (TIR) domain, a sequence of 200 amino acids with subtle differences between member subgroups. The TIR domain can be further divided into three main functional sub-domains, which are highly conserved within the group; a central 'core' region, present in all IL-1R and Toll-receptors and two additional regions, which are critical for signalling [110]. ST2 also expresses an extracellular tri-Ig sequence [101], another hallmark of the IL-1R superfamily. Whilst both ST2 and sST2 express a tri-Ig sequence, sST2 does not express a TIR domain or a transmembrane domain. The existence of two similar proteins encoding the same Ig-like sequence, of which only one appears capable of conferring an intracellular signal, mirrors that of other members of the IL-1 family [111]. This suggests the possibility that sST2 could exist as a decoy receptor for the ST2 ligand whilst ST2L provided the signalling receptor.

Whilst the search for the ST2 ligand continued, the expression of ST2 was identified in a large variety of cell-types. These are summarised in Table 1-1.

Table 1-1 ST2-expressing cells

Non-immune cells	Mouse	Human	Reference
Fibroblast	RNA and protein	-	[101, 112]
Epithelial cell	-	RNA and protein	[113, 114]
Osteoblasts	RNA	-	[115]
Endothelial cell	RNA and protein	RNA and protein	[113, 116, 117]
Immune cells	**Mouse**	**Human**	**Reference**
T helper 2 cell	RNA and protein	RNA and protein	[118-122]
Cytotoxic T cell	RNA and protein		[123, 124]
B cell	RNA	RNA and protein	[118, 125, 126]
NK cells	Protein	RNA	[126, 127]
Basophils	Protein	RNA and Protein	[128-130]
Monocyte	RNA	Protein	[118, 131]
Macrophage	RNA and protein	Protein	[125, 132, 133]
Mast cell	RNA and protein	Protein	[82, 134]
Eosinophil	Protein	RNA and protein	[44, 135, 136]
Dendritic cell	RNA and protein	-	[137]
Neutrophil	RNA and protein	-	[138]
Innate lymphoid cell	Protein	RNA and protein	[139-141]

1.2.4 ST2 function

Since its discovery, ST2 has been found to be expressed in numerous cell types. However, the initial finding that ST2 was preferentially expressed in the CD4 Th2 lymphocyte cell line D10, but not in Th1 cell lines, suggested a role for this receptor in type-2 immune responses [118]. Interestingly, Yanagisawa *et al*. demonstrated that the expression of ST2L was found in unstimulated D10 cells. However stimulation with phorbol ester and ionomycin induced secretion of sST2, suggesting differential regulation and roles for the two proteins. Thereafter, Xu *et al*. and Lohning *et al* demonstrated that ST2 was only found in type-2 cytokine-secreting T helper cells and not in interferon-γ-producing Th1 cells [119, 120], further corroborating the possible role of ST2 in type-2 immunity. This possibility was also supported by the fact that during an ova-induced allergic airway disease model, levels of ST2 expression were enhanced in the lung following antigen challenge [142].

Using blocking antibodies, the role of ST2 both *in vitro* [143] and *in vivo* [119, 144, 145] was further assessed. These studies supported the importance of ST2 in Th2-driven models, whereby antigen-specific Th2 cell-mediated induction of eosinophilic airway inflammation could be blocked by anti-ST2 antibody [119, 144]. Additionally, in mice undergoing a collagen-induced arthritis model, treatment with an sST2 monoclonal antibody appeared to attenuate disease by decreasing tumour necrosis factor (TNF)-α and IL-6 production [145].

The evidence for a role for ST2 in type-2 immune-mediated diseases was also corroborated in human studies. The selective expression of ST2 in activated Th2 cells rather than in Th1 cells or regulatory T cells was also confirmed in human samples [122]. More so, sST2 was found to be elevated in the sera of asthmatic patients suffering of acute exacerbations [146] and in the bronchoalveolar lavage (BAL) of patients with eosinophilic bronchitis [147]. Additionally, mirroring the results in murine models, sST2 was found to be elevated in the sera of patients suffering a variety of autoimmune conditions, including rheumatoid arthritis (RA), when compared to healthy controls [148].

The generation of an *St2* knockout ($^{-/-}$) mouse greatly enhanced the understanding of the role of ST2 *in vivo*. Two groups generated mutant mice lacking *St2* [149, 150]. Hoshino *et al*. demonstrated that Th2 cells were able to differentiate normally in *St2*$^{-/-}$ mice [149]. However, further studies using a pulmonary parasite-driven model of granuloma-formation demonstrated that the function of the antigen-stimulated Th2 cells in ST2-deficient mice was impaired. Moreover, these Th2 cells produced reduced amounts of type-2 cytokines (IL-4, IL-5 and IL-13) in response to *Schistosoma mansoni* following both primary and secondary challenge with the parasite [150].

The importance of ST2 in other inflammatory conditions was also ascertained using *St2*$^{-/-}$ mice. Interestingly, macrophages from *St2*$^{-/-}$ mice produced greater amounts of inflammatory cytokines *in vitro* in response to bacterial lipolysaccharide (LPS) and IL-1 [132]. Additionally, mice deficient in ST2 were unable to develop resistance to endotoxin shock demonstrating an important role for ST2-induction in LPS-tolerance. The exact mechanism whereby ST2 regulated LPS-tolerance was not determined. However, over-expression of ST2 in macrophages blocked IL-1- and LPS-induced nuclear factor (NF) κB activation, an important signalling kinase in IL-1 and TLR responses. This effect was found to be via ST2 sequestration of molecules upstream of NFκB, including myeloid differentiation primary response gene 88 (MyD88) [132]. These findings suggest ST2 may also play an important role in immune regulation during microbial infection.

1.2.5 IL-33, in the beginning

The elucidation of the roles of ST2 in the immune system progressed substantially since its discovery in 1989. However, the identification of its ligand, IL-33, was an important breakthrough in understanding the physiological role of this receptor. Prior to its discovery as a ligand for ST2, IL-33 was described as a nuclear factor in high endothelial venules (NF-HEV) [151]. High endothelial venules are of interest as they form important entry and exit sites for lymphocytes homing in an out of secondary lymphoid tissue, and are lined with specialised endothelial cells [152]. Baekkevold *et al*. determined that NF-HEV was localised to the nuclei of endothelial cells and that it expressed a putative deoxyribonucleic acid- (DNA) binding site [151]. Its function in the nucleus was, however, unclear. A decade after this discovery, computational database searches for the ligand for ST2 found a match with NF-HEV and Schmitz *et al*. renamed it IL-33. They went on to demonstrate that IL-33 was able to bind ST2L and to confer an intracellular signal, with NFκB activation [82].

1.2.6 IL-33 gene and product

IL33 was identified on human chromosome 9 with the murine counterpart being located on chromosome 19. The mRNA sequences of *IL33* and *Il33* encode 270 and 260 amino acid polypeptides respectively, producing full-length proteins of 30 kiloDalton (kDa) and 29kDa mass, for the human and mouse cytokines, respectively [82]. Human and mouse orthologs are 55% identical at amino acid level [82]. Whilst it was initially proposed that IL-33 was cleaved to an active form from its full-length protein by caspase 1, in keeping with other IL-1 family members [82], this has since been disputed [153-156]. Schmitz *et al* proposed that IL-33 was cleaved by caspase 1 before the IL-1-like domain producing an 18kDa mature IL-33 protein [82]. Cayrol *et al*, however, demonstrated that caspase 1 cleaved IL-33 within the IL-1-like domain [154], producing a 20-22kDa product. Disruption by caspase 1 at this site rendered the 'mature' protein inactive. More so, using immunoprecipitation methods, un-cleaved IL-33 was shown to bind ST2 and signal through NFκB [154]. In a further publication, it was demonstrated that the 'mature' cleaved form of IL-33 existed intracellularly, independently of caspase 1 activation, suggesting that processing of full length IL-33 was likely due to other proteases [156]. In keeping with this

finding, two groups demonstrated that full-length IL-33 was cleaved by caspase 3 [153, 154], an apoptotic caspase [157]. Since IL-33 lacks a signal sequence for secretion, it was speculated that active, full-length IL-33 was released from cells following damage, and was in turn inactivated by apoptotic caspases during apoptosis [153, 154]. With this hypothesis in mind, IL-33 was considered an 'alarmin' which could alert other cells of danger when released from necrotic cells in a 'necrocrine' manner [158]. More recent publications, however, have suggested that the maturation of IL-33 differs depending on the cell-type assessed. Since IL-33 plays an important role in both innate and adaptive immunity (see later), the possibility that other innate cell-derived proteases could cleave IL-33 into biologically functional forms was assessed. Looking specifically at neutrophil serine proteases, the investigators showed that both cathepsin G and neutrophil elastase were able to cleave IL-33 into smaller proteins of ~18-21kDa in size [155]. Interestingly, these 'mature' forms of IL-33 appeared to induce increased cytokine production from an ST2-expressing mouse mast cell line, MC-9 [155] suggesting that neutrophil proteases played an important role in IL-33-mediated inflammation.

The comparison of IL-33 with other well-described alarmins has been made, in particular, its similarity to high motility group box-1 (HMGB1), a nuclear factor that affects transcriptional regulation. Similarly to IL-33, HMGB1 localises to the nucleus. However, it is a potent alarmin when released from the cell [159]. Studies assessing the release of IL-33 from fibroblasts have demonstrated that IL-33 resides in the nucleus when first formed, but it subsequently translocates into cytoplasmic vesicles via nuclear pores where it remains until the cell is stimulated. Furthermore, mechanical strain allows IL-33 secretion from the living cell, a mechanism that is enhanced by microtubule disruption [160]. It therefore appears that, as well as resembling HMGB1, IL-33 also bears similarities with IL-1α, since both cytokines are responsive to mechanical stress [161].

1.2.7 IL-33 expression

The expression of IL-33 in a variety of organs and tissues has been determined. Interestingly, whilst the expression of ST2 appeared to be tightly regulated and restricted, IL-33 appears to be expressed more widely [82]. IL-33 is expressed in

numerous organs (Table 1-2). Additionally, individual cell-types have been assessed for their expression and production of IL-33 and these are also summarized below (Table 1-3). Whilst ST2 is expressed in numerous immune cells, IL-33 expression appears to be mainly in stromal cells.

Table 1-2 Organs expressing IL-33

Organ	Mouse	Human	Reference
Eye	RNA and protein	RNA and protein	[162],[163]
Liver	RNA	-	[164]
Colon	RNA and protein	RNA and protein	[165],[166-168]
Small bowel	RNA and protein	-	[114]
Stomach	RNA	Protein	[82, 169]
Joints	RNA and protein	Protein	[170-172]
Lungs	RNA and protein	RNA and protein	[82, 169, 173-176]
Heart	RNA and protein	-	[82, 116, 177]
Skin	RNA and protein	RNA and protein	[82, 125, 169, 178]
Central nervous system	RNA and protein	RNA and protein	[82, 179-181]
Spleen	RNA	-	[82, 164]
Kidney	RNA	Protein	[82, 169]
Lymph node	RNA	RNA and protein	[82, 151, 169]

Table 1-3 Cell-types expressing IL-33

Immune cells	Mouse	Human	Reference
Macrophages	RNA and protein	RNA	[82, 164, 168, 174, 182]
Dendritic cells	RNA and protein	RNA	[82, 164, 182, 183]
Mast cells	RNA and protein	-	[182, 184]
Non-immune cells	**Mouse**	**Human**	**Reference**
Epithelial cells	-	RNA and protein	[82, 114, 133, 169]
Smooth muscle cells	RNA	RNA and protein	[82, 169, 175]
Fibroblasts	RNA and protein	Protein	[164, 177]
Myofibroblasts	-	RNA and protein	[166, 167]
Endothelial cells	RNA and protein	RNA and protein	[116, 151, 169, 185]
Glial cells	RNA and protein	-	[179]
Osteoblasts	RNA and protein	RNA	[115, 186]
Adipocytes	-	RNA	[187]

1.2.8 IL-33 signalling

As a member of the IL-1 superfamily, IL-33 shares numerous features with other members of this family, including induction of a similar signalling pathway. The functional receptor for IL-33 is composed of a heterodimer of ST2 and the IL-1 receptor accessory protein (IL-1RAcP) [188, 189]. Interestingly, whilst sST2 binds IL-33, soluble IL-1RAcP enhances this neutralising function, suggesting that the heterodimer binds IL-33 more efficiently [189] than sST2 alone. As aforementioned, ST2 expresses a TIR domain. Similar to IL-1 and IL-18, recruitment of the TIR adaptor MyD88 to the membrane-associated receptor

complex is critical for IL-33-induced cytokine production [190]. Following this step IL-1R associated kinase (IRAK) 1 and 4 are recruited. This leads to the downstream phosphorylation of numerous signalling molecules including extracellular signal-regulated kinase 1/2 (ERK 1/2), p38 and inhibitor of NF-κB-α (IκB-α), which in turn allows NF-κB activation [82]. NF-κB activation results in target gene activation. Since a multiplicity of cells respond to IL-33 it is perhaps not surprising that cell-type variations exist in the signalling pathway of IL-33. One such example includes Th2 cells, whereby activation of signal transducer and activator of transcription 5 (STAT5) by IL-2 enhances IL-33-induced IL-13 production [191]. Similarly, mast cells have been shown to require mast/stem cell growth factor receptor Kit (c-kit) stimulation with stem cell factor (SCF) for optimum IL-33 signalling [192] and signal amplification can be achieved by activation of the phosphatase calcineurin via concurrent antigen stimulation [193]. Calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), allowing NFAT translocation to the nucleus and gene transcription [194]. Similarly, both endothelial cells [117] and fibroblasts [112] have been shown to depend on TNF receptor-associated factor 6 (TRAF6) phosphorylation for IL-33 signalling, a step which is not necessary for IL-33-induced basophil activation [190]

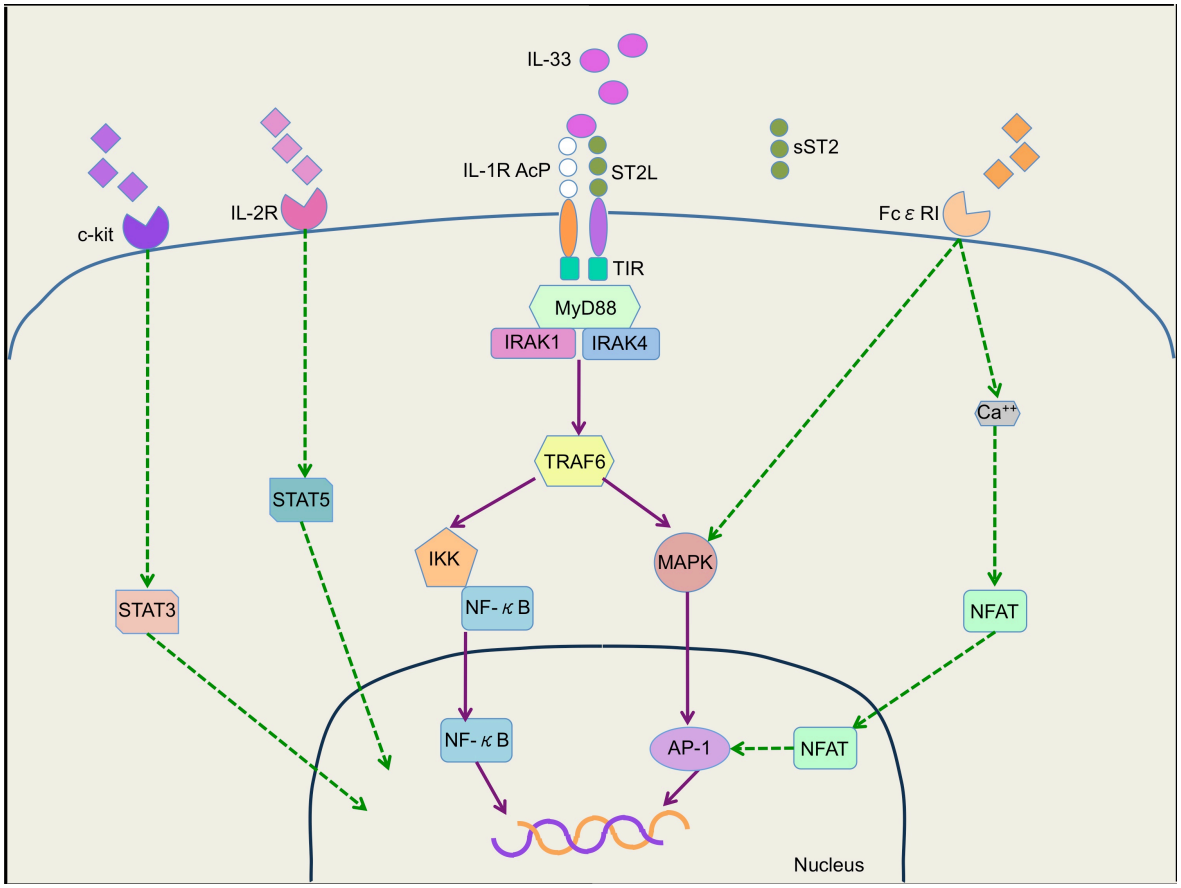

Figure 1-3 IL-33 signalling pathway

1.2.9 IL-33 function

1.2.9.1 IL-33 as a nuclear factor

IL-33 was found to be associated with heterochromatin *in vivo* and in live cells [185]. Heterochromatin has repressor functions within the nucleus, suggesting that IL-33-binding to it may be involved in these functions. Over-expression of IL-33 in the nuclei of cells in which rates of transcription were measured with a luciferase reporter construct demonstrated that the presence of IL-33, but not control protein, in the nucleus decreased transcription [185]. More evidence for the role of IL-33 as a nuclear factor arose when Roussel *et al*. demonstrated striking similarity between a short form of IL-33 and the latency-associated nuclear antigen (LANA) in Kaposi sarcoma herpesvirus (KSHV) [195]. They demonstrated that this form of IL-33 was able to bind and compact chromatin using the acidic-pocket formed between a histone dimer, in the same way the Kaposi herpesvirus does [195]. This suggested that the virus' ability to affect transcription might have evolved by molecular mimicry of IL-33. Furthermore,

co-immunoprecipitation of cytosolic and nuclear compartments of cells transfected with IL-33 demonstrated that IL-33 was able to interact with p50 and p65 NF-κB subunits. Moreover, IL-33 binding to the p65 subunit, prevented its association with DNA, hence affecting gene transcription and the function of NF-κB as a transcription factor [196]. This confirms the function of IL-33 as a repressor of transcription. Interestingly, Sanada *et al*. demonstrated that IL-33 inhibited the pro-hypertrophic effects of angiotensin in fibroblasts by inhibiting their activation of NF-κB [177] and it is possible that the mechanism described above is accountable for this effect.

1.2.9.2 IL-33 as a classical cytokine

As discussed above, upon release from cells, IL-33 is biologically active both as a full-length protein as well as in its cleaved form. IL-33 functions as a classical cytokine driving a variety of responses depending on the cell-type assessed. The effects of IL-33 on these cell types are detailed below.

1.2.9.2.1 IL-33 and innate cells

As described in Table 1-1, numerous cell-types express ST2 and hence IL-33 can affect both the innate and the adaptive immune systems. Within the innate immune system, the role of IL-33 in macrophage function has been assessed. *In vitro* treatment of macrophages with IL-33 enhances their response to LPS via an increase in their expression of Toll-like receptor (TLR) 4 [197]. As aforementioned, similarly to the dichotomous description of the Th1 and Th2 phenotypes of CD4 T cells, a comparable paradigm can be applied to macrophage polarisation. Classically-activated macrophages produce IL-1β and are able to eliminate phagocytosed pathogens through oxidative burst. This type of macrophage is induced by IFNγ. Type-2 cytokines such as IL-4 and IL-13 induce an alternatively-activated phenotype on macrophages, with reduced IL-1β production but enhanced major histocompatibility complex (MHC) II expression [198]. These alternatively-activated macrophages (AAM) have been implicated in tissue repair [198] but they also appear to contribute to airway inflammation in an allergic airways disease mouse model [133]. Importantly, in combination with IL-4 or IL-13, IL-33 enhances differentiation of macrophages to an AAM phenotype [133]. Interestingly, *in vitro* treatment of human monocytes with IL-33, in the presence of macrophage-colony stimulating factor (M-CSF), a

monocyte growth factor, induces their differentiation into functional osteoclasts [131]. This phenomenon links inflammation with the development of osteoporosis, since bone resorbtion by osteoclasts drives this disorder [199].

DC have also been shown to express ST2 and *in vitro* stimulation of DC with IL-33 induces cytokine and chemokine production, as well enhancing the expression of co-stimulatory molecules such as OX40L [200]. Interestingly, OX40-OX40L interactions have been implicated in allergic airways disease and asthma [201]. IL-33-stimulated DC co-cultured with naïve Th cells promote atypical Th2 cells which produce IL-5 and IL-13, but no IL-4 [137]. *In vivo*, IL-33-activation of DC has been shown to be important to the induction of allergic airways inflammation in a mouse ova-induced model [200] since $ST2^{-/-}$ mice have reduced airway inflammation as well as impaired DC activation and migration.

Mast cells express ST2 highly, making them exquisitely sensitive to IL-33 stimulation [82, 202]. *In vitro* stimulation of CD34-expressing cells (a marker of haematopoietic precursor cells) with IL-33 enhanced mast cell development and induced IL-13 production from mature mast cells [202]. Interestingly, the effect of IL-33 on mast cell cytokine production is independent of IgE binding of FcεRI [134, 203], yet IL-33 stimulation is insufficient to induce either human or mouse mast cell degranulation [134, 203]. Notably, the combination of IgE and IL-33 synergistically enhances both mast cell and basophil cytokine production [129]. Murine basophils treated with IL-33 *in vitro* secrete inflammatory cytokines, including IL-6, and histamine [128] and mice treated with IL-33 *in vivo* have increased numbers of basophils in their bone marrow [128].

One of the most notable effects of treating mice with IL-33 *in vivo* is a potent induction of systemic eosinophilia and splenomegaly [82]. Eosinophils have consequently become a hallmark of IL-33-induced inflammation. *In vitro* stimulation of mouse eosinophils with IL-33 induces IL-6 and IL-13 production as well as thymus and activation regulated chemokine (TARC) release [44]. Additionally, IL-33 treatment of bone marrow haematopoietic precursor cells with IL-33 induces eosinophil differentiation [44]. *In vivo*, IL-33-activated eosinophils drive macrophage polarisation to AAM phenotype, leading to airway inflammation [44]. Human eosinophils also respond to IL-33 *in vitro* [130, 204],

leading to cytokine production, an effect that is enhanced by combining IL-33 with either IL-1β or IL-18 [204].

1.2.9.2.2 IL-33 and adaptive immune cells

Since ST2 had initially been described as a stable marker for Th2 cells [120, 205], much research has focused on the effects of IL-33 on T cells. The culture of CD4 T cells with IL-33 *in vitro*, in the presence of antigen, induces an atypical subtype of Th2 cells, which express IL-5 and IL-13, but fail to express IL-4 [173]. Interestingly, this is the same phenotype of Th cell obtained by culturing naïve CD4 cells with IL-33-stimulated DC [137]. Human polarised Th2 cells also produce a variety of cytokines in response to IL-33 *in vitro*. Most notably, however, IL-4 is not detected in these cultures [126]. Interestingly, IL-33 also appears to act as a chemoattractant for ST2-expressing Th2 cells [206], similar to its effects on neutrophils [172].

The importance of the cytokine milieu in defining CD4 T cell profiles has increasingly been understood and importantly, Blom *et al*. demonstrate that a combination of transforming growth factor (TGF)-β with IL-33 induces IL-9 expression and secretion from Th2 cells [207]. This suggests that the effects of IL-33 on T cells can vary depending on the cytokine environment.

Whilst the effect of IL-33 on T cells has mainly focused on its effects on CD4 T cells, recently, CD8 T cells have also been shown to be IL-33-responsive. Somewhat unexpectedly, cytotoxic CD8 T cells (Tc) express ST2 in a T-box expressed in T cells (T-bet)-dependent manner[124]. T-bet is a transcription factor which has been shown to be vital for Th1 development and IFN-γ expression [208]. Additionally, IL-33 synergises with both IL-12 and T cell receptor (TCR) activation *in vitro* enhancing Tc1 cell IFN-γ production [124]. This is particularly interesting since IFN-γ is an important cytokine for anti-viral immunity [209] and IL-33 has been shown to be increased in alveolar macrophages and epithelial cells during lung viral illness [141, 174], suggesting a mechanistic link for IL-33 in virus immunity. Intriguingly however, exposing mice to the TLR3 ligand polyinosinic:polycytidylic acid (poly i:c), which resembles viral double-stranded RNA, prior to IL-33 exposure, impairs CD8 T cell up-regulation of ST2 and consequently impairs the synergy observed between IL-12 and IL-33 in CD8 IFN-γ production [210].

B cells can also express ST2. The B1 subset of cells has been shown to express ST2 and to produce IgM, IL-5 and IL-13 in response to IL-33 *in vitro* [211]. Additionally, IL-33 administration *in vivo* enhances B1 cell numbers in an IL-5-dependent manner and IL-33-activated B1 cells are sufficient to exacerbate cutaneous inflammation in a contact-dermatitis model [211]. Additionally, IL-33 has been shown to enhance B-cell immunoglobulin class-switch to IgE *in vivo* in an IL-4-dependent manner [212].

1.2.9.2.3 IL-33 and type-2 innate lymphoid cells (ILC)

In the last two years, the discovery of novel IL-33-responsive subsets of innate lymphoid cells has invigorated the field of IL-33. The first description of these cells arose from the assessment of a population of non-B, non-T cells found in intra-abdominal, peri-adipose lymphoid tissue, which the authors called fat-associated lymphoid clusters (FALC). These cells were found to express ST2 and they were able to produce large amounts of type-2 cytokines (IL-5 and IL-13) in response to IL-33 both *in vitro* and *in vivo* [213]. They relied on IL-7 for their survival *in vitro* and interestingly, they were found to support B1 cell proliferation *in vivo*. They were therefore named natural helper cells (NHC) [213]. Their importance in the immune system was determined by performing an adoptive transfer into mice lacking the common-γ chain (γc) receptor and Rag2 genes ($\gamma c^{-/-}$*x*$Rag2^{-/-}$), which lacked FALC. Using a model of *Nippostrongylus* infection, NHC were found to be important for parasite clearance [213].

Shortly after this discovery, two different groups nearly simultaneously, yet using different reporter mice, identified IL-33-responsive innate lymphoid cells that were devoid of common lineage markers [139, 214] in the gut. Whilst the MacKenzie lab used IL-13 reporter mice [139], the Locksley laboratory identified this novel population using an IL-4-reporter mouse [214]. The use of these mice allowed the identification of IL-13- or IL-4-expressing cells, respectively, by their expression of green fluorescence protein (GFP) inserted at the promoter regions of these genes. Since they were first described in IL-13-reporter mice, the McKenzie group named these cells nuocytes (Nu=13^{th} letter of the Greek alphabet) [139]. They demonstrated that nuocytes expanded *in vivo* in response to IL-33 and IL-25. Additionally, they showed that in a model of helminth infection (*N. brasiliensis*) nuocytes were the main source of IL-13. More so nuocytes were sufficient to drive worm expulsion in this model, when they were

transferred into *Il-17R*$^{-/-}$ *x St2*$^{-/-}$ mice, in an IL-13-dependent manner [139]. Similarly to NHC, these cells were also shown to proliferate in response to IL-7 *in vitro* and their response to IL-33 stimulation was similar to that of NHC, with increased production of IL-5 and IL-13 [139]. These findings were similar to those described by Price *et al.*, since they demonstrated that these lineage negative lymphoid cells were key for worm expulsion and were the main source of type-2 cytokines in this model. They named the cells innate type-2 helper cells (Ih2), which were shown to be systemically dispersed in mice treated with IL-33 i.p. [214]. Ih2, however, did not expand in response to γc-binding cytokines such as IL-2, IL-4, IL-9 IL-13, IL-15 or IL-21 [214]. Interestingly, transcriptome analysis of Ih2 compared to Th2 cells and basophils, clearly demonstrated that these cells were a distinct, novel cell type [214]. Whilst neither nuocytes and Ih2 expressed common lineage markers, they did share the expression of a small number of surface markers, suggesting that these two cell types were very similar and possibly the same cell type. Interestingly however, Ih2 cells did not express the stem cell marker, stem cell antigen (Sca) -1 [214], which nuocytes did [139].

In these initial seminal studies, the role of ILC was mainly studied in the gut. Since Price *et al*. had demonstrated that these cells were systemically dispersed, the focus shifted onto the role of ILC in the lung. In a highly informative paper, Chang *et al*. demonstrated that IL-33 was produced by alveolar macrophages in response to lung influenza infection, which in turn stimulated the expansion of a population of lineage negative ILC [174]. Additionally, these cells were an important source of type-2 cytokines in this model and were indeed sufficient to drive influenza-induced airway hyperresponsiveness, using an adoptive transfer model into *Il-13*$^{-/-}$ mice [174]. This paper addressed the important issue of virus-induced asthma exacerbation [215] and suggested that both IL-33 and ILC were key to the mechanism for this phenomenon.

Further evidence supporting the pathogenic role of ILC in the lung was demonstrated in mouse models of glycolipid-[216], ova-[217] and papain-induced [218] airway inflammation models. Interestingly, ILC were shown to synergise with IL-33 in combination with other stromal-cell-derived cytokines, such as TSLP, to produce type-2 cytokines [218], indicating the importance of the

cytokine milieu in the activation of ILC cytokine production. In addition, Wilhelm *et al*. demonstrated, using IL-9 fate-reporter mice, that ILC were the main source for IL-9 *in vivo* in a papain-induced model of inflammation [219]. They also demonstrated that IL-2 was essential to drive ILC IL-9 production in this model, firmly linking ILC responses with the adaptive immune system in the lung [219].

The importance of type-2 immune responses in tissue repair has been extensively described (reviewed in [220]). Importantly, a role for ILC in tissue homeostasis and repair has been described. In fact, the Artis lab demonstrated that, using a similar lung influenza model of inflammation as Chang *et al* [141], ILC were necessary to drive adequate epithelial repair following a viral insult. ILC-mediated lung repair was in part via the production of amphiregulin, a member of the epithelial growth factor family [221]. By treating *Rag2*$^{-/-}$ mice, lacking B and T cells, with a CD90.2 antibody, this group attempted to eliminate all ILC in these mice, since ILC express this marker [139, 213]. Subsequently, some of these mice had their ILC population reconstituted by adoptive transfer of CD90.1^{+} ILC. The mice were then used in the aforementioned influenza-driven lung inflammation model and pulmonary repair was measured. Lung function and repair was severely impaired in the mice devoid of all ILC and these functions were rescued with the ILC transfer [219], consistent with a requirement for ILC in post-viral lung repair.

Much effort has focused on identifying phenotypes of these innate cells and whilst they all lack expression of common lineage markers, express ST2 and respond similarly to IL-33-stimulation, the question of whether they are indeed the same cell-type at different stages of differentiation or different cell-types, remains. NHC have been shown to be of lymphocytic origin, since they are dependent on fms-like tyrosine kinase-3 (Flt3) expression for their development [222]. Flt3 is a cytokine receptor, that binds Flt3 ligand, and is highly expressed in bone marrow lymphoid progenitors as well as rare myeloid progenitors with lymphoid potential [223]. Yang *et al*. demonstrated, not only that Flt3 expression was essential for NHC development, but that these cells could arise from lymphoid-primed multipotent lymphoid progenitors (LMMP) or common-lymphoid progenitor (CLP) cells [222]. The finding that nuocytes also arise from CLP cells in the bone marrow [224] further supports the fact that all these novel

cells are in fact one cell-type. Interestingly, Wong *et al*. demonstrated that nuocyte development required cell-intrinsic expression of the transcription factor retinoic-orphan receptor (ROR)-α [224]. This differentiated the development of nuocytes from IL-22-producing gut ILC which are dependent on ROR-γ expression [225]. An interesting finding from a large genome-wide association study (GWAS) looking at asthma-associated genes has highlighted not only the importance of *IL33* in asthma, but to a lesser degree of association, *RORA*, suggesting that ILC may indeed be key players in asthma development. Perhaps unsurprisingly, given their type-2 immune phenotype, nuocytes [139], NHC [213] and Ih2 [214] all express the Th2 key transcription factor GATA-binding transcription factor(GATA)-3 [226], however, the role of GATA-3 in ILC development and function is yet unknown. A further transcription factor, inhibitor of differentiation 2 (Id2), which has roles in peripheral lymphoid tissue and natural killer cell development [227], has been shown to be important in ILC development, since ILC are absent in *Id2*$^{-/-}$ mice [141].

The above studies were all performed in mice. However, cells resembling murine type-2 ILC have been described in humans [140, 141]. These cells have been shown to respond to IL-33 to produce IL-5 and IL-13 [140, 141] and have been detected in resting lung tissue [141] and foetal gut, as well as nasal polyps [140]. The presence of ILC in the nasal polyps of allergic patients further supports their role in allergy-driven inflammation. The description of IL-13^{+} cells, in lung biopsies of severe asthmatics, which are negative for T cell, mast cell and eosinophil markers suggests the possibility that these cells could be type-2 ILC [72]. Their increased number in severe asthmatic lungs compared to mild asthma or healthy control lungs enticingly suggest that these cells may be mediators or markers of severe, rather than mild, asthma [72] but further characterisation of these cells is required.

1.2.10 IL-33 in disease

Important roles for IL-33 in disease are increasingly being described, demonstrating its function can be deleterious or protective, depending on the disease process involved.

1.2.10.1 Allergy and asthma

The discovery that *IL33* is an important gene associated with asthma in two separate GWAS studies has conclusively linked this cytokine with this condition [228, 229]. *IL33* polymorphisms have also been associated with allergic disease [230]. In addition to this evidence, however, numerous studies have assessed the importance of the ST2-IL-33 axis in allergy and allergic airways disease.

IL-33 levels are increased in the lungs of asthmatic patients, compared to healthy controls [175, 176] and these levels appear to positively correlate with disease severity [176]. More so, increased levels of IL-33 have been found in the sera of patients with allergic pollinosis [230]. Furthermore, the mRNA levels of *IL33* in nasal biopsies of allergic individuals, unlike those of *IL5*, were shown to remain elevated during antigen exposure, despite prophylactic antihistamine treatment [231].

As discussed previously, direct inoculation of IL-33 into mouse airways induced many changes associated with atopic asthma, namely eosinophilia, AHR and mucus hypersecretion [232]. This effect did not require adaptive immune responses and this led to subsequent studies assessing the roles of various innate cells' contributions to allergic airway inflammation in the context of IL-33-mediated polarisation or stimulation [44, 133, 200, 233]. More recently, in a model of allergic rhinitis, *Il13*$^{-/-}$ mice sensitised to pollen failed to mount both an early (sneezing) or late (basophilic and eosinophilic inflammation) response to the antigen challenge [234]. Whilst IL-33 induced profound eosinophilic inflammation in the absence of B or T cells, it also enhanced the adaptive immune response. Thus, IL-33 has been shown to induce a Th2 phenotype on antigen-specific T cells, which can exacerbate allergen-driven lung disease in mice [173].

It is perhaps unsurprising that IL-33 can influence the pathobiology of both allergy and asthma as most of the cells implicated in these diseases express the IL-33 receptor, ST2 (Table 1-1). Recent studies into IL-33 biology have demonstrated its role in the induction of a novel cell type, ILC, in the lung and their function in allergy has been discussed previously. Interestingly, prior to the discovery of ILC, circulating haemopoeitic progenitor CD34^{+} cells were shown

to respond vigorously to IL-33 by producing large amounts of type-2 cytokines *in vitro* [235]. Additionally, these cells could be found in the sputa of allergic individuals and their number was enhanced within 48 hours of antigen challenge [235], suggesting their involvement in antigen-driven inflammation. Similarly, IL-33-responsive cells resembling ILC have been described in the nasal polyps of patients with allergic rhinitis in increased numbers compared to healthy controls [140].

Infections are common triggers for asthma exacerbations and a role for macrophage-derived IL-33 in viral induced AHR has been shown in mouse studies [174]. More so, IL-33 has been shown to modulate cytotoxic T cell (CD8)-mediated anti-viral responses by either enhancing them [123, 124] or hindering them [210], depending on the concurrent immune stimuli. Additionally, fungal and bacterial infections can also precipitate deteriorations in asthma and IL-33 has been implicated in the response to these pathogens too (Table 1-4). Sensitisation to the fungal pathogen *Alternaria alternata* has been associated with increased asthma severity and worsening of asthma symptoms have been reported when its environmental spore-counts are high [236]. It is therefore interesting that this same pathogen induces ILC via IL-33-production in the lungs, which drive pulmonary inflammation in a mouse fungus-induced allergy model [74].

The studies so far suggest a mainly deleterious role for IL-33 in the lung. However, IL-33-induced ILCs have also been implicated in tissue repair and homeostasis [141], as discussed earlier.

1.2.10.2 Other diseases

The role of the IL-33/ST2 axis has been investigated in numerous other diseases, a summary of some of these can be found in Table 1-4.

Table 1-4 Disease-specific roles of IL-33/ST2

Disease	Role of IL-33	Reference
Cardiovascular disease	• IL-33 is highly expressed in endothelial cells • sST2 is a biomarker of heart failure (HF) • sST2 levels correlate with risk of developing HF • IL-33 is cardioprotective on human cardiac fibroblasts and antihypertrophic in mouse myocardial infarct (MI) models • IL-33 reduces foam cell and artherosclerotic plaque formation	[116, 151, 177, 237-240]
Parasite infection	• Exogenous IL-33 aids parasite clearance • IL-33 induces ILC which promote parasite clearance • Parasite epithelial damage induces IL-33 production via trefoil factor 2 • *St2*$^{-/-}$ mice have increased inflammation in *Toxoplasma gondii* encephalitis	[139, 165, 181, 213, 214, 241, 242]
Bacterial infection	• IL-33 enhances macrophage response to LPS • IL-33 improves neutrophil migration to sites of sepsis • Improves inflammation and bacterial clearance in infective keratitis model	[138, 163, 197]
Colitis	• Increased IL-33 expression in colonocytes in ulcerative colitis (UC) • IL-33 levels reduced by treating UC with anti-tumour necrosis factor (TNF) -α antibody • Colitis model is more severe in *Il-33*$^{-/-}$ mice • Enhanced IL-33 levels in TGF-β-deficient mice with colitis leading to worsened pathology	[114, 168, 243, 244]
Arthritis	• Exogenous IL-33 exacerbates inflammation in murine arthritis models • IL-33-enhanced arthritis inflammation is anti-TNF therapy-responsive • Elevated IL-33 levels measured in sera and synovial fluid of rheumatoid arthritis patients • IL-33 is also increased in ankylosing spondylitis • IL-33 levels correlate with disease activity in humans	[170-172, 245, 246]
Nervous system disorders	• Increased IL-33 expression in activated glial cells • Increased IL-33 levels in experimental encephalitis • IL-33 gene polymorphisms are associated with Alzheimer's disease • Exogenous IL-33 attenuates a mouse encephalitis model by promoting AAM differentiation	[179-181, 247, 248]

1.3 Rapamycin and the mammalian target of rapamycin (mTOR)

1.3.1 In the beginning

Rapamycin is a macrolide antibiotic that was first discovered in Easter Island (Rapa Nui) in 1975. It was extracted from *Streptomyces hygroscopicus* and was initially described as an antifungal [249]. Rapamycin was subsequently found to have immunosuppressive effects that were initially attributed to its inhibitory

effect on cell cycle progression [250]. The search for a mechanism for this effect led to the discovery of the target of rapamycin (TOR) in *Saccharomyces cervesiae* [250]. Mutations in *TOR1* and *TOR2* genes led to blocking of the effects of rapamycin on growth cycle arrest in these cells, demonstrating the importance of these genes for rapamycin function. This discovery was followed subsequently by the detection of the mammalian ortholog of TOR, named mechanistic (or mammalian) target of rapamycin (mTOR) [251], an atypical serine-threonine kinase of the phosphoinositide 3-kinase (PI3K) family [252]. Whilst in the fungus two genes encoded the targets of rapamycin [250], only one gene was found for the mammalian equivalent [251]. The single *MTOR* gene encodes a protein that signals through two distinct complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 contains the rapamycin-sensitive scaffolding protein regulatory-associated protein of mTOR (raptor) [253], mammalian lethal with Sec13 protein 8 (mLST8), proline-rich AKT substrate 40kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein (Deptor) [254]. mTORC2 instead contains rapamycin-insensitive companion of mTOR (Rictor) [255], along with mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1) [254]. Additionally, mTORC2 shares two proteins with mTORC1, mLST8 and Deptor [255, 256] (Figure 1-4).

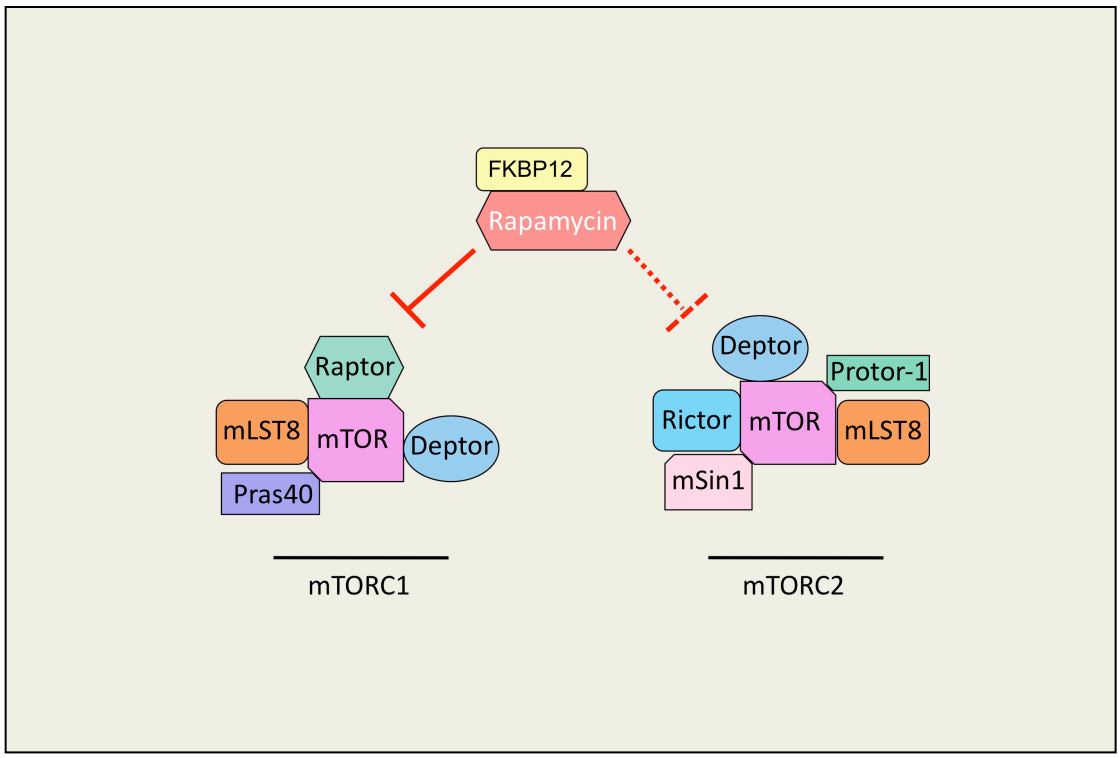

Figure 1-4 mTOR complexes

mTORC1 has numerous substrates, however, the best understood are ribosomal protein S6 kinase (S6K) and eukaryote translation initiation factor 4E-binding protein 1 (4E-BP1) [257]. S6K phosphorylation by mTORC1 induces phosphorylation of the eukaryotic initiator factor 4B (eIF4B), which in turn allows initiation of translation via the activation of eukaryotic initiator factor A (eIF4A) [258]. 4E-BP1 is a repressor of translation, which mTORC1 phosphorylates, inhibiting its function and allowing translation to occur [259]. By these two mechanisms, mTORC1 can alter protein synthesis and signalling pathways.

Rapamycin binds the cytoplasmic protein FK506-binding protein of 12kDa (FKBP12) [260] forming a complex which specifically binds to mTORC1, weakening its structural integrity and abolishing its ability to phosphorylate its substrates [261]. Interestingly, recent cryo-electron-micoscopy of the mTORC1 crystal structure has demonstrated that rapamycin interference with this complex's stability affects its ability to phosphorylate 4E-BP1 and S6K differently. In fact, Yip *et al* propose that the FKBP12-rapamycin complex prevents large molecules such as S6K from accessing the mTOR active site,

whilst complete dissociation of raptor from mTOR inhibits 4E-BP1 phosphorylation [262]. Whilst initially mTORC2 was found to be resistant to inhibition by rapamycin [255], it was later demonstrated that prolonged treatment with rapamycin inhibited mTORC2 assembly and therefore could also inhibit mTORC2-driven functions in certain cell types [263]. Despite this finding, the sensitivity of rapamycin for mTORC1 inhibition in short-term assays has been since been thoroughly tested and confirmed [264].

1.3.2 mTOR and the immune system

Our understanding of the function of mTOR has, to a great extent, come from experiments studying the biological effects of rapamycin. They will therefore be discussed in combination. As aforementioned, one of the first roles described for mTOR was its function in cell cycle regulation [251]. It was initially believed that rapamycin-induced inhibition of B cell and T cell proliferation was sufficient to explain the immunosuppressive effects seen with this drug [265]. Since then however, mTOR has been shown to regulate numerous cellular pathways, demonstrating the complexity of the role of mTOR in immune regulation. The use of both rapamycin and gene-targeting techniques has greatly enhanced the understanding in this field.

1.3.2.1 mTOR in the adaptive immune system

Much of the understanding of the importance of mTORC1 activation in the immune system originates from studies on T cells. mTORC1 is activated in numerous pathways in the adaptive immune system, including TCR activation, an effect which is further enhanced by T cell activation of other co-stimulatory molecules such as CD28 or OX40 [266]. Additionally, mTORC1 activation has been shown to be essential in the differentiation of CD4 helper T cells into their effector subsets. Whilst complete deletion of mTOR leads to defects in the differentiation of all Th subsets, mTORC1 appears to be essential for Th1- and Th17- cell differentiation, but not Th2 polarisation [267]. Selective deletion of Rictor in mouse T cells and the consequent abrogation of mTORC2 functions prevents Th2 polarisation, demonstrating the non-redundant functions of the two distinct mTOR complexes [267]. In addition, mTORC1 is activated by a variety of cytokines that influence T cell activation and differentiation. mTOR is

activated during IL-2 stimulation of Th cells, allowing T cell proliferation [268], whilst IL-7-induced mTOR activation prevents T cell atrophy and maintains naïve T cell size [269]. Interestingly, whilst mTORC1 activity appears not to be involved in Th2 differentiation, its activation is essential for IL-4-induced proliferation of Th2 cells, since Th2 pre-treatment with rapamycin blocked the proliferative effect of IL-4 [270]. The Th1 cytokine, IFNγ, also signals through the mTOR-S6K pathway to induce gene transcription [271].

Interestingly, whilst rapamycin inhibits naïve T cell proliferation, it selectively allows the proliferation of regulatory T cells (Treg) [272]. Whilst Sauer *et al*. demonstrated that this was a consequence of rapamycin inhibition of TCR signalling through mTOR, which in turn enhanced the expression of the Treg key transcription factor, *Forkhead box P3* (*Foxp3)* [273], Haxhinasto and colleagues showed that the induction of FoxP3 in Th cells by TGFβ was under the control of Akt and mTOR [274].

The effects of mTOR on cytotoxic CD8 T cell activation have also been assessed. In particular, mTORC1 activation has been shown to be important in orchestrating anti-viral responses induced by CD8 T cells. Interestingly, administration of low doses of rapamycin enhanced CD8 T differentiation to a memory phenotype in a viral-induced *in vivo* model, demonstrating the importance of mTOR in driving effector CD8 T cell fate decisions [275]. By contrast, high doses of rapamycin diminished anti-viral responses [269]. The effect of IL-12 on CD8 T cells on the upregulation of the transcription factor T-bet is also mTOR-dependent. Rao *et al*. reported that rapamycin-treatment of CD8 T cells stimulated with IL-12 inhibits T-bet expression in favour of eomesodermin [276], an important transcription factor involved in memory CD8 T cell differentiation [277].

Less is known about the role of mTOR in B cell activation, however studies looking at the role of Sin1, an essential component of mTORC2, demonstrate that its deletion in haematopoietic cells prevents the normal development of B cells. The lack of mTORC2 activity disrupts the ability of immature B cells to downregulate *Rag* expression, preventing them from maturing into pre-B cells [278]. On the other hand, deletion of *Tuberose sclerosis complex (Tsc) 1*, an mTORC1 inhibitor, leads to enhanced mTORC1 activation which partially blocks B

cell maturation and prevents marginal zone B cell development, suggesting that controlled mTORC1 activation is essential for this process [279]. These results must be interpreted with caution since deletion of *Tsc1* and *Tsc2* can also enhance mTORC2 activation [280].

1.3.2.2 mTOR and the innate immune system

The role of mTOR in DC function has been extensively investigated via the use of rapamycin. The maturation of DC appears to be regulated by rapamycin, since blocking mTOR activation both *in vitro* and *in vivo* reduced DC proliferation and maturation [281]. Additionally, rapamycin has been shown to regulate the functions of different subsets of mature DC too. Haidinger and colleagues showed that rapamycin treatment of human monocyte-derived and myeloid DC inhibited their ability to produce pro-inflammatory cytokines in response TLR stimuli [282]. Interestingly, whilst DC expression of co-stimulatory molecules was reduced by blocking mTOR, the ability of DC to present antigen was not diminished, demonstrating the specificity of the roles of mTOR in these cells [282]. In fact, some reports demonstrate that inhibition of mTOR with rapamycin may improve DC antigen-presentation functions by enhancing autophagy [283], a process that is negatively regulated by mTOR [284] (see below).

The role of mTORC2 in these processes is less well understood, however, loss of mTORC2 function by the deletion of *Rictor* leads to increased inflammatory responses to LPS in DC [285]. Interestingly, macrophage treatment with rapamycin in the presence of LPS activation also enhances pro-inflammatory cytokine production via deregulated IL-10 production [286], suggesting that both mTOR complexes are important in regulating innate responses to bacterial products. The effects of mTOR inhibition with rapamycin in the context of LPS-induced acute lung injury, has however, given conflicting results. Whilst Feilhaber *et al*. demonstrated that pre-treating mice with rapamycin prior to LPS challenge led to reduced inflammation in a STAT1-dependent manner [287], Lorne and colleagues found that rapamycin enhanced the effects of LPS in the lung, reflecting the *in vitro* data on macrophage and DC responses in the presence of mTOR inhibition [288]. The differences between these two studies might be explained by the different protocols for the pre-treatment of mice with

rapamcyin, suggesting that the timing of mTOR inhibition is crucial to its effects in this model.

1.3.3 Other important mTOR functions

Since its discovery, descriptions of the cellular processes in which mTOR plays a role have been increasing at a rapid pace. Whilst these functions are not directly linked to the immune response, they too have an effect in the immune system. As discussed earlier, mTOR inhibition leads to cell cycle arrest. Cell growth is a metabolically demanding cellular function, as is T cell activation. Increasingly it is believed that one of mTOR's roles is as a regulator of these functions by acting as a sensor for the availability of nutrients and cellular energy stores. In this regard, mTOR activation is very sensitive to amino acid depletion [289]. Additionally, mTORC1 activation is also hindered by low energy reserves, since diminished adenosine triphosphate (ATP) cellular stores opposes TSC2 phosphorylation, which in turn enhances TSC-dependent inhibition of mTORC1 activity [290]. Similarly, mTORC1 is downstream of numerous growth factors pathways, such as insulin and insulin-like growth factor [291], and has a role in cellular lipid metabolism. As such, deletion of the mTORC1 protein, Raptor, leads to decreased white adipose tissue and leaner mice *in vivo* [292] and failure of adipogenesis *in vitro* [292].

mTORC2 also has roles in lipid metabolism since deletion of the mTORC2 protein, Rictor, in adipose cells impairs their response to insulin *in vitro* whilst *in vivo* this deletion leads to deregulated lipolysis and insulin resistance [293]. In similar mice, lacking Rictor in adipose cells, organomegaly was noted, suggesting that mTORC2 is also important in cell size regulation [294]. Additionally, mTORC2 has been implicated in regulating the formation of the cellular cytoskeleton, by modulating actin functions [256].

Autophagy is a process that is closely linked to growth and metabolic activity. Autophagy is a catabolic 'recycling' process that allows cells to release intracellular stores of nutrients by degradation in lysosomes [254]. Additionally, autophagy is an important defensive immune mechanism against intracellular infections [295]. mTORC1 is a negative regulator of autophagy, preventing its initiation when nutrients are plentiful [296]. Autophagy related 1 (Atg1), a

serine/threonine kinase important in autophagosome formation, is downstream of mTORC1 [297] and its phosphorylation by mTORC1 reduces its function [297]. The importance of autophagy in disease is increasingly being understood with roles being described not only in infection [298] and metabolic disease [299], but also cancer [300] and pulmonary disorders [301], suggesting potential roles for mTOR in disease.

1.3.4 mTOR in the clinic

Since mTOR is involved in such a variety of important cellular physiological functions, it is unsurprising that it has been implicated in a variety of diseases. This section will focus on the roles of mTOR in the lung and clinical uses of rapamycin with brief discussion of the role of mTOR in other conditions.

1.3.4.1 mTOR in the lung

With increasing understanding of the importance of Th2 cells in allergic asthma, there has been much interest in the use of immunosuppressants in the treatment of this condition [19]. The most common use of rapamycin in clinical practice is as an immunosuppressant to prevent solid organ rejection (see below) and increasingly, rapamycin and derivatives are being approved for use in cancer therapy, including lung cancer [302]. Rapamycin remains one of the most efficacious and tolerated immunosuppressants and its potential application in the treatment of allergic airways disease is being increasingly investigated. Most recently, using an acute and chronic mouse model of allergic airway inflammation induced by house dust mite (HDM), systemic administration of rapamycin reduced lung inflammation and AHR [303]. Additionally, using a transgenic mouse model of airway remodelling and AHR, systemic rapamycin treatment was also beneficial [304]. Intriguingly, however, Fredricksson *et al*. demonstrated that, whilst rapamycin administration during the induction of HDM-induced airway inflammation was beneficial, it was detrimental when given as a treatment during established disease [305]. The conflicting results in these models would suggest that, whilst mTOR activation is important for the onset of airway inflammation, it exerts anti-inflammatory effects once the inflammation is established. More research is required to elucidate the exact mechanisms driving these differences.

An important cause of respiratory disease is cigarette smoking and as discussed in section 1.1.2, it has significant detrimental effects on the effectiveness of asthma therapy. mTOR activation has been shown to be essential for the anti-inflammatory effects of glucocorticoids on myeloid cells [306] and interestingly, the mTOR pathway has recently been shown to be vital in cigarette smoke-induced lung damage [307]. In fact, cigarette smoke induced HIF-1 activated RTP801 (RTP801), which in turn stabilises the mTOR repressor TSC, inhibiting mTOR function and this pathway was responsible for smoke-induced emphysema in a mouse model [307]. These two publications link two important aspects of clinical asthma with mTOR.

One of the drawbacks of clinical use of rapamycin has been its association with the risk of developing of acute interstitial pneumonitis [308], an inflammatory process of the distal airways. This condition has been reported in up to a fifth of treated patients in some studies [309], with severity ranging from asymptomatic disease to severe respiratory failure [309]. The condition typically resolves following drug withdrawal, suggesting a causal relationship between mTOR inhibition and pulmonary inflammation [308]. This reiterates the need for better understanding of the roles of mTOR in the lung in order to allow its therapeutic use in lung diseases with reduced side effects.

1.3.4.2 The role of mTOR in extra-pulmonary conditions

The increasing understanding of the myriad of roles of mTOR has allowed this pathway to be investigated and targeted in ever-increasing numbers of diseases. The main conditions in which mTOR has been implicated are summarised in Table 1-5.

Table 1-5 The roles of mTOR in non-pulmonary disease

Disease/ system	Role of mTOR	References
Cancer/ tumourgenesis	• Mutations of TSC (which regulates mTOR function) cause familial cancer disorders • mTORC1 is downstream of the commonly mutated tumour suppressor gene (TSG) p53 • increased mTORC2 activity human gliomas and in mouse glioma models • mTORC2 involved in murine prostate cancer model	[310-315]
Diabetes/ Metabolic disease	• Insulin signals via mTOR • Chronic rapamycin treatment induces insulin resistance and hyperlipidaemia • mTOR regulates peroxisome proliferator-activated receptor-gamma activity in adipogenesis, a target of anti-diabetic drugs • Mutation of kinase suppressor of Ras 2 (KSR2), a gene implicated in obesity and diabetes, induces mouse obesity via hyperphagia in an mTOR-dependent manner	[316-319]
Neurodegeneration	• Rapamycin rescues neuronal plasticity and attenuates cognitive impairment in a mouse model of tuberose sclerosis • In a mouse Alzheimer's disease model, aberrant neuronal cell cycling is mediated by the PI3K-Akt-mTOR pathway and is improved by rapamycin treatment • Rapamycin improves outcomes in Parkinson's and Huntington disease models	[320-322]

1.3.4.3 Clinical uses of rapamycin

Despite being first described as a fungicide [249], the potential of this agent as an immunosuppressant became apparent with its effects on B cell cycle regulation [250]. The Foods and Drugs Agency (FDA) approved rapamycin for use in transplant immunosuppression in 1999 and since then, its applications as a steroid sparing, cyclosporine-replacement treatment has extended from uses in solid organ transplantation to treatment of graft-versus-host disease [323]. Since it is potentially toxic, therapeutic drug monitoring is required and its use has been associated with a variety of side effects including pneumonitis [308] and mucositis [324]. Despite this, however, it appears to be better tolerated than other agents and it is associated with improved transplant survival in long-term studies [325]. One of the key advantages of using rapamycin and its derivatives is that, unlike other immunosuppressants, it does not increase the risk of cancer-development [326]. More so, the use of rapamycin and its analogs as anti-cancer agents is expanding since its approval in metastatic renal cell carcinoma [327], leading to large clinical studies looking at efficacy of this agent in other malignancies [328].

One of the most recent uses of rapamycin is its use in cardiovascular disease. Whilst all immunosuppressants, including rapamycin, are associated with the risk of developing hypercholesterolaemia [325], the use of mTOR inhibitor-coated coronary artery stents have improved survival and reduced arterial re-stenosis in longterm studies [329].

With increasing knowledge of the mTOR pathway and the ability to develop more target-specific drugs, it is likely that the clinical applications of mTOR regulation will continue to develop at this swift pace. However, greater understanding of the mechanisms of the side effects observed with this treatment is imperative if mTOR manipulation is to be used more widely.

1.4 Objectives

In the preceding introduction the current understanding of asthma immunopathogenesis and IL-33 biology have been described, as well as the functions of mTOR in the immune system. Important ongoing questions remain, as well as a large unmet clinical need in the significant population of asthma patients requiring better treatments for this disease. The use of immunosuppressants in asthma is mainly restricted to CS, and better understanding of the mechanisms disrupted by clinically available drugs is an important avenue yet to be fully investigated. Hence, assessing the role of mTOR in IL-33-induced functions is an important question with potentially clinically relevant answers.

The recent description of ILC as IL-33-responding cells that appear to be important in the lung immune response has raised further questions regarding their roles in IL-33-induced airway inflammation and their functional responses to this cytokine *in vitro*. Additionally, their interactions with the adaptive immune response are unknown.

As such, this thesis aims to address three main hypotheses:

1. mTOR activation is important for IL-33-induced airway inflammation

2. IL-33-driven ILC functions are mTOR-dependent *in vitro* and ILC are sufficient to drive IL-33-induced airway inflammation *in vivo* in an mTOR-dependent manner

3. ILC and Th cells interact *in vitro* and *in vivo* to drive type-2 immune responses

By performing experiments to address these hypotheses this thesis aims to demonstrate that IL-33 plays an important role in the airway and that mTOR is an important regulator of its function. More so, these experiments also attempt to demonstrate the importance of IL-33-induced ILC in lung immune functions.

2 Methods

2.1 Reagents and buffers

Recombinant mouse IL-33 was obtained from Biolegend (endotoxin <0.01ng/μg). All other cytokines and reagents were sourced as indicated. Commonly used reagents and buffers are indicated in Table 2-1.

Table 2-1 Commonly used reagents and buffers

Reagent	Composition
Phosphate buffered saline (PBS)	8g NaCl, 1.16g Na2HPO4, 0.2g KCl, 0.2g KH2PO4 in 1 litre distilled water, pH 7.4
Medium	RPMI (Invitrogen)+ 100IU pen strep/ml
Complete medium (CM)	RPMI (Invitrogen), 10% heat inactivated fetal calf serum (FBS), 100IU pen strep/ml, 2mM L-glutamine, 50 μM β-mercaptoethanol (all Invitrogen)
FACS buffer	PBS, 2% FCS, 5mM EDTA
Wash buffer	0.05% Tween-20 in PBS pH 7.4
Assay diluent buffer	Ebioscience Assay diluent x1 (diluted from x5 stock in distilled water)
Lysis buffer	RIPA buffer (Thermo Scientific) containing sodium orthovanadate (1μM) + protease inhibitor cocktail (Calbiochem)
Sample buffer	NuPage sample buffer (Invitrogen) + 100mM dithiothreitol
Stop buffer	90mls distilled water+ 6mls of sulphuric acid
Avertin	1:1 weight:volume solution of 2,2,2-tribromoethanol in tert-amyl alcohol

2.2 In vivo methods

Balb/c mice were purchased from Harlan, UK. *St2*$^{-/-}$ mice were originally a kind gift from Dr A MacKenzie (Cambridge) and a colony was bred in the Glasgow University Biological facilities. *DO11xRag2*$^{-/-}$ mice were obtained from Prof Garside (Glasgow). All animals were kept in pathogen-free conditions in facilities managed by Biological services staff, University of Glasgow under strict accordance of the regulations described by the United Kingdom Home Office Animals (Scientific procedures) act 1986. All procedures were performed under Project Licence Number 60/3791, procedure 5.

2.2.1 Intranasal (i.n.) dosing

Balb/c wild-type (WT) or *St2*$^{-/-}$ mice were anaesthetised using 3% isofluorane in an induction chamber. Intranasal administration was performed with the animal held in the upright position with gentle pressure at the lower mandible to prevent swallowing. Using a 200μL pipette 30μL of solution was instilled into the nostrils allowing inhalation of the reagent. When both cells and reagent were dosed into the mice, a final volume of 50μL was instilled. The mice were allowed to recover from the anaesthetic spontaneously, in the recovery position.

2.2.2 Intravenous (i.v.) dosing

Mice were placed in a heat box at 38°C for 20-30 minutes prior to injection. They were placed in a suitable restrainer followed by inoculation of 200μL of PBS i.v. containing the appropriate numbers of cells, using a 1ml insulin syringe. The puncture site was treated with direct pressure to prevent further bleeding and the mice were then released.

2.2.3 IL-33-induced airway inflammation model

1μg of IL-33 (or PBS) ± 1mg/kg rapamycin (Calbiochem) was instilled for 5 consecutive days using the method described in section 2.2.1 into BALB/c or *St2*$^{-/-}$ mice. The mice were killed 24 hours after the last inoculation. Samples were collected as described below.

2.2.4 Adoptive transfer model

Type 2 innate lymphoid cells (ILC) were sorted from the lungs of intranasally IL-33-treated mice as described below. 1x10^6 ILC were transferred intranasally into *St2*$^{-/-}$ mice as described above, in 20μL PBS. The mice were then treated immediately after with PBS or IL-33 ± rapamycin in 30μL PBS for 5 consecutive days. The mice were then killed by schedule 1 method on day 6.

2.2.5 Double adoptive transfer model

CD4 T cells were sorted from DO11.10x*Rag2*$^{-/-}$ mice using magnetic bead sorting methods as described below (section 2.4.4). WT ILC were sorted as described in

section 2.4.3. $0.5x10^6$ DO11.10 CD4 T cells ± $1x10^6$ ILC were transferred i.v. in 200μL PBS as described above. Immediately after, mice were treated with intransal IL-33 (1μg) and ovalbumin (ova) (100μg) in 30μL PBS as described in section 1.2.1. The mice were killed by cardiac puncture under terminal anaesthesia on day 5 after the transfer.

2.2.6 Airway hyperresponsiveness measurement

Non-invasive plethysmography was used to measure enhanced pause (Penh) in mice following 5 days of i.n. inoculations. Briefly, mice were placed individually into chambers of an 8 chamber plethysmography unit (EMMS, England, UK) for a 30 minute acclimatisation period. Basal recordings for enhanced pause were made followed by recordings over 2 minutes during which the mice received nebulised saline or increasing concentrations of methacholine (Sigma) (1%, 3%, 5%, 10%, 30%). Following Penh measurements, the mice were killed by cardiac puncture under terminal anaesthesia and samples collected.

2.2.7 Sample collection and processing

2.2.7.1 Serum collection

Mice were terminally anaesthetised with a single 500μL intraperitoneal (i.p.) Avertin injection (1:40 dilution of Avertin stock in PBS). Following induction of complete anaesthesia, the heart was exposed and cardiac puncture using a 23G needle and 1ml syringe was performed. The blood was allowed to clot at room temperature and serum was separated by centrifugation at 11000g for 30 minutes. The serum was collected and stored at -20°C until required.

2.2.7.2 Bronchoalveolar lavage (BAL)

After termination by exsanguination under terminal anaesthesia the trachea was exposed and a small incision was made in its proximal end. Cannulation using a 1ml syringe attached to a 23G needle sheathed with polythene tubing (0.58mm ID, 0.78mm OD, VWR International) was performed. 800μL of PBS was instilled into the lungs whilst providing a seal with forceps pressure at the tracheal insertion site, ensuring lung re-expansion with the instillation. The fluid was aspirated following 10 seconds and the BAL fluid was collected in a 1.5ml tube

and placed on ice. The process was repeated with a further 800μL PBS and the samples were kept separately.

BAL samples were weighed to assess individual sample collection efficiency and the fluid was centrifuged at 380g at 4°C for 6 minutes to allow the cells to pellet. The supernatant was collected from individual samples separately and kept at -20° until further processing. The cells from individual mice were resuspended in 1ml of PBS and live cell counts were counts were performed using a Neubauer haemocytometer (Weber Scientific International Ltd, Teddington, UK). Samples were diluted 1:2 in 4% trypan blue solution. The remaining cells were either used to perform cytospins or used for FACS analysis (see below).

2.2.7.3 Cytospin preparation

$1x10^5$ cells were spun onto glass slides using a Shandon Cytospin3 (Thermo Shandon, Runcorn, UK) at 450 rpm for 6 minutes. The slides were allowed to air-dry and were fixed in methanol for 10 minutes. They were subsequently stained using the Romanovsky method using eosin and methylene blue stains (Raymond A Lamb, Eastborne, UK) and coverslides were adhered using the synthetic resin, DPX for protection. The cells were counted at x100 magnification under oil immersion.

2.2.7.4 Mediastinal lymph node (mLN) collection

Following BAL collection, the heart and lung was removed *en bloc* from the thoracic cavity and the mLN located and placed in 1.5ml tubes containing media. These were placed on ice until further processing. The mLN for individual mice were passed through 40μM cell strainers (BD) with the aid of a syringe plunger, and centrifuged at 300*g* for 10 minutes. The pellet was then resuspended in medium for counting. The remaining cells were used in culture or for FACS analysis (see below).

2.2.7.5 Lung collection

The lungs were collected using different methods depending on the processing required. When lungs were required for histology, the heart and lung block were placed in 5ml of buffered formalin solution for paraffin embedding.

When the lungs were used to perform whole lung digests, the lobes were dissected away from the main bronchi and placed in medium, on ice, for further processing.

For RNA extraction, the upper lobe of the left lung was removed from each animal and placed in a bijou which was immediately snap-frozen in liquid nitrogen and subsequently stored at -80°C for further use.

2.2.7.6 Lung digestion

Whole lungs were immersed in 1ml of sterile-filtered digest media (RPMI+100IU/ml pen/Strep solution, 0.1mg/ml DNAse 1 (Roche), 50μL of Liberase TL at 28 Wunsch units/ml (Roche)) in a bijou and mechanically dissociated. The bijous were then placed in a mechanical rotator for 1 hour at 37°C. The digested material was then passed through a 100μM filter and medium added to stop the digestion reaction. Any remaining lung tissue fragments were passed through the filter with the aid of a syringe plunger. The cells were centrifuged at 300g for 10 minutes and the cell pellet was resuspended in 1.5ml of red-cell lysis buffer (Sigma) for 1 minute. The cells were flooded with media and centrifuged again. The single cell suspension was resuspended in 25mls of complete medium for counting and filtered again using a 40μM filter. The cells were then incubated in a 75cm^2 flask at 37°C for 1 hour to remove adherant cells.

Following incubation, the media containing non-adherant cells was collected and the flask was rinsed several times with FACS buffer to collect remaining cells, being careful not to scrape off any adherant cells in the process. The cells were washed in FACS buffer at 340*g* for 5mins at 4°C and resuspended in 2ml FACS buffer and stained as per FACS protocol (see below).

2.2.7.7 Lymphoid tissue collection for CD4 T cells

Mice were killed by schedule 1 procedures in keeping with Home Office recommendations. Peripheral lymph nodes (LN) were collected from cervical, axillary, femoral and popliteal sites into bijous containing 1.5ml medium. The abdominal cavity was exposed and the mesenteric LN and spleen were located and collected. Tissues were kept on ice until further processing.

2.2.8 Histology

Lungs collected in 10% buffered formalin were embedded in paraffin using a Shandon citadel 1000 tissue processor (Thermo Scientific) and submerged into paraffin blocks. These were cut into 5μm sections using a microtome, onto histology slides (VWR). They were then deparaffinised and rehydrated using graded alcohol solutions (Xylene, 100% ethanol, 70% ethanol and distilled water), ready for staining.

2.2.8.1 Haematoxylin and eosin staining (HE)

Where sections were stained with HE, the slides were stained with Harris haematoxylin for 2 minutes and excess was washed off with water. The stain was enhanced with 1% acid/alcohol solution and Scots tap water substitute. Finally, the slides were counterstained with eosin for a final 2 minutes with excess washed off with water. The sections were then dehydrated with increasing concentrations of alcohol (70% ethanol, 100% ethanol, xylene) and the tissue was covered with Di-n-butylPhthalate in Xylene (DPX) mountant (Sigma) and a coverslide.

2.2.8.2 Periodic-acid Schiff (PAS) staining

Periodic acid Schiff stains glycopeptides, a major component of lung mucus [330]. Slides were dehydrated as described above (section 2.2.8). A Sigma kit was used to stain the slides following the manufacturer's instructions. Briefly, the slides were submerged in acid solution for 5 minutes followed by several washed with distilled water. The slides were immersed in Schiff reagent for 15 minutes and again, washed in running water for 5 minutes. Finally, the slides were counter-stained with Harris haematoxylin for 90 seconds followed by a

brief water wash to remove excess stain. The slides were dehydrated as before and the sections were covered with DPX mountant and a coverslide, ready for viewing.

2.2.8.3 Histology scoring method

All slides were assessed by 2 blinded assessors. An arbitrary scoring system was used, scoring from 0 (no inflammation) to 3 (severe inflammation) in a minimum of 5 high power field areas of the HE-stained lung sections. A score of 3 was given where peri-vascular and peri-bronchial inflammation was seen in the same field. Scores were averaged for individual mice.

Similarly, scoring for extent of PAS staining was performed by 2 blinded assessors scoring a minimum of 5 high power fields in each lung section. Areas were marked arbitrarily 0 (no PAS staining) to 3 (high levels of PAS staining associated with goblet cell hyperplasia). Scores for each slide were summed and averaged to provide a final score for that mouse.

2.3 *In vitro* techniques

All cell culture work was performed under sterile conditions and cultures were incubated in a humidified incubator at 37°C, supplemented with 5% carbon dioxide (CO_2). Centrifugation was performed at 380*g* for 5 minutes at 4°C (Jouan CR3i centrifuge), unless otherwise stated. Cell counts were performed using a haemocytometer and cells were diluted in varying dilutions (ranging from 1:2 to 1:10) of 0.4% trypan blue solution (Sigma).

2.3.1 Type 2 innate lymphoid cell culture

FACS sorted ILC were rested overnight in 1ml of CM at a density of 4-5 $x10^6$ cells/ ml for 16 hours. Cells were collected the next day, centrifuged in media and resuspended in CM ready for counting. Whenever cells were used for adoptive transfer, cells were resuspended in PBS.

2.3.2 Cell culture for cytokine quantification by ELISA

Cells were cultured in 96 well flat-bottomed plates for the specified time periods and conditions. Cytokines (IL-2, IL-7, IL-12, Thymic stromal lymhopoeitin (TSLP) all from Peprotech), were added at the indicated concentrations. Supernatants were collected and care was taken not to collect the cells in this process. The samples were frozen at -20°C until further analysis was possible.

2.3.3 Cell culture for signalling assays

Since the incubation periods for signalling assays was very short, cells were cultured in sterile 1.5ml tubes to facilitate efficient processing within the specified time periods. $1x10^6$ cells were used for each condition and where indicated, cells were pre-incubated with rapamycin (100nM) for 30 minutes prior to cytokine stimulation. Following the required incubation periods, the cells were centrifuged at 1200*g* at 4°C for 3 minutes and the supernatant carefully discarded.

2.3.4 Thymidine proliferation assays

Cells were cultured as before in 96 well plates for 72 hours. In the final 16 hours of culture, the cells were pulsed with 0.037MBq ^{3}H-thymidine. Following the culture period, the cells were harvested onto a membrane using a Perkin Elmer Filter mate cell harvester following manufacturers' instructions. The membrane was allowed to dry and it was then covered with scintillation fluid and radioactivity was measured using a Perkin Elmer LSC Luminescence counter 1450.

2.3.5 Fluorescent-labelled cell proliferation assay

Cells were washed in PBS. Where cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen), a 1ml solution containing 1μL of the stock CFSE solution was made to a final concentration of 25ng/ml. The cell pellet was resuspended in this solution and incubated at room temperature for 10 minutes in the dark prior to the addition of 5mls of FBS to stop the reaction. The cells were then washed twice with CM. Where cells were stained with cell proliferation dye efluor 670 (ebioscience) a working solution made by diluting

1μL of stock dye in 1ml of PBS was made to a final concentration of 5nM. The cells were resuspended in 800μL of PBS and 200μL of this stock solution was added. The cells were incubated for 15 minutes at 37°C in the dark and subsequently washed twice with CM. Cells were then cultured as before for 72 hours at which point the cells were collected, washed with FACS buffer and fluorescence dilution assessed by FACS.

2.3.6 Cell stimulation for intracellular cytokine measurement

Lymph node cells and lung digest cells were cultured in 24 well plates at $2x10^6$ cells/ well in complete medium in the presence of phorbol 12-myristate 13-acetate (PMA) (50ng/ml) (Sigma), Ionomycin (0.9nM) (Sigma) and Golgi-stop (6.7μL/ml) (BD) for 4 hours at 37°C. The cells were then collected using a cell scraper (Corning) to allow collection of adherent cells. The cells were placed in individual 6ml tubes and washed with PBS, followed by centrifugation at 380g for 5 minutes.

2.3.7 Ova peptide recall assay

Whole mLN and lung cells were cultured in U-bottomed 96 well plates at a density of 1.5 $x10^5$ per well, in triplicate, with CM alone or either 0.5μM or 5μM ova peptide 323-339 (Invivogen). The cells were cultured for 72 hours and supernatants were collected for cytokine ELISA measurement.

2.3.8 Naïve T helper cell and ILC co-culture

ILC were sorted from lungs as described below. The ILC were rested overnight in CM, washed and resuspended in CM ready for counting and culture. $CD4^+CD44lo$/intermediate cells (naïve) were also sorted by FACS, washed and resuspended in CM. 96 well flat-bottom plates were coated with anti-CD3ε antibody (1.5μg/ml) and anti-CD28 (3μg/ml) diluted in PBS. The plates were incubated for 1.5 hours at 37°C. Following this incubation the plates were washed once with PBS prior to use for culture. $1x10^5$ of each cell type was used for co-culture experiments.

2.3.9 Semi-permeable membrane-separated co-culture

96 well receiver plates were coated with anti-CD3/CD28 as above. CD4 T cells were added to the culture plates first to ensure contact with the antibodies was achieved. Where co-culture was to be separated by a semi-permeable membrane, ILC were added once the Transwell 0.4μm porous membrane (Corning) was placed. ILC were added to the upper compartment (Figure 2-1)

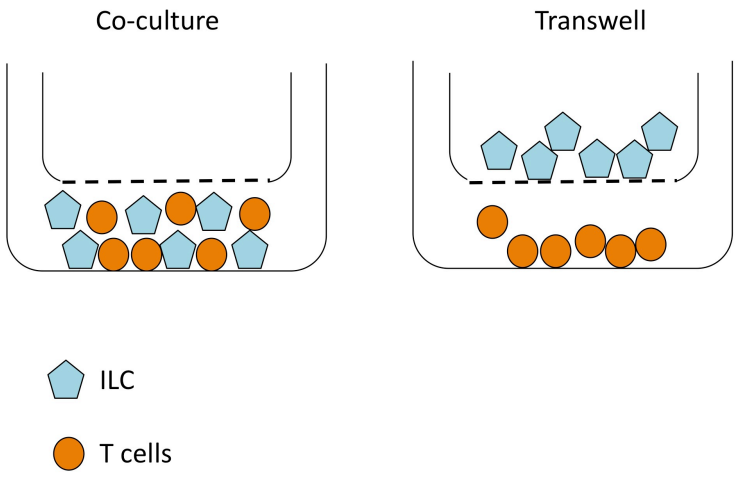

Figure 2-1 T cell and ILC co-culture

2.4 Assays and analysis

2.4.1 Flow Cytometry (FACS) I – cell surface staining

The cell pellets formed in section 2.2.7.6 were resuspended in 100μL of FACS buffer, FcR Block (ebioscience) was applied at 1:100 dilution and incubated at 4°C for 15 minutes. The cells were subsequently stained for surface markers at 1:200 dilutions in a final volume of 100μL for 25 minutes in the dark at 4°C. During the final 5 minutes of incubation, DAPI was added at 1:200 dilutions to the cells, which were subsequently washed with FACS buffer twice and resuspended in FACS buffer ready for analysis. Whenever this protocol was used for the preparation of cells for cell sorting, up to $150x10^6$ cells were stained in 2ml of FACS buffer using antibodies at the aforementioned dilutions. All FACS antibodies used are found in Table 2-2.

2.4.2 Flow Cytometry (FACS) II - intracellular staining of fixed cells

The cell pellets were washed in PBS twice after collection to ensure all traces of FBS were washed off. Each sample was then resuspended in 1ml PBS containing 1μL of live-dead Aqua-dye (Invitrogen) stock solution (stock solution made fresh by adding 50μL DMSO to the fluorescent dye) and vortexing the cells. The cells were then incubated at 4°C for 30 minutes. Subsequently, the cells were washed in PBS and surface marker staining performed as above. The cells were washed with PBS following staining and resuspended in 300μL BD cyto/fix solution, incubating the cells in the dark at 4°C for 30 minutes. Once permeabilised and fixed, the cells were washed twice with BD cyto/perm solution (made from stock x10 solution, diluted in distilled H_2O) and intracellular staining performed using firstly 1:100 FcR blocking agent (ebioscience) for 15 minutes at 4°C followed by appropriate cytokine staining at 1:100 dilution for 30 minutes at 4°C in the dark. The cells were washed once more in BD cyto/perm solution and finally in FACS buffer prior to acquisition.

BD FACS calibur and LSRII machines were used for acquisition. Whenever cells were sorted using the BD FACS Aria machine or lung digests were acquired, the cells were filtered through 40μm filters prior to acquisition to prevent blockages in the FACS machines. FACS data was analysed using FlowJo 8.8.4 analysis software.

Table 2-2 FACS antibodies

Antibody	Clone	Isotype	Source
B220	RA3-6B2	Rat IgG2aκ	eBioscience
CD3	145-2C11	Armenian hamster IgG	eBioscience
CD4	GK1.5	Rat IgG2aκ	eBioscience
CD8	53-6.7	Rat IgG2aκ	BD biosciences
CD25	PC61	Rat IgG1λ	BD biosciences
CD44	IM7	Rat IgG2bκ	eBioscience
CD45	30-F11	Rat IgG2b	eBioscience
CD11b	M1/70	Rat IgG2bκ	BD biosciences
CD11c	N418	Armenian hamster IgG	eBioscience
CD127	SB199	Rat IgG2aκ	BD biosciences
C-kit	ACK45	Rat IgG2b	BD biosciences
ICOS	C398.4A	Armenian hamster IgG	BioLegend
FcεRI	MAR-1	Armenian hamster IgG	eBioscience
ICAM	YN1.1.7.4.	Rat IgG2bκ	eBioscience
IL-2	JES6-5H4	Rat IgG2bκ	BD biosciences
IL-4	11B11	Rat IgG1	BD biosciences
IL-5	TRFK5	Rat IgG1κ	BD biosciences
IL-9	RM9A4	Rat IgG1	BioLegend
IL-13	eBio13A	Rat IgG1κ	eBioscience
IFNγ	XMG1.2	Rat IgG1	BD biosciences
LFA-1	2D7	Rat IgG2aκ	BD biosciences
NK1.1	PK136	Mouse IgG2a	eBioscience
Gr1	RB6-8CS	Rat IgG2bκ	eBioscience
OX40L	RM134L	Rat IgG2bκ	eBioscience
Siglec F	E50-2440	Rat IgG2a	BD biosciences
ST2	DJ8	Rat IgG1	MD Biosciences
TCRαβ	H57-597	Armenian hamster IgG	eBioscience
TCRγδ	ebioGL3	Armenian hamster IgG	eBioscience

2.4.3 FACS cell sorting

Cells were sorted from lung digests or lymphoid tissue following staining as detailed in section 2.4.1 using a BD FACS Aria machine. The gating strategy for ILC is further described in chapter 4. Cells were collected into 15 centrifuge falcons containing CM. Following collection cells were centrifuged at 300*g* for 10

minutes, 4°C. Cell pellets of ILC were resuspended in CM and cultured in a 24 well plate at a concentration of 3-5 x10^6 overnight at 37°C. Alternatively, the ILC were resuspended in PBS, for *in vivo* transfer experiments. When CD4 T cell were sorted, naïve CD4 T cells were gated upon live $CD4^+$ cells followed by CD44 low/ intermediate expression ($CD4^+CD44^{lo}$), based on a histogram analysis of fluorescence. Following sorting, naïve CD4 T cells were centrifuged as described above and resuspended in CM, ready for counting and *in vitro* assays. Routine purities for both ILC and $CD4^+CD44^{lo}$ cells were >95%.

2.4.4 Magnetic bead CD4 cell sorting

LN and spleen were dissociated separately using the plunger of a 5ml syringe and 100μm filter to obtain a single cell suspension. The LN cells were counted and kept on ice. Since *DO11 TCR x Rag2*$^{-/-}$ mice were used, no CD4 T separation was necessary for the LN cells, since 90% of the cells were $CD4^+$. Following centrifugation, the spleen pellet was resuspended in red blood cell lysis buffer and incubated for 1 minute. Lysis was stopped with media and the cells were centrifuged again. The cells were washed with FACS buffer and cells were counted. Up to 100x10^6 cells were resuspended in 400μL of FACS buffer and 100μL of antibody cocktail (CD4 T cell enrichment kit, Miltenyi). This was incubated at 4°C for 15 minutes after which a further 300μL of FACS buffer was added to the cells plus 200μL of Miltenyi magnetic beads. The cells were incubated at 4°C for a final 15 minutes and then washed once with FACS buffer. Using a Milteny automacs machine, the cells, resuspended in 5ml of FACS buffer were sorted by negative selection into $CD4^+$ and $CD4^-$ cells. The $CD4^+$ cells were collected, washed, resuspended in PBS and counted. The cells were then adjusted to the required concentration, ready for adoptive transfer. Purity checks were performed for both the spleen-separated CD4 cells as well as the LN cells by FACS ($CD4^+KJ126^+$). Routine purity was 90%.

2.4.5 Western blot for signalling kinases

The cell pellets obtained as described in section 2.3.3 were resuspended in 30μL of RIPA cell lysis buffer (Pierce) and left on ice for 15 minutes. They were subsequently centrifuged at 11000g for 5 minutes at 4°C and the supernatants

were collected into a new tube. Sample buffer was added to the supernatants at a 1:3 ratio and the samples were stored at -20°C.

For gel electrophoresis, the cell lysates were defrosted and incubated at 80°C for 5 minutes to denature the proteins. 20μL of the samples were subsequently loaded into 10 lane 4-12% NuPage SDS-PAGE gels (Invitrogen) alongside 7 μL of a molecular weight marker (See-blue Plus 2, Invitrogen). The gels were placed into a western blot tank filled with x1 MES buffer (Invitrogen) and a charge of 125 watts applied for 2 hours or until the samples had run up to the bottom edge of the gel.

Using the iBlot Dry Blotting system (Invitrogen), the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. To ensure successful transfer, each membrane was briefly coated with Ponceau's solution and checked for the presence of protein bands. This solution was briefly washed off using wash buffer, and the membrane blocked with 10ml of 5% BSA (Sigma) made up in wash buffer. The container was gently agitated at room temperature for 1 hour. The membrane was then incubated overnight at 4°C in 5 ml of 5% BSA solution containing a 1:1000 dilution of the required antibody. The membrane was washed at least 3 times for a minimum of 20 minutes with wash buffer and the blot was incubated for a minimum of 1 hour with PBS containing 1:2000 dilution of the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Following three wash steps with wash buffer the membrane was covered with 750μL of each of buffers A and B Amersham ECL chemiluminsecent reagent for 3 minutes. The membrane was placed in a cassette and exposed to medical X-ray (Kodak).

2.4.6 Cytokine immunoassays

Enzyme linked immunoabsorbant assays (ELISA) were performed for cytokine quantification using paired antibodies and kits as specified in Table 2-3 following the manufacturers' instructions. Briefly, ELISA 96 well plates (Corning) were coated with 50μL/well with capture antibody, diluted in PBS, overnight at 4°C. All wash steps were done using wash buffer. Following three washes, the plates were blocked for 1h at room temperature using 200μL of x1 assay diluent buffer (eBioscience) in each well. The plates were then washed and 50μL of each

sample was added to the appropriate wells. A minimum of 7 serial dilutions of the standards in duplicate was performed for each plate using assay diluent buffer. A minimum of two wells were incubated with assay diluent buffer alone, to provide 'blank' wells. The plates were then incubated at room temperature for a minimum of 2 hours. Following another three washes, 50μL of detection antibody diluted in assay diluent buffer were added and plates incubated for 1h at room temperature. The plates were washed again and HRP-streptavidin (Sigma) diluted 1:1000 into assay diluent buffer was added to the wells and incubated in the dark for 30 minutes. Six washes were performed and 100μL of TMB substrate solution was added to each well. Checking the plates regularly, they were incubated in the dark until developed. The reaction was stopped by the addition of 100μL of stop buffer to each well. The optical density was measured immediately after using a 450nm filter in a Sunrise absorbance plate reader machine.

Table 2-3 ELISA kits used and sources

Cytokine/chemokine	Supplier
IL-2	BD biosciences OptEIA kit
IL-4	Ebioscience Ready-set-go! kit
IL-5	BD biosciences OptEIA kit
IL-6	Ebioscience Ready-set-go! kit
IL-9	Biologend
IL-13	Ebioscience Ready-set-go! kit
Eotaxin-2	RnD paired antibodies
MIP-1α	RnD duoset
GM-CSF	Ebioscience Ready-set-go! kit
Interferonγ	Ebioscience Ready-set-go! kit

2.4.7 RNA isolation and quantitative polymerase chain reaction

Cell pellets, obtained by centrifugation, were flash-frozen in liquid nitrogen and stored at -20°C until further processing was possible. The pellets were allowed to reach room temperature and RNA extraction was performed using Qiagen RNeasy minikits as per the manufacturers' instructions. RNA quantification was performed using a Nanodrop 1000 machine and 1μg of RNA was used to make complementary DNA (cDNA) by polymerase chain reaction (PCR) using

MultiScribe reverse transcriptase kit (Applied Biosystems) as per manufacturers' instructions. Real time quantitative PCR was performed in triplicate for each sample using Taqman probes, primer sets and an ABI Prism 7900 Sequence Detection System instrument real-time PCR system (Applied Biosystems).

2.5 Statistics

Statistics comparing groups was performed using Student's T tests or 1-way ANOVA, depending on the number of groups to be compared using GraphPad v4 software.

3 Rapamycin inhibits IL-33-induced airway inflammation

3.1 Introduction

Experiments in our laboratory had shown that IL-33-induced cytokine production in T helper (Th) 2 cells required mammalian target of rapamycin (mTOR) activation *in vitro*. The hypothesis that IL-33-dependent responses *in vivo* would also require mTOR activation was proposed. Experiments described in this chapter aim to address this hypothesis by assessing whether IL-33 signalling requires mTOR signalling *in vivo*, using a model of IL-33-induced airway inflammation. This model had been extensively investigated within the lab group. The reasons to use an airway inflammation model were two-fold. Firstly, Th2 cells are induced by IL-33 and had been shown to play an important role in airway inflammation [173]. Secondly, my area of interest as a respiratory physician is airway inflammation and allergic airways diseases, in which IL-33 plays a role in both murine models and human asthma [175, 176, 232].

When IL-33 was first described as the ligand for ST2, Schmitz *et al*. demonstrated that, in mice, daily intraperitoneal (i.p) injections of IL-33 at varying doses (0.4-4 μg/injection) for seven days induced substantial systemic inflammation characterised by peritoneal eosinophilia and splenomegaly [82]. Subsequently, Kondo *et al*. showed that intranasal administration of 1 μg IL-33 in BALB/c mice daily for 4 consecutive days [232] led to profound airway inflammation with significant airway eosinophilia and mucus production. This demonstrated that IL-33 could be used to induce airway inflammation in the absence of antigen sensitisation or challenge.

Within our lab group, numerous researchers had previously dissected the effect of intranasal administration in both BALB/c and C57BL/6 mice. Dose responses and kinetic effects of IL-33 administration had shown that, when IL-33 was given daily, airway inflammation and cellular recruitment to the lung increased gradually from day 3 onwards. The peak in cytokine production, however, was measured following 5 daily intranasal IL-33 inoculations. A 2 μg dose of intranasal IL-33 was given daily in these models as recombinant human IL-33 was used and had been shown in *in vitro* studies to have half the bioactivity of murine IL-33 on murine cells (M. Kurowska-Storlarska *unpublished data*).

3.2 Results

3.2.1 Defining the IL-33 induced airway inflammation model

BALB/c mice were used for the IL-33-induced airway inflammation model. The decision to use this mouse strain was based on the fact that the initial results showing a role for mTOR in IL-33 signalling had been shown in BALB/c-derived Th2 cells. More so, BALB/c mice have been shown to have an intrinsically Th2-skewed response in airway inflammation models, compared with mice of other genetic backgrounds [331]. Finally, the effect of IL-33 in Th2 responses *in vivo* had been shown previously in BALB/c mice [173]. The model was defined as per Figure 3-1. A daily dose of 1 μg IL-33 was administered intranasally based on the fact that recombinant murine IL-33 was used, rather than human IL-33 as in previous studies. This dose was also in keeping with that used by our group in recent publications [44]. A 5-day model was employed as it had been shown previously that cytokine levels peaked on this day of the model, this being an important read-out for the current work.

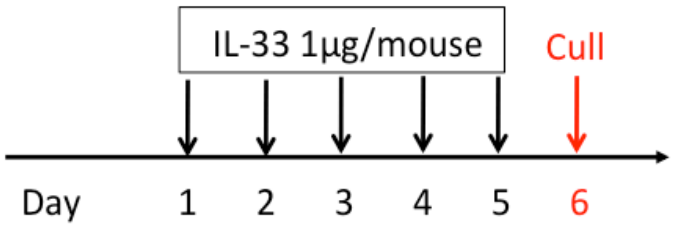

Figure 3-1 The IL-33-induced model of airway inflammation

BALB/c mice were treated with PBS or 1μg recombinant mouse IL-33 intranasally in 30μL PBS daily for 5 consecutive days. The mice were then killed on day 6.

3.2.2 The effect of intranasal IL-33 on airway inflammation

Initial experiments were performed in order to robustly replicate the results of previously described IL-33-induced airway inflammation models as per Figure 3-1. Intranasal administration of IL-33 for 5 consecutive days in BALB/c mice significantly increased the total number of bronchoalveolar lavage (BAL) cells from a baseline of 2.8 x 10^5 cells in PBS-treated mice to an average of 31.3 x 10^5

cells in IL-33-treated mice (Figure 3-2A). Additionally, the differential cells counts of the BAL following haematoxylin and eosin (HE) staining and microscopic examination revealed a marked change in the cell types infiltrating the lung with a significant increase in numbers of all cell types analysed. In particular, a substantial increase was observed in the proportion of eosinophils, from virtually none in the PBS-treated group, to an average of 19.4 x 10^5 cells/ml of BAL (p=0.0000096) (Figure 3-2B).

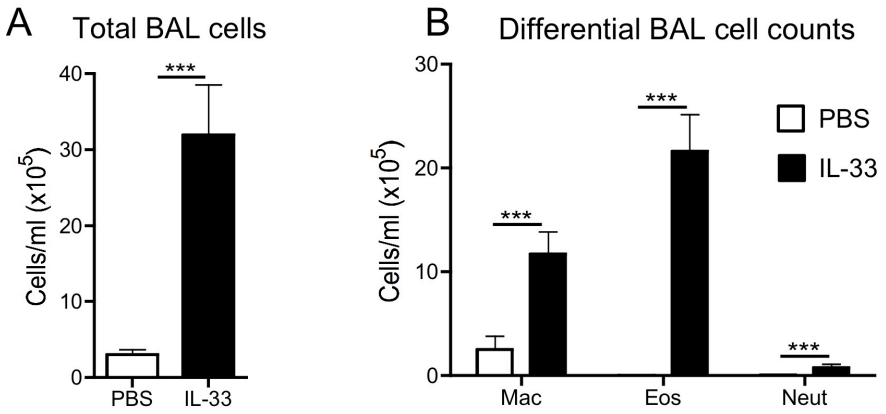

Figure 3-2 IL-33 increases total BAL cell numbers though an increase in BAL granulocytes

BALB/c mice were treated with IL-33 or PBS as per Fig 2-1 model. Following cardiac puncture under terminal anaesthesia, BAL was performed and total cell counts analysed for each individual mouse (A). Differential cell counts were performed on the BAL based on microscopic morphology of the cell as described previously (B). The data are representative of 2 experiments with 4-5 mice per group. Error bars represent SD. ***p<0.001 when compared to PBS control.

3.2.3 The effect of intranasal IL-33 on lung cytokine production

IL-33 induces type-2 cytokine production from numerous cell types in the lung including T helper cells [173], mast cells and basophils [232]. Therefore, levels of these cytokines in BAL fluid were measured by ELISA. Whilst IL-4 was not detectable in the BAL fluid (*data not shown*), high amounts of IL-5 and IL-13 were measured (Figure 3-3A). Compared with PBS-treated mice, IL-33 greatly enhanced the production of both IL-5 and IL-13, from barely detectable levels in control mice to over 1 ng/ml of IL-5 and an average of 600 pg/ml IL-13 in IL-33-treated animals. Levels of Eotaxin-2 (C-C motif ligand (CCL) 24), which is important for eosinophil recruitment, and macrophage inflammatory protein (MIP)-1α (CCL3), an important chemokine involved in granulocytic chemotaxis were also measured (Figure 3-3B). An increase in both CCL24 and CCL3 in the IL-

33-treated group was apparent, reflecting the cellular recruitment in the BAL fluid. These data demonstrate that the model was in keeping with our group's previous results and published data [232].

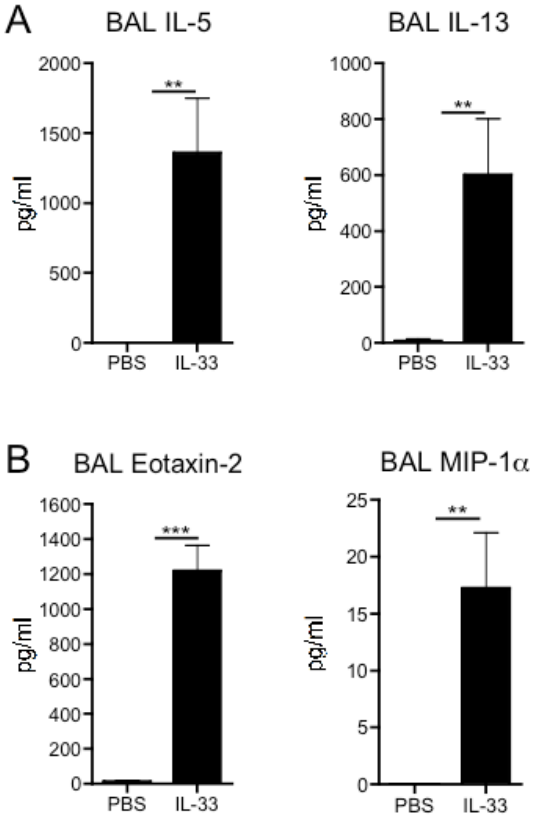

Figure 3-3 Intranasal IL-33 increases type-2 cytokines and chemokines in BAL fluid

Cytokine ELISA was performed for IL-5 and IL-13 on the BAL obtained from each mouse (A). Chemokine levels for Eotaxin-2 and MIP-1α were measured on the first BAL sample (B). Samples were measured for each individual mouse with 4-5 mice per group. The data are representative of 2 experiments. Error bars represent SD. **p<0.01, ***p<0.001 when compared to PBS group.

3.2.4 The effect of intranasal IL-33 on lung architecture and goblet cell mucus production

One of the key features of IL-33-induced airway inflammation is the induction of goblet cell hyperplasia and lung architecture distortion as a consequence of cellular infiltration [232]. This has been shown to occur following i.p. IL-33 injections, in the absence of the adaptive immune system as it can be replicated in *Rag2*$^{-/-}$ mice, which lack B and T cells. These processes are dependent on the induction of IL-13 production in the lung and signal transducer and activator of transcription (STAT) 6 activation [232]. In order to ascertain if the intranasal

model fulfilled the same histological characteristics, the lungs of the mice that had been treated intranasally for 5 days with IL-33 or PBS were collected and sections from the same lobes were assessed for architecture distortion and cellular infiltration on HE-stained slides, as well as mucus staining on Periodic-acid Schiff (PAS)-treated tissue. When comparing lung sections of the control mice versus the IL-33-treated mice, there was a clear increase in the cellular infiltration of the lung (Figure 3-4A). The cellular infiltration predominated in the peri-bronchial and peri-vascular areas. However, there were also some alveolar areas that appeared distorted due to increased cellular infiltration. The distortion of alveolar areas was patchy and unevenly distributed. Mucus production was also increased in IL-33-treated mice compared to control and associated with this there was increased goblet hyperplasia within the larger bronchi (Figure 3-4A). With the aid of an additional assessor, these changes were quantified blind using an arbitrary scale looking for three main changes: overall change in lung architecture, cellular infiltration and mucus staining (Figure 3-4B). The effect of IL-33 treatment on all the above parameters, compared to control, was significant (architecture score $p=0.0016$, cellular infiltration score $p=0.0019$, mucus staining score $p=0.046$).

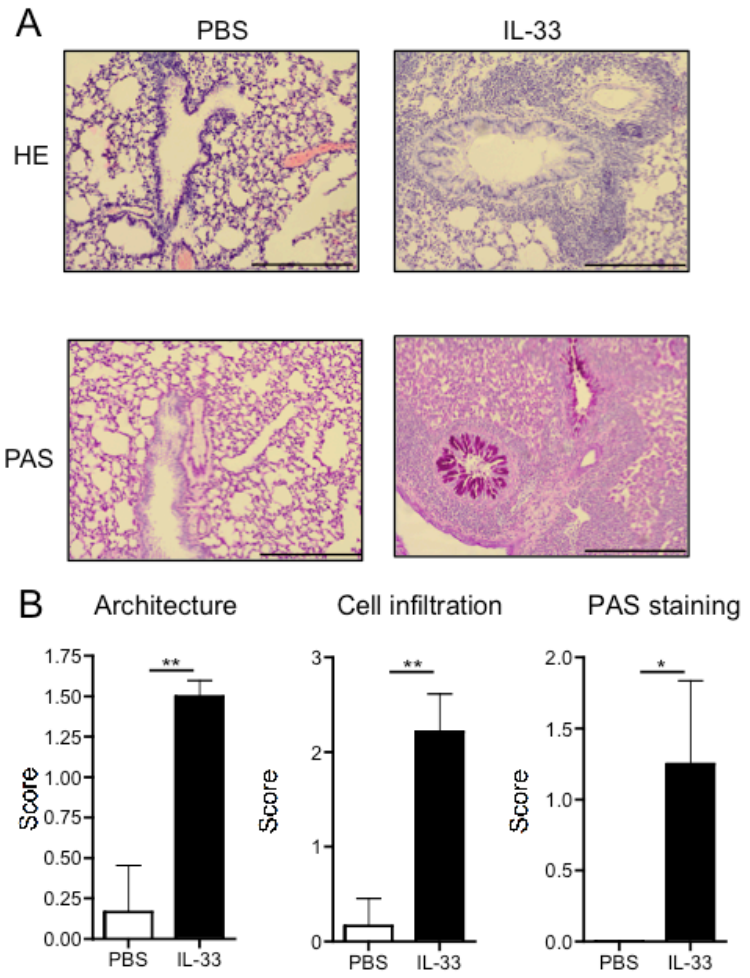

Figure 3-4 IL-33 induces lung inflammation, architecture distortion and mucus hypersecretion

The right lung was collected, fixed in formalin and embedded in paraffin as described previously. 5 μm sections were cut and stained with HE for cell infiltration and architectural distortion assessment. Sections were also stained with PAS for mucus quantification and goblet hyperplasia assessment (A). The slides are representative of their group. Bar represents 200 μm. With the aid of an independent investigator, the sections were assessed across 5 high powered fields for changes in the architecture of the lung, cell infiltration quantification and extent of mucus staining using an arbitrary scoring system as described in chapter 2 (B). 4-5 mice were used per group. Error bars represent SEM. *p<0.05, **p<0.01 when compared to PBS group.

3.2.5 The effect of rapamycin on IL-33-induced airway inflammation

Having validated the *in vivo* model, the working hypothesis that IL-33-induced airway inflammation required mTOR activation *in vivo* was tested. This hypothesis was based on the finding that *in vitro*, Th2 cells treated with IL-33 in the presence of the mTOR inhibitor rapamycin produced significantly less IL-5

and IL-13 than when treated with IL-33 alone [75]. To demonstrate a role for mTOR signalling in IL-33-mediated airway inflammation, the mTOR inhibitor rapamycin was delivered intranasally alongside IL-33 or alone, as a control. The inflammation model was the same as in Figure 3-1 and rapamycin was administered at a concentration of 1 mg/kg in keeping with the dose used by others using similar compounds and this drug delivery method [332].

3.2.5.1 Rapamycin inhibits IL-33-induced lung cell infiltration

Following 5 days of intranasal IL-33 ± rapamycin, mice were killed by cardiac puncture under terminal anaesthesia and BAL was performed. Total cell counts in the BAL fluid demonstrated a robust inflammatory response to the IL-33 in keeping with previous results described (Figure 3-2). In turn, concomitant rapamycin treatment reduced the number of cells collected in the BAL by an average of 37% (Figure 3-5A) when compared to IL-33-only-treated mice. The differential cell counts of the BAL fluid revealed that rapamycin was extremely efficacious at reducing the number of macrophages and eosinophils in the BAL (Figure 3-5B). Intriguingly, rapamycin treatment significantly ($p=0.0064$) increased the number of neutrophils recruited into the lung. It must be noted that, although the effect of rapamycin on neutrophil numbers was significant, proportionally they only accounted for less than 10% of the BAL cells recovered. However, whilst the total BAL cells remained unchanged in the group treated with rapamycin only, a tendency for an increase in neutrophil numbers was observed in this group too (Figure 3-5B).

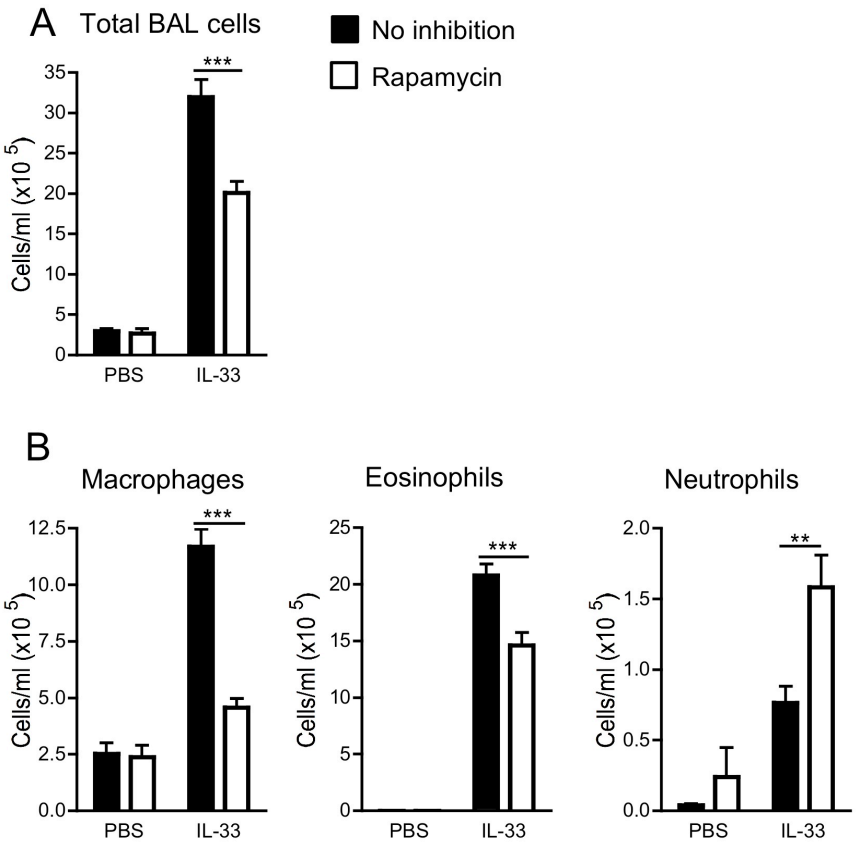

Figure 3-5 Rapamycin inhibits IL-33-induced airway inflammation and cell recruitment

BAL was performed as before and total cell counts were measured (A). Rapamycin significantly decreases the number of cells recovered in BAL fluid, compared to mice treated with IL-33 alone. mTOR inhibition significantly decreases the number of macrophages and eosinophils in the BAL fluid with an increase in BAL neutrophil numbers (B). Data representative of 2 pooled experiments with 7-9 mice per group. Error bars represent SD. **p<0.01, ***p<0.001.

3.2.5.2 mTOR inhibition reduces IL-33-induced type-2 cytokine production in the lung

One of the striking features of IL-33-induced airway inflammation is an increase in type-2 cytokines measurable in the lungs (Figure 3-3). Therefore, levels of these cytokines in BAL fluid as well as in the sera from IL-33-treated mice were measured. Similarly to the reduction in inflammatory cells in the BAL fluid, rapamycin treatment decreased the amount of both IL-5 and IL-13 present in the BAL fluid of these mice (Figure 3-6A). The effect of rapamycin appeared to be restricted to the lung, as, although intranasal administration of IL-33 resulted in increased levels of these cytokines in the sera of the treated mice, this was not significantly altered by concomitant rapamycin treatment. Although the levels of IL-13 in the sera suggest that rapamycin was able to reduce the level of this

cytokine in the blood, the amounts measured were at the very limit of the detection of the test.

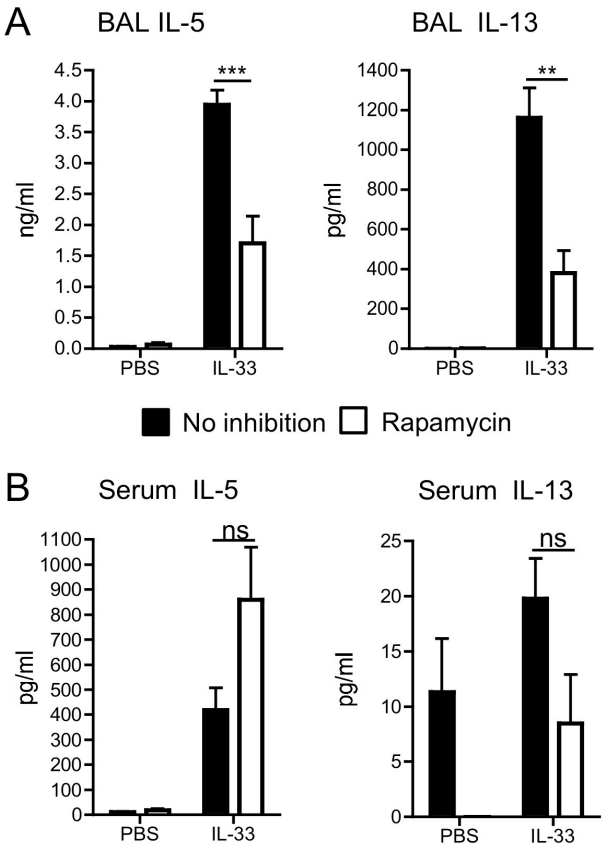

Figure 3-6 Rapamycin inhibits IL-33-induced cytokine production in the lung but does not affect IL-33-induced changes in serum cytokine levels

BAL IL-5 and IL-13 levels were measured by ELISA (A). Rapamycin decreases IL-33-mediated IL-5 and IL-13 production in the lung. Rapamycin does not affect levels of IL-5 and IL-13 induced by IL-33-treatment in the sera of mice (B). Cytokine levels were measured for individual mice with 3-5 mice per group. Data are representative of 3 experiments. Error bars represent SD. **p<0.01, ***p<0.001, ns=not significant, when IL-33+rapamycin is compared to IL-33 treatment alone.

Eotaxin-2 and MIP-1α were also measured in BAL of mice treated with IL-33 in the presence or absence of rapamycin. Whilst IL-33 had been shown to elevate the levels of these chemokines in the lung, rapamycin did not inhibit their production as measured by ELISA (Figure 3-7).

Taken together, the reduction in BAL cytokines measured in IL-33+rapamcyin-treated mice, when compared to IL-33-treated mice, further supports the hypothesis that mTOR signalling is important for IL-33-induced cytokine production *in vivo*. The results from the BAL chemokine measurements suggest,

however, that mTOR may not be essential for IL-33-driven chemokine production in this model.

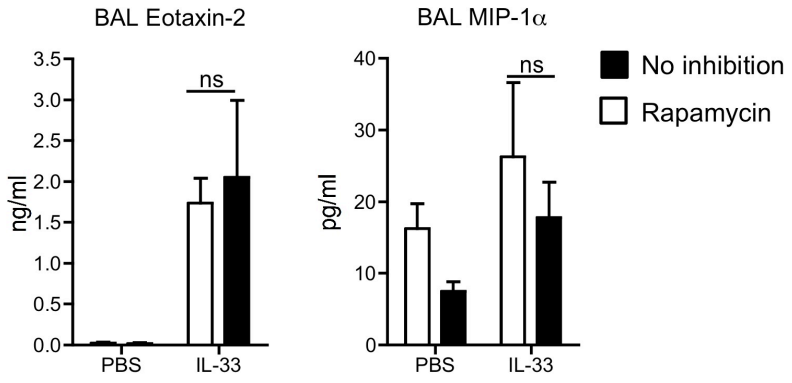

Figure 3-7 Rapamycin does not inhibit IL-33-induced lung chemokine production

Mice were treated with 5 days of intranasal IL-33 in the presence or absence of rapamycin. Eotaxin-1 and MIP-1α was measured by ELISA. BAL from individual mice was measured with 3-5 mice per group. Data are representative of 2 experiments. Error bars represent SD, ns=not significant.

3.2.5.3 Rapamycin inhibits IL-33-induced lung inflammation and mucus hypersecretion

As previously discussed, intranasal IL-33 increases cellular recruitment in the peri-bronchial and peri-vascular areas of the lung, in addition to causing mucus hypersecretion in an IL-13-dependant manner [232]. Since concomitant rapamycin treatment had been shown to reduce IL-33-driven cytokine production, experiments to assess the effect of mTOR inhibition on these histological changes in the lung were performed. Rapamycin was found to decrease IL-33-induced cell infiltration in the lung significantly ($p=0.0252$) (Figure 3-8B). Rapamycin did not abolish cell infiltration into the lung fully, and the majority of the cell recruitment was seen in the peri-vascular areas with sparing of the peri-bronchial areas (Figure 3-8A). Goblet cell hyperplasia and mucus hypersecretion was also decreased in the IL-33+rapamycin-treated mice (Figure 3-8A).

The changes in the histological appearance of the lung were scored using an arbitrary score as previously described (Figure 3-8B). Interestingly, whilst rapamycin clearly reduced cell infiltration and mucus hypersecretion (Figure 3-8B) in the lungs of IL-33-treated mice, the effect on lung architecture were

less clear. The variability observed in this histological parameter ensured that, although there was a trend towards a reduction in the architectural distortion, this did not reach statistical significance.

These results support the inhibitory effect of rapamycin on IL-33-driven inflammation, providing further evidence of the importance of mTOR signalling in IL-33-induced airway disease.

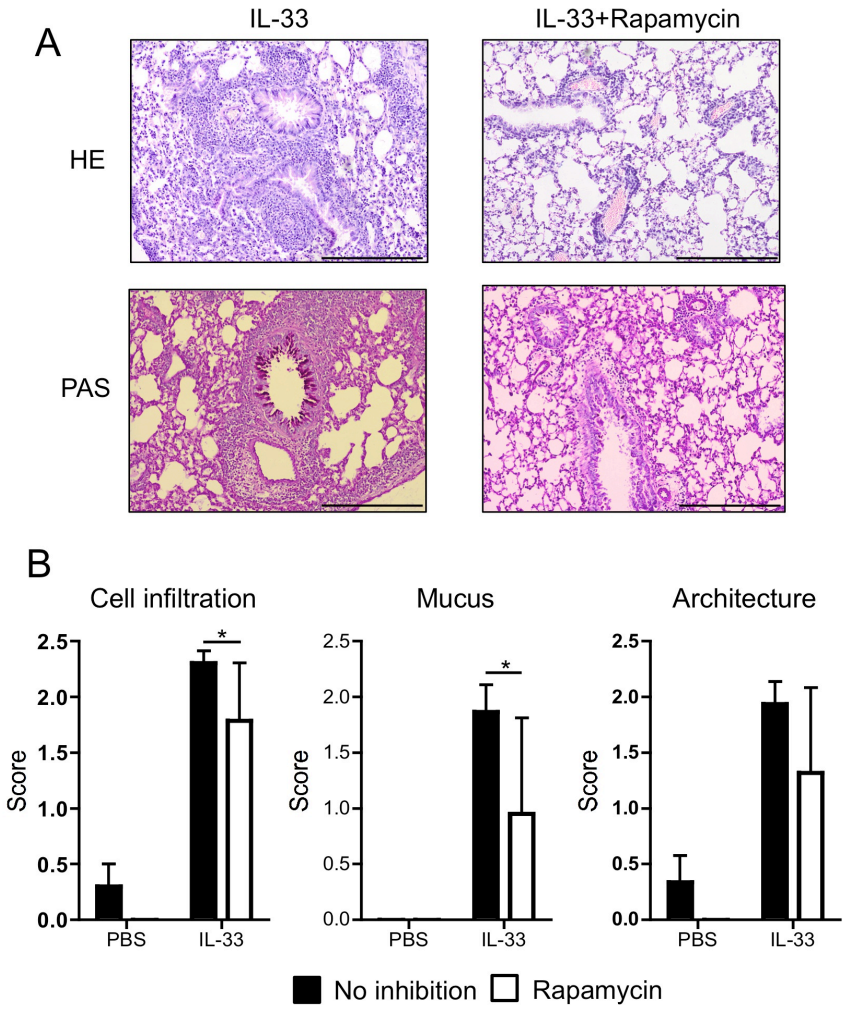

Figure 3-8 Concomitant rapamycin treatment reduces IL-33-induced lung inflammation and mucus secretion.

Cell infiltration and lung architecture were assessed as described previously on the right lobe of treated mice using HE-stained sections. Mucus staining was assessed in PAS-stained slides (A). Bar represents 200 μm. The sections are representative of each group. With the assistance of an independent assessor, lung sections were scored using a pre-defined, previously described method for cell infiltration, mucus staining and change in lung architecture (B). Data are representative of 2 pooled experiments. Error bars represent SEM of 7-9 mice per group. *p<0.05.

3.2.6 IL-33 increases lung granulocyte numbers but these are not the main source of IL-5

There are a large number of cell types present in the lung that express ST2 (Table 1-1). Since rapamycin treatment reduced IL-33-induced airway inflammation, it was important to determine which cells were responding to IL-33 in this *in vivo* model to allow further assessment of the effect of rapamycin on IL-33-induced inflammation. As demonstrated in section 3.2.3, IL-5 production in the lung is increased by IL-33 treatment. Indeed, we and others have shown this cytokine being produced by a number of different cell types *in vitro* in direct response to IL-33 [126, 173, 211, 333]. Therefore, IL-5 production by cells from the lungs was used as a marker of IL-33 responses.

The number of IL-5-producing cells in the lung digests of PBS- or IL-33-treated mice were determined by intracellular staining following stimulation by phorbol-myristate acetate and ionomycin (PMA/I) and flow cytometry. As expected from the BAL measurements, the number of IL-5^+ cells in the lung were vastly increased by IL-33 treatment (Figure 3-9A). The forward-scatter (FSC) and side-scatter (SSC) profiles demonstrated that there was a clear increase in the percentage of granulocytes in the IL-33-treated mice, in keeping with the BAL differential counts (Figure 3-9B). However, when gating on these cells, although some of these cells were IL-5^+, granulocytes did not appear to be the main source of IL-5 in the lung (Figure 3-9C).

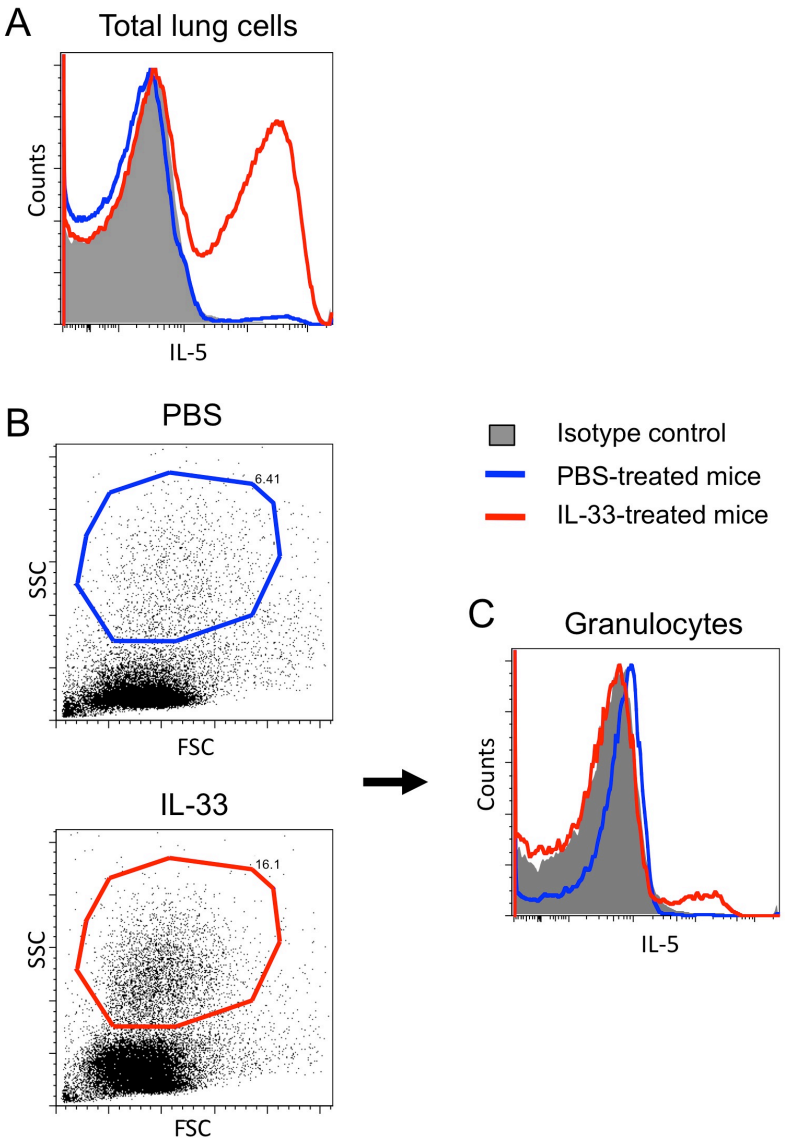

Figure 3-9 IL-5^{+} cells are increased in IL-33-treated mice but granulocytes are not the source of IL-5.

Whole lungs of PBS-or IL-33-treated mice were obtained and following mechanical disruption and chemical digestion, single cell suspensions were cultured with PMA-I+GS for 4 hours. Cells were collected and stained as previously described. The level of IL-5^{+} fluorescence in total lung cells in each group was determined (A) following exclusion of dead cells (Aqua-dye^{+}) and including single cells only (data not shown). Granulocytes were gated based on FSC and SSC characteristics in PBS- and IL-33-treated mice (B) and their expression of IL-5 was determined (C). Plots representative of each group with 3 mice per group. Data are representative of 3 experiments.

3.2.7 CD4^{-} Lymphocytes are the main source of IL-5

Since the effect of rapamycin on IL-33-induced functions had been shown in Th2 cells *in vitro* and granulocytes had been demonstrated not to be the source of IL-5 in the lung, pulmonary lymphocytes were assessed. Indeed, FSC and SSC views of whole lung digests in PBS- and IL-33-treated mice showed an additional

population in the IL-33-treated mice that were absent in the control lungs (Figure 3-10A). Gating on these cells demonstrated that these cells were capable of producing IL-5 in large quantities (Figure 3-10B) in the IL-33-treated mice. Interestingly, these cells were $CD4^-$ suggesting that they were not Th2 cells (Figure 3-10C).

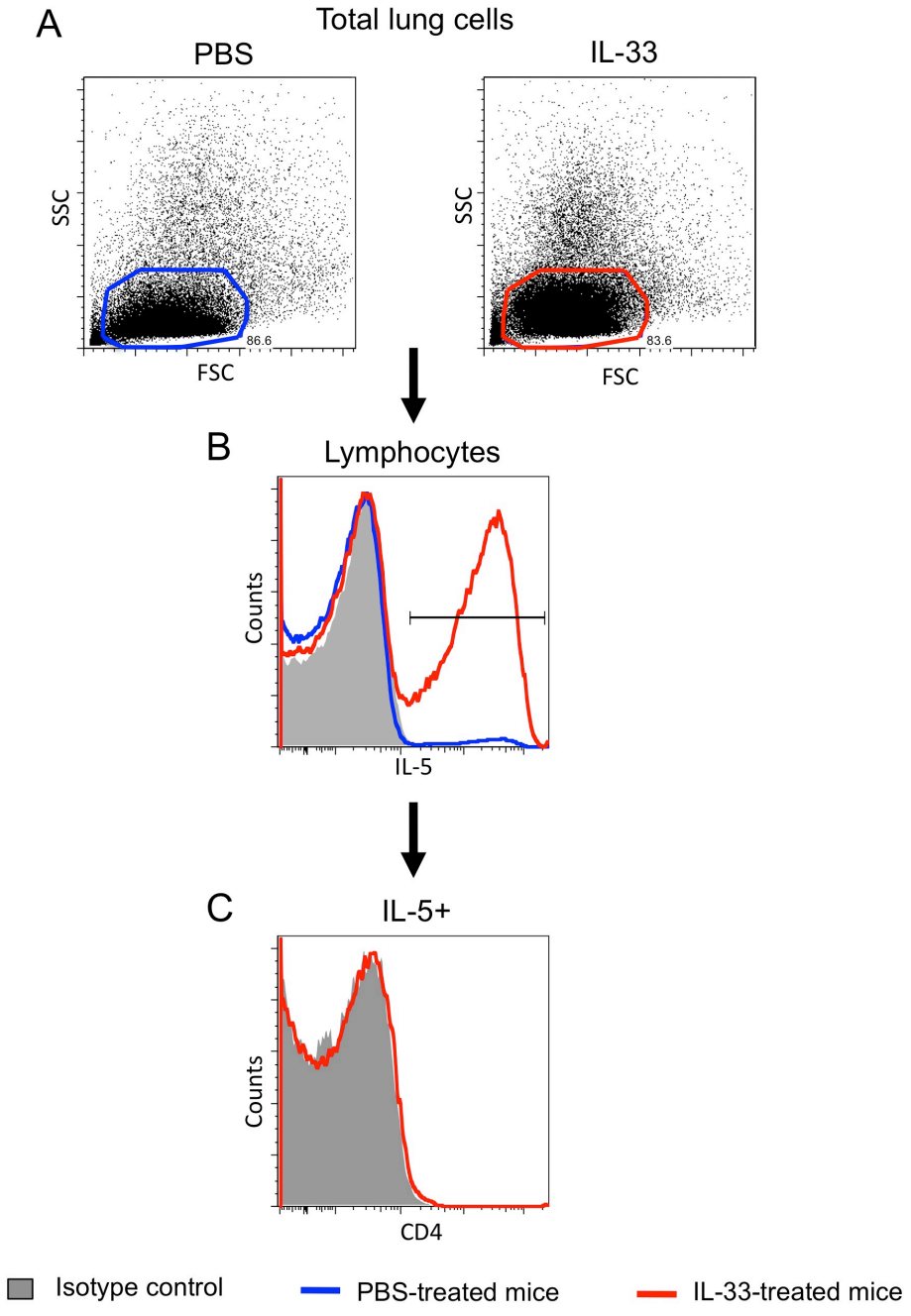

Figure 3-10 $CD4^-$ lymphocytes are the source of IL-5 in IL-33-treated mice

Total lung cells were stimulated *ex-vivo* and stained as previously described. Lymphocytes were gated based on FCS and SSC parameters on PBS- or IL-33-treated mice (A) IL-5 fluorescence in the gated lymphocytes was determined (B). Gating on $IL\text{-}5^+$ cells, their expression of CD4 was measured (C). Panels are representative of each group with 1-3 mice per group. Data are representative of 2 experiments.

3.2.8 Lineage negative lymphoid cells are the main source of IL-5 and IL-13 in the lung in IL-33-induced airway inflammation

Several recent publications have described novel innate lymphoid cell (ILC)-types which are induced by IL-25 (IL-17E) and IL-33 and produce type-2 cytokines [139, 213, 214]. These cells are characterised by a lack of expression of lineage-specific markers (CD3, CD11b, CD11c, Siglec F, γδTCR, FcεRI)[139] and express the IL-25 receptor, IL-17BR and ST2 as well as cell surface markers such as CD45 and ICOS. In order to ascertain if ILC were the source of IL-5 in this model, the cells were stained with a panel of antibodies following *in-vitro* stimulation with PMA/I. Sequential gating on lymphocytes, using FSC and SSC (Figure 3-11A) parameters and subsequently assessing the level of IL-5$^+$ fluorescence identified the IL-5$^+$ cells (Figure 3-11B). Using a panel of lineage surface markers as described by Neill *et al* [139], including CD3ε, B220, CD11b and FcεRI, as well as CD45, the IL-5$^+$ cells were shown to be negative for all the lineage markers used, yet were CD45$^+$ (Figure 3-11C). More so, further analysis indicated that these cells co-expressed ICOS and ST2 in keeping with previously described ILC populations [139] (Figure 3-11D).

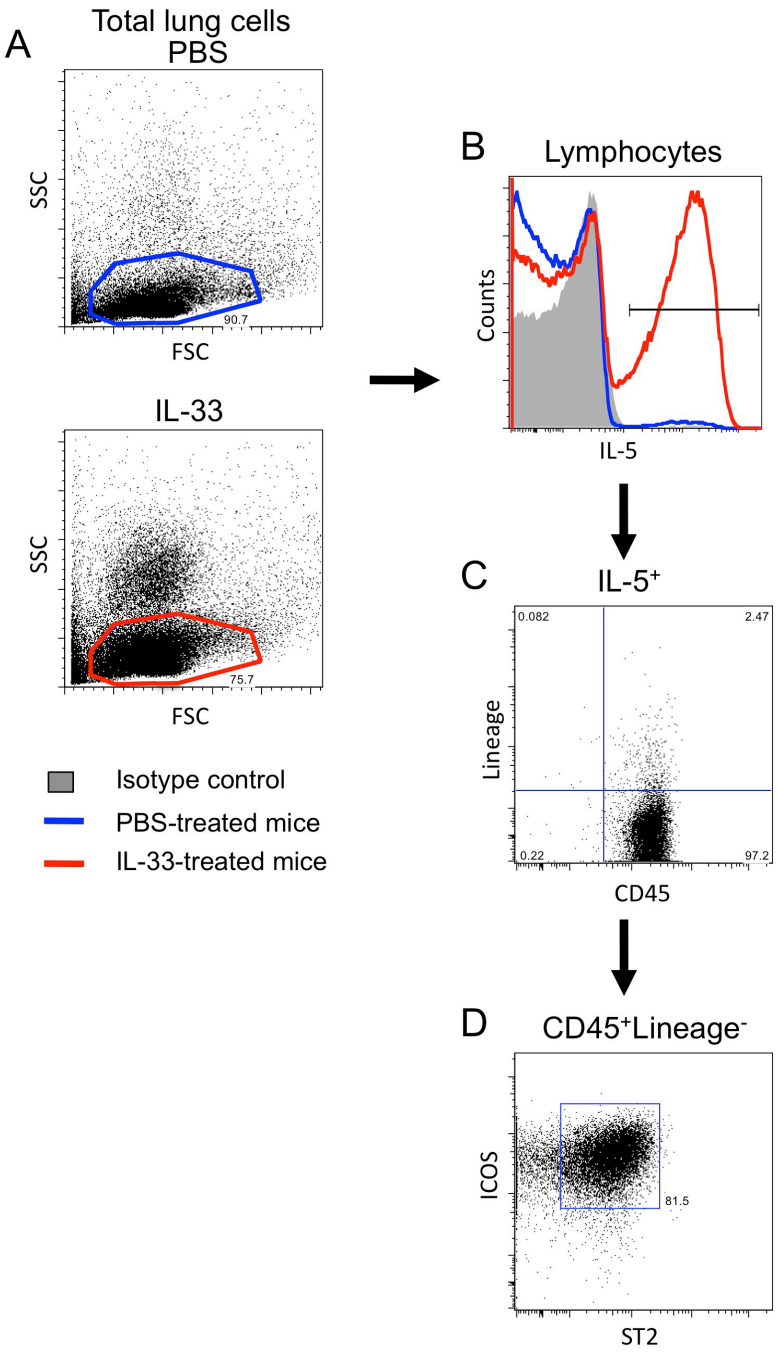

Figure 3-11 Lineage negative lymphoid cells are the main IL-5-producing cells in IL-33-induced airway inflammation

Whole lung digests were performed as before and cells were stimulated and stained as aforementioned. Lung lymphocytes were gated based on FSC and SSC characteristics in PBS-treated and IL-33-treated mice (A) after exclusion of dead cells (Aqua dye^{+}) and inclusion of single cells only. IL-5 fluorescence intensity was determined in the gated population (B) and expression of common lineage markers (CD3ε, B220, CD11b and FcεRI) and CD45 was assessed (C). Gating on lineage^{-}CD45^{+} cells, their expression of ICOS and ST2 was determined (D). Panels are representative of each group, with 3 mice per group. Data are representative of 3 experiments.

IL-33 stimulates IL-13 production by numerous cell types [44, 126, 173, 190, 203], including type 2 ILC [139, 214]. The BAL cytokine measurements

confirmed that IL-13 production in the lung was increased by intranasal administration of IL-33 (Figure 3-3A). Therefore, the role of the ILC population in IL-33-mediated IL-13 production was assessed. Using the same method as for IL-5 staining, cells from lung digests were stained for IL-13. Following gating of the lymphocyte populations (Figure 3-12A), IL-13 fluorescence levels were measured (Figure 3-12B). These experiments clearly showed that, in keeping with the IL-5 results, IL-13-producing cells in IL-33-treated mice were lymphocytic. Additionally, the vast majority of these cells were lineage$^-$CD45$^+$ (Figure 3-12C) and co-expressed ICOS and ST2 (Figure 3-12D).

These data further confirm that ILC respond to IL-33 *in vivo* to produce both IL-5 and IL-13 in the lung and are therefore an important contributor to IL-33-driven airway inflammation.

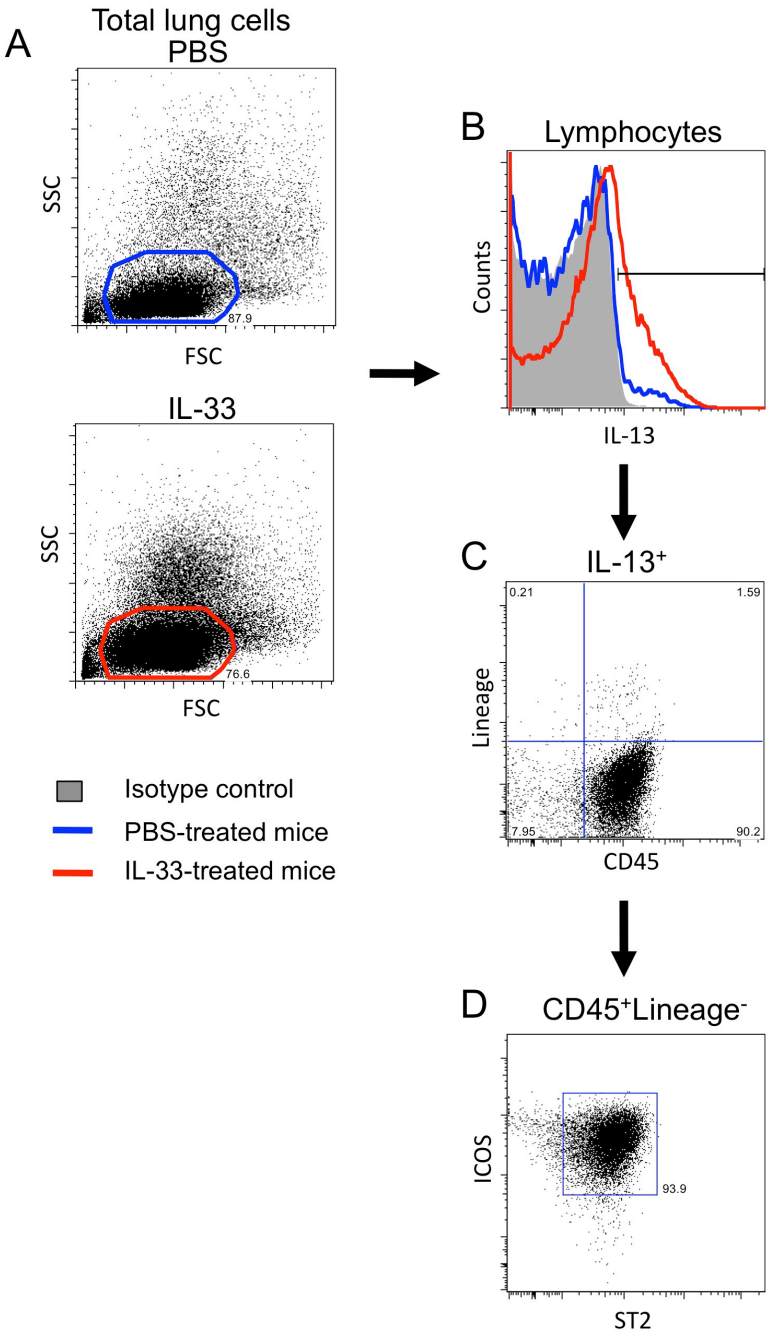

Figure 3-12 Lineage negative ILC are the main IL-13$^+$ population in IL-33-induced pulmonary inflammation

Following whole lung digestion and stimulation, cells were stained as described in Fig 2-9. Lymphocytes were gated following exclusion of dead cells (Aqua dye$^+$) and including single cells only in PBS- and IL-33-treated mice (A). IL-13 fluorescence was measured in gated lymphocytes (B). IL-13$^+$ cells are predominantly lineage$^-$CD45$^+$ (C) and ICOS$^+$ST2$^+$ (D). Data are representative of 2 experiments with 3 mice per group.

3.2.9 Intranasal rapamycin inhibits IL-33-induced ILC accumulation in the lung

Having determined the role of ILC in IL-33-induced production of type-2 cytokines in the lung, the effects of intranasal rapamycin on these cells were assessed. As before, mice were treated for 5 consecutive days with intranasal IL-33 in the presence or absence of rapamycin. The lungs were harvested on day 6 and intracellular IL-5 and IL-13 expression in ILC analysed by FACS. The results of these experiments indicated that IL-33 greatly enhanced the number of ILC in the lung, although a small number of ILC were also present in PBS-treated mice. Importantly, the number of ILC induced by IL-33 was significantly ($p=0.02$) reduced by rapamycin treatment (Figure 3-13A). Additionally, rapamycin treatment also resulted in a reduction in the absolute number of IL-5^+(Figure 3-13B) and IL-13^+(Figure 3-13C) suggesting that the reduction in cytokines by rapamycin measured in the BAL may be a consequence of reduced ILC numbers in the lung.

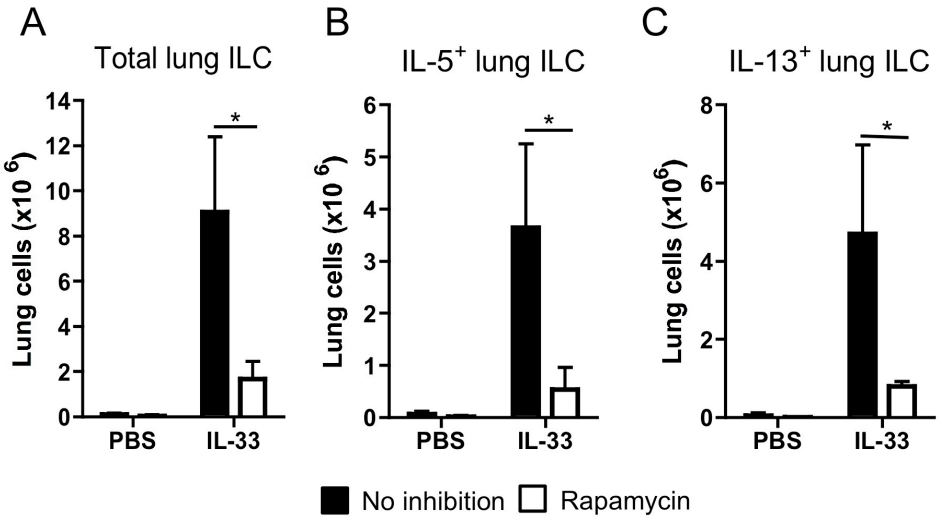

Figure 3-13 Rapamycin inhibits IL-33-induced ILC in the lung

Mice were treated with intranasal IL-33 +/- rapamycin and lung digests were performed as before. ILC were gated as single cells that were $Aqua^-Lineage^-CD45^+ST2^+ICOS^+$. Total ILC are increased in IL-33-treated mice and reduced by concomitant rapamycin treatment (A). IL-5^+ ILC (B) and IL-13^+ (C) are reduced by mTOR inhibition in IL-33-treated mice. Error bars represent SD for 3-4 mice per group. Data are representative of 5 (Total ILC and IL-5^+ ILC) and 2 (IL-13^+ ILC) experiments. *$p<0.05$.

3.2.10 IL-33 induces AHR which is not inhibited by rapamycin

Intranasal IL-33 has been shown to drive airway hyperresponsiveness (AHR) in the absence of the adaptive immune system [232]. However, the cells responsible for this effect have remained elusive until now. Since the discovery of ILC, a number of publications have demonstrated a role for these cells in respiratory function. Chang *et al* demonstrated that these cells were responsible for IL-13-driven AHR in an influenza model of airway inflammation [174]. Additionally, the role of ILC in AHR in a variety of allergic airway inflammation models, including a glycolipid-induced and an ova allergic airway inflammation model, has been shown [74, 217]. Interestingly, rapamycin has also been shown to have therapeutic effects on AHR when given systemically in a transgenic model of airway remodelling [304] and in mice in which a house-dust mite (HDM)-induced model of airway inflammation was assessed [303].

Enhanced pause (Penh) measurement is a non-invasive method using whole body plethysmography to measure AHR. Mice were administered IL-33 intranasally for 5 days in the presence or absence of rapamycin and Penh measured on day 6 following methacholine nebulisation to induce AHR. The results showed that IL-33-treated mice presented with substantial AHR in response to increasing doses of methacholine. Whilst concurrent rapamycin treatment did not have an effect on AHR at lower methacholine doses, it appeared to reduce the average Penh measured in those mice at higher challenge doses (Figure 3-14). This did not reach statistical significance. Interestingly, rapamycin-only treated mice had an enhanced response to methacholine when compared to PBS-treated mice and this difference did achieve significance ($p=0.0017$ at 5mg/ml methacholine, $p=0.00011$ at 10mg/ml methacholine, $p=0.011$ at 30mg/ml methacholine, when compared to PBS control).

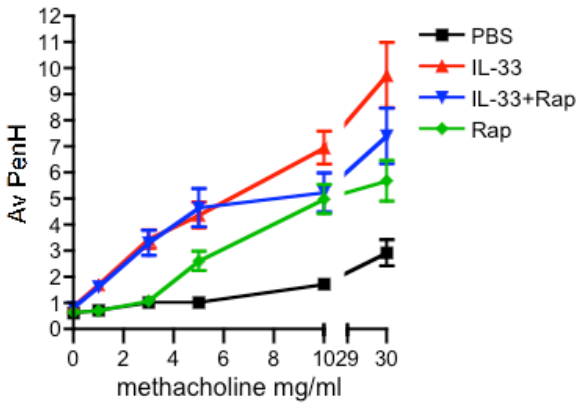

Figure 3-14 IL-33 induces increased Penh, which is not inhibited by rapamycin

Mice were treated with intranasal IL-33 +/- rapamycin for 5 consecutive days. On day 6, the mice were exposed to increasing concentrations of methacholine as discussed in chapter 2 and Penh was measured. Data represents 2 pooled experiments with 7-14 mice per group.

3.3 Conclusions

From these data it is clear that intranasal administration of IL-33 induces profound airway inflammation. IL-33 drives the recruitment of numerous inflammatory cells into the lung, which is reflected by increases in the BAL total cell number and differential cell types. Eosinophils are the main cell-type found in the BAL following IL-33 administration and are a hallmark of IL-33-induced inflammation [232]. Eosinophils have been shown to differentiate from haematopoietic precursors in the bone marrow with IL-33 in an IL-5-dependent manner [44]. Additionally, IL-33 increases expression of C-C motif receptor (CCR) 3 on eosinophils [44], a chemokine receptor involved in both cell trafficking out of the bone marrow as well as a marker of eosinophils activation [334]. CCR3 binds eotaxin-1, -2 and -3 to induce eosinophil chemotaxis [37]. Furthermore, when eotaxin-2 is given exogenously, it co-operates with IL-5 to induce IL-13 production in the lung and AHR [335]. Interestingly, the numbers of macrophages in BAL are also increased in the lung in response to IL-33. IL-33, in combination with IL-13, has been shown to induce an alternatively activated phenotype on macrophages [133]. These macrophages were able to produce eotaxin-1 and -2 and induced eosinophil recruitment in IL-33-induced airway inflammation [133]. Whilst the phenotype of the macrophages obtained in the

BAL in these studies was not ascertained, it is likely that these are alternatively activated, in keeping with previous publications.

Work in our group has demonstrated that cellular recruitment into the lung is preceded by an increase in chemokines (N. Pitman Thesis 2009). Alveolar macrophages expressing ST2 and stimulated by IL-33 have been shown to produce eotaxin-1 and -2 as well as well as thymus and activation regulated chemokine (TARC) (CCL17). Macrophages have also been shown to produce MIP-1α (CCL3) in response to IL-33, which was important for neutrophil recruitment in a model of inflammatory joint disease [172]. Mast cells, which are also found in the lung and express ST2, have been shown to produce monocyte chemotactic protein (MCP)-1 (CCL2) in response to IL-33 [336]. Whilst MCP-1 was not detected in the BAL (*data not shown*), this may be due to the time point selected for the sacrifice, which was optimised for cytokine measurement but is late for peak chemokine measurement (N. Pitman Thesis 2009). This may also explain the low, yet significantly elevated, levels of MIP-1α measured in the IL-33-treated mice when compared to control. Eotaxin-2 levels, however, remained high despite the late time point, suggesting different kinetics for the secretion of these chemokines.

In addition to enhanced cell recruitment and chemokine production, IL-5 and IL-13 production was also increased by IL-33 treatment. IL-13 has been shown to mediate IL-33-induced goblet hyperplasia and mucus production [232]. Since levels of IL-13 were found to be elevated in the BAL fluid of treated mice, it is not surprising to find substantial increases in mucus secretion, as noted by PAS staining, in their lungs. The histology further confirms the effect of IL-33 on cell recruitment, which extends beyond the alveoli and is mainly peri-bronchial and peri-vascular in nature.

Rapamycin is a specific inhibitor of mTOR that, as discussed in chapter 1, is in clinical use as an immunosuppressant. The choice of mTOR inhibitor was made on the basis that rapamycin is clinically relevant, since it is already in use in humans, therefore making it a safe drug for *in vivo* modulation of mTOR. Moreover, rapamycin targets mTOR very specifically compared to other mTOR inhibitors. In short-term studies, the effect of rapamycin on mTOR inhibition is restricted to suppression of the mTORC1 pathway. However, prolonged

treatment with rapamycin blocks all newly-formed, un-complexed mTOR, hence having an effect on the mTORC2 pathway too [263]. Whilst other mTOR inhibitors such as PP242 have recently been shown to have off-target effects on other kinases such as Janus kinase (JAK) 1, 2 and 3 [264], studies have demonstrated the exquisite specificity of rapamycin [337]. In keeping with the *in vitro* effects of mTOR inhibition on Th2 cells, rapamycin treatment significantly reduced IL-33-mediated airway inflammation. The effect of rapamycin on eosinophil numbers was substantial. This reduction may be due to the reduced amounts of IL-5 produced in the lung, which enhances survival, cell trafficking and effector functions in eosinophils [338]. Interestingly, inhibition of mTOR by rapamycin has been shown to inhibit IL-5-mediated eosinophil survival *in vitro* [339]. Rapamycin also affected the production of IL-13 in response to IL-33, which in turn inhibited goblet cell hyperplasia and mucus production in the lung, a process which has been shown to be IL-13-dependent in this model [232].

The effect of rapamycin on the number of alveolar macrophages in the lung was particularly striking. Thus, there was a marked reduction in the number of macrophages in the BAL of mice co-administered rapamycin and IL-33 as compared to mice receiving IL-33 alone. It had been believed that, being resident cells, alveolar macrophages did not proliferate in the lung. However, a recent paper challenged that view demonstrating that in the presence of a type-2 immune response, alveolar macrophages proliferated in the lung in an IL-4-dependent manner [340]. Further experiments to assess whether rapamycin affects alveolar macrophage proliferation would be interesting in view of the pathological role that IL-33-induced alternatively macrophages have been shown to play in allergic airway inflammation [133].

Interestingly, rapamycin significantly increased the number of neutrophils found in the lung. The number of neutrophils was very small by comparison to the other cell types in the BAL. The mechanism underlying the effects of rapamycin on neutrophil numbers is at present uncertain. IL-33 has been shown to enhance neutrophil migration to sites of sepsis and to prevent the down-regulation of interleukin 8 receptor beta (C-X-C chemokine receptor (CXCR) 2) expression in response to lipopolysaccharide (LPS) [138]. On the other hand, in a model of house dust mite (HDM)-induced airway inflammation, systemic rapamycin

decreased lung neutrophil recruitment [303]. MCP-1 is an important chemotacic factor for neutrophils. However this was not measurable in the BAL at the time of sacrifice. This may be due to the kinetics of IL-33-induced chemokine production in the lung (N Pitman thesis, 2009). In order to ascertain in the increase in neutrophils in response to rapamycin is due to an effect on chemokine secretion, measurements at earlier time-points could prove insightful. Interestingly, a number of groups investigating the anti-rejection properties of rapamycin in transplant models in rats have shown a reduction in chemokine secretion with rapamycin [341], suggesting that this may not be the mechanism for this observation. An additional candidate would be IL-8 (C-X-C chemokine ligand (CXCL) 8), which is produced in the lung by human epithelial cells (*data not shown*) as well as eosinophils [135], basophils [130] and endothelial cells [342] in response to IL-33 and is also elevated in IL-33-induced airway inflammation (N Pitman thesis, 2009). IL-8 is a chemoattractant for neutrophils [343] and analysis of the effect of rapamycin on IL-33-induced IL-8 levels in the lungs would be an important experiment to clarify this result.

Whilst rapamycin reduced the production of cytokines in the lung significantly, it did not affect the levels of any of the chemokines measured. The levels of MIP-1α were very low and within the limits of the detection of the ELISA kit. However, the levels of eotaxin-2, however, remained high despite rapamycin. Whilst the timing for measurement of IL-33-induced chemokines was not optimal as previously discussed, the lack of effect of rapamycin on eotaxin-2 levels suggests that these IL-33-driven effects may not be mTOR-dependant. Further experiments performed during optimised timepoints would be beneficial to clarify this.

As discussed in chapter 1, there are a large number of cells in the lung that express ST2 and are hence IL-33-responsive (Table 1-1). Using multi-parameter FACS, it appeared that neither granulocytes nor $CD4^+$ T cells were the main source of IL-5 and IL-13 in IL-33-treated lungs. The recent description of IL-33 responsive fat-[213] and gut-associated [139] ILC suggested the possibility that these novel cells could be important in IL-33-induced airway inflammation. These cells were negative for common lineage markers but expressed a small number of other surface markers, including ST2, IL-17BR, CD45 and ICOS [139].

Using a combination of lineage markers previously described [139], the IL-5- and IL13-producing cells in the lung were found to indeed be lineage-negative and, intranasal rapamycin administration significantly reduced the numbers of ILC found in the lung. The mechanism for this reduction was unclear and a number of possibilities existed. The effects of mTOR inhibition on the proliferation of numerous cell types have been investigated, including B cells [344], NK cells [345] and T cells [346]. The possibility of rapamycin having a similarly direct effect on ILC function is the focus of the experiments described in Chapter 4. In the future, experiments investigating whether ILC are resident cells which proliferate *in situ*, similarly to alveolar macrophages, or that are recruited from extrapulmonary sites would be insightful.

The effect of rapamycin on IL-33-induced cytokine production in the lung might be sufficient to explain the significant reduction in ILC in the lung. Experiments described in Chapter 4 aimed to clarify whether mTOR inhibition has a direct effect on ILC or whether a reduction in total numbers solely accounts for the effect of rapamycin on BAL cytokine levels. The reduction in cytokines measured in the lung during concomitant rapamycin treatment is significant, yet not complete. Whilst rapamycin has been shown to affect mTORC2 pathways *in vitro,* rapamycin derivatives also appear to be able to reduce mTORC2 signalling in leukaemic cells *in vivo* [347]. *In vitro* data using Th2 cells has demonstrated that IL-33 phosphorylates both S6K1 and Akt, suggesting both mTORC1 and mTORC2 are activated [75]. The effect of rapamycin in inhibiting mTORC2 *in vivo* in this model was not assessed and further experiments using either mTOR inhibitors such as Torin-1, which inhibits both mTORC1 and mTORC2 [264] would be useful to assess whether the incomplete effect of rapamycin was due to suboptimal mTORC2 inhibition. An additional explanation for these data could result from suboptimal dosing of rapamycin and additional experiments to assess whether increased doses could further reduce the cytokine production could be informative.

Systemic rapamycin was recently shown to inhibit AHR in a transgenic airway inflammation model [304], as well as in a HDM-induced model of allergic airways disease [303]. Since AHR in IL-33-induced airway inflammation had been shown to be dependent on IL-13 [232] and rapamycin appeared to inhibit its production in the lung, it seemed plausible that mTOR inhibition would be sufficient to

reduce IL-33-induced AHR. More so, ILC induced in a model of pulmonary influenza infection had been shown to drive AHR [174] and in our model, rapamycin was able to reduce the number of ILC in the lung. In combination, these results hinted at the possibility of rapamycin being sufficient to reduce IL-33-induced AHR. From these results it is clear that IL-33 increases the Penh value in mice, compared to control, however there appears to be no difference when mice are also treated with rapamycin. Additionally, mice receiving rapamycin alone have higher Penh than control mice. This suggests that rapamycin may be having an effect in the lungs, driving AHR. Whilst this is unexpected, there are a number of possible causes to explain the results. Firstly, when comparing these results to those from other groups assessing the effect of rapamycin on AHR, the method of delivery differ [303, 304]. It may be that direct inoculation of rapamycin in the lung has a toxic effect driving AHR. Whilst there were no obvious changes noted in the histology of rapamycin-only treated mice, subtle abnormalities could be missed.

A potentially important caveat to the analyses of AHR in IL-33-treated mice is the fact that several groups have questioned the reliability of Penh as a measure of hyperresponsiveness. The measurement of Penh was first described by Hammelman *et al.*[94] as a non-invasive method to measure AHR in mice. In this paper, the authors demonstrated the correlation of enhanced pause with changes in pleural pressure during AHR as well as an increase in Penh in both an allergic model of airway inflammation and in response to methacholine Since the publication of this paper, numerous groups have used this method of whole body plethysmography (WBP) to measure AHR in mice [348, 349]. Despite its widespread use, Penh has increasingly been criticised by a variety of groups questioning the value of this derived measurement as a measure of airway reactivity. The main question posed by these authors is whether Penh is really derived from a respiratory parameter at all. The pressure changes induced by a change in the airway calibre are extremely small and many feel that deriving a number such as Penh from this flux is not scientifically robust. This then has a knock-on effect on all numbers derived thereafter, including the comparison of changes of Penh from baseline, putting into question the value of Penh as a measure of AHR [96]. Unfortunately due to Home Office constraints and local expertise, measurement by Penh was the only available option. In future

experiments, it would therefore be ideal to measure AHR in IL-33-treated mice by measuring airway resistance invasively in the gold-standard fashion [93] to make robust conclusions on this matter.

In conclusion, data in this chapter demonstrate that IL-33 induces profound airway inflammation characterised by increased chemokine and type-2 cytokine secretion. Additionally, ILC are the main source of IL-33-driven lung IL-5 and IL-13 production. Intranasal treatment of mice receiving both IL-33 and rapamycin have substantially less cellular infiltration, cytokine amounts and ILC in the lung, pointing at an important role for mTOR signalling in IL-33-driven biological effects *in vivo*.

4 The role of mTOR in IL-33-induced innate lymphoid cell functions *in vitro* and *in vivo*

4.1 Introduction

In chapter 3 it was shown that the mTOR inhibitor rapamycin inhibits IL-33-induced airway inflammation, suggesting a role for mTOR signalling in the *in vivo* functions of IL-33. The reduction in cytokine production observed in the bronchoalveolar lavage (BAL) was mirrored by significantly fewer type-2 innate lymphoid cells (ILC) in the lung. Since ILC were demonstrated to be the main producers of IL-5 and IL-13 in the lung under the influence of IL-33, it was possible that the inhibitory effect of rapamycin on BAL cytokines was indirect, via a reduction in ILC numbers, rather than a direct inhibition of mTOR signalling induced by IL-33. Experiments described in this chapter were designed to answer this question. ILC from IL-33-treated lungs were isolated and *in vitro* experiments were performed to assess the effects of rapamycin on IL-33-induced effects directly. Experiments in this chapter also assessed the biological effects of IL-33 on ILC *in vitro* and the importance of mTOR activation in these processes. Finally, the contribution of ILC to IL-33-induced airway inflammation was determined using an adoptive transfer model into $ST2^{-/-}$ mice in order to isolate the effects of IL-33-stimulated ILC *in vivo*.

4.2 Results

4.2.1 ILC sorting from IL-33-treated lungs

Using multi-parameter FACS, ILC were isolated from lungs. Since the number of ILC found in naive mice had been shown to be very small (Figure 3-13) the decision to use mice treated with IL-33, as described in Chapter 3, to induce a larger population of ILC, was made. Following lung digestion, single cell suspensions were incubated in complete medium (CM) for 1 hour to remove adherent cells, such as macrophages and epithelial cells. The cells were subsequently stained with a panel of antibodies as previously described [139] and sorted using a BD FACS Aria flow cytometer following the gating strategy shown (Figure 4-1A). Post-sort purity was checked and shown to routinely be >96% (Figure 4-1B).

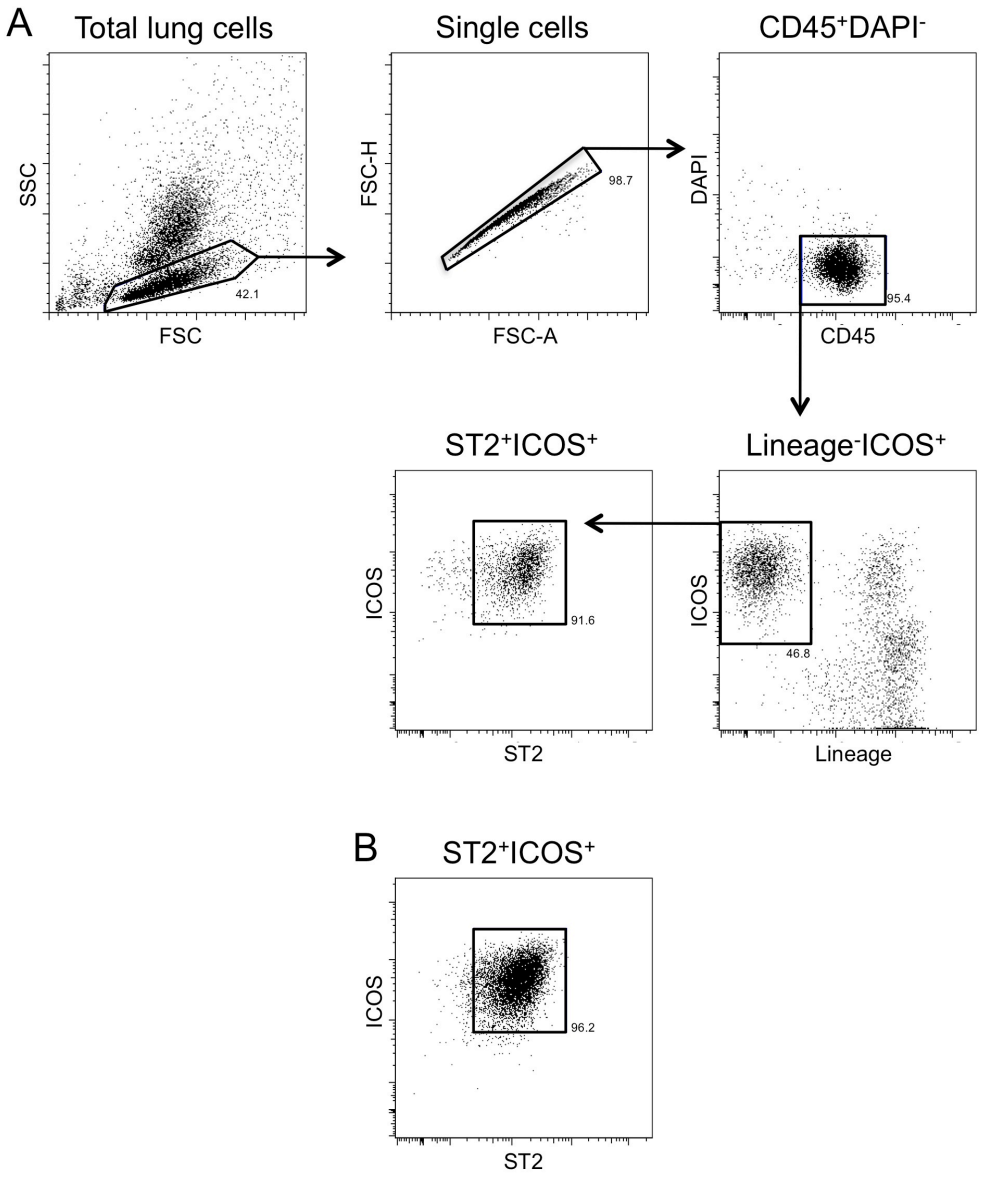

Figure 4-1 Gating strategy for ILC sort

BALB/c mice were treated with 1μg IL-33 for 5 days. Lungs (3-4) were digested as before and adherent cells were removed. Cells were stained with FITC ST2, PE Lineage (CD3, FCεRI, B220, CD11b), PerCP-Cy5.5 ICOS, AF700 CD45 and V450 DAPI. Cells were gated (A) and post-sort purity was checked (B).

4.2.2 Characterisation of sorted ILC

ILC populations have been described by a number of different groups. The evidence suggests that these populations are very phenotypically similar and are probably the same cell type. However subtle differences in surface marker expression have been shown [74, 139, 213, 214] and hence the surface markers expressed by sorted ILC were determined. During the sorting process it was

established that IL-33-induced lung ILC did not express CD3, B220, CD11b and FCεRI. Additionally, freshly sorted ILC did not express CD11c, NK1.1, TCRγδ or TCRβ (Figure 4-2A). These results are in keeping with the surface phenotypes of described ILC[74, 139, 213]. Additionally, ILC did express a number of surface markers in addition to ST2, ICOS and CD45 (surface markers used for gating strategy). These included CD25, CD127 and c-Kit (Figure 4-2B).

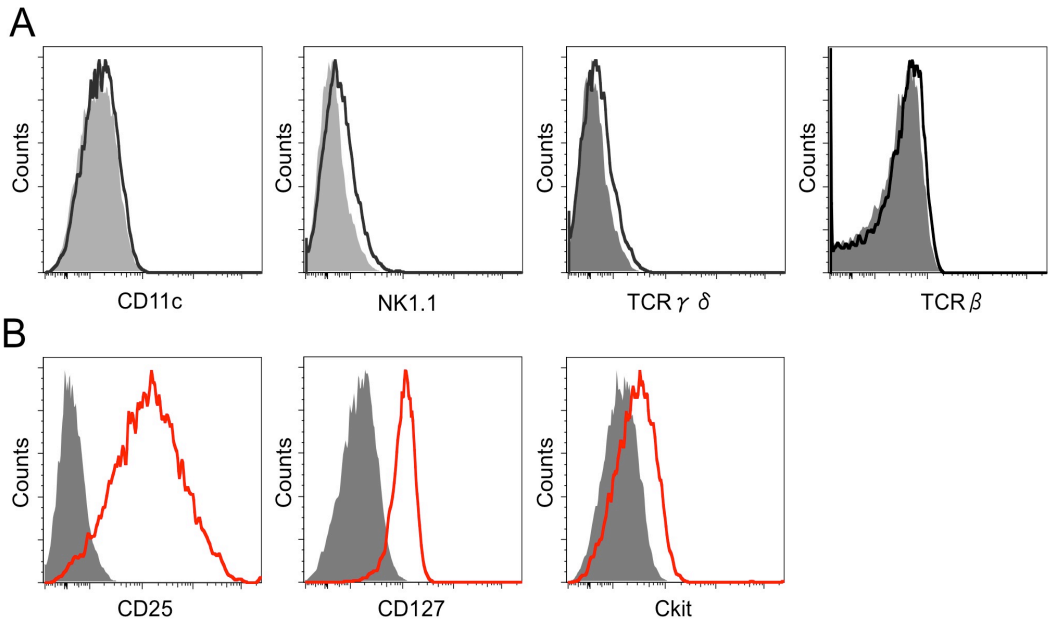

Figure 4-2 ILC surface markers

ILC were sorted as previously described. Expression of specific lineage markers were measured on freshly sorted ILC (A). Expression of additional surface receptors was also measured (B). Filled gray histograms represent isotype controls, ILC represented in solid black line (A) or red (B).

4.2.3 The effect of IL-33 on ILC in vitro

4.2.3.1 IL-33 induces IL-5 and IL-13 production from ILC

From the FACS data shown in Chapter 3 it was clear that ILC represented the major IL-5- and IL-13-producing cell type present in the lungs of IL-33-treated mice. Therefore, initial experiments were performed in order to confirm the ability of ILC to respond directly to IL-33. The sorted ILC were rested overnight in complete medium (CM) alone prior to stimulation as initial experiments using freshly sorted cells demonstrated that their baseline cytokine production remained very high when measured shortly after the sort (*data not shown*). This was likely to be a consequence of their recent exposure *in vivo* to large amounts

of IL-33 in the previous 24 hours. Following resting, ILC were cultured with varying amounts of IL-33 for 24h and IL-4, IL-5 and IL-13 in culture supernatants were measured. ILC produced substantial amounts of IL-5 (Figure 4-3A) and IL-13 (Figure 4-3B) in response to even small amounts of IL-33. No IL-4 was detected (*data not shown*). From these data, it appeared that 1 ng/ml IL-33 was sufficient to drive peak IL-13 production from ILC. However, the amount of IL-5 production continued to increase with incremental doses. Based on these results, the decision to use 10 ng/ml IL-33 for *in vitro* ILC stimulation was made.

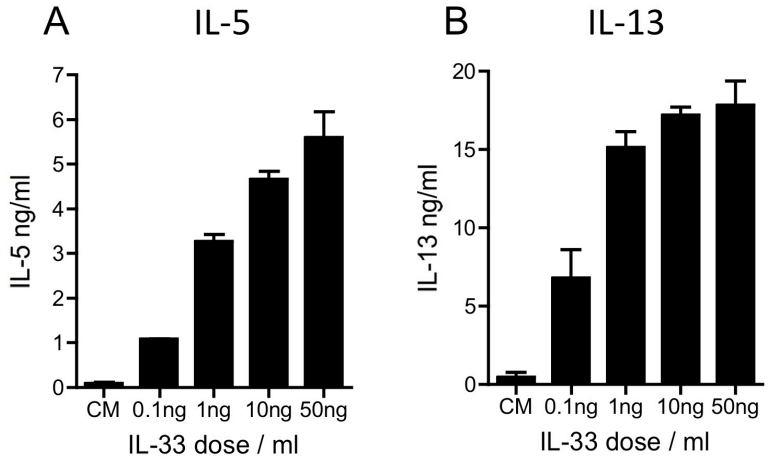

Figure 4-3 Incremental doses of IL-33 induce increasing amounts of type-2 cytokines from ILC

Rested sorted ILC ($1x10^5$) were cultured with incremental doses of recombinant IL-33 in triplicate and supernatants were collected after 72hrs. IL-5 (A) and IL-13 (B) were measured by ELISA. Error bars represent SD for experimental triplicates. CM=complete medium.

4.2.3.2 IL-33 induces p38 phosphorylation and IκB-α degradation

The IL-33-induced signalling pathway has been extensively investigated in numerous cell-types [82, 112, 117, 188-190, 350]. Similar to other members of the IL-1 family of cytokines, IL-33 recruits myeloid differentiation primary response gene 88 (MyD88) and induces phosphorylation of mitogen-activated protein (MAP) kinases, including p38 and also induces inhibitor of kappa B-alpha (IκB-α) degradation, leading to nuclear factor kappa B (NFκB) activation [82]. In order to ascertain if IL-33 also induced activation of these canonical pathways in ILC, these cells were treated with 10 ng/ml IL-33 for the described times and lysates prepared for western blot (WB) analysis of phosphorylated p38 (pp38)

(Figure 4-4A) and IκB-α degradation (Figure 4-4B). IL-33 treatment resulted in elevated levels of p38 phosphorylation that increased from 5 minutes to 30 minutes stimulation and had started to decrease by 60 minutes. IL-33-induced IκB-α degradation appeared to peak at 30 minutes. From the data obtained, it was clear that IL-33 induced these canonical pathways in ILC, in keeping with data from other cell types.

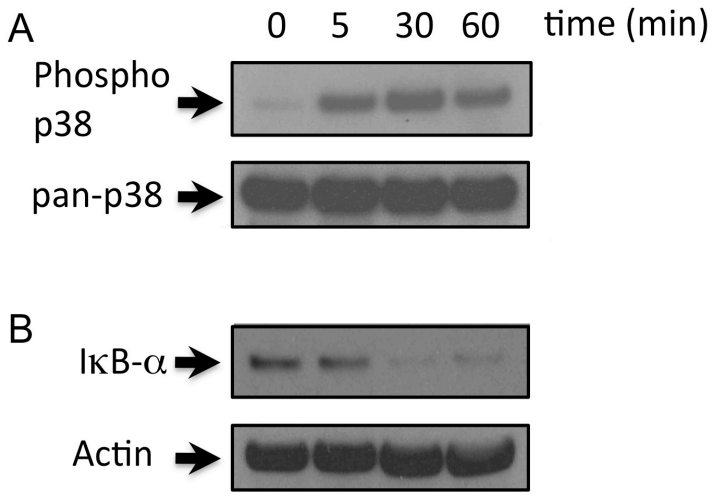

Figure 4-4 IL-33 induces p38 phosphorylation and IκB-α degradation

Sorted ILC (5 x 10^5 cells) were treated for the indicated time with 10ng/ml IL-33. Cell lysates were collected as previously described and WB performed blotting for phosphorylated p38 (A) and pan-p38 for loading control. Blots were stripped and re-blotted for IκB-α (B) with actin as loading control.

4.2.4 The effect of rapamycin on IL-33-induced ILC function in vitro

The above data confirmed that IL-33 directly stimulated cytokine production and activation of the IL-33 canonical pathway in isolated ILC. It was therefore important to determine the role of mTOR activation in IL-33-dependent ILC responses *in vitro*.

4.2.4.1 Rapamycin inhibits IL-33-induced S6 phosphorylation but does not affect IκB-α degradation

The role of mTOR in IL-33-induced ILC activation was assessed first by measuring S6 phosphorylation levels in IL-33-treated ILC in the presence or absence of the

mTOR inhibitor, rapamycin. As discussed in Chapter 1, mTORC1 activation leads to phosphorylation of S6 kinase 1 (S6K), which in turns phosphorylates the ribosomal protein S6 (S6). S6 phosphorylation has therefore been widely used as a marker of mTORC1 activity [253]. Whilst previous experiments using Th2 cells [75] had demonstrated increased S6 phosphorylation induced by IL-33, it was important to establish whether IL-33 was able to enhance S6 phosphorylation in ILC. *Ex-vivo* ILC were therefore pre-treated with rapamycin (100nM) for 30 minutes prior to stimulation followed by IL-33 for 60 minutes. Whole cell lysates were prepared and WB performed, blotting for phosphorylated S6 (pS6) (Ser 240/244) and actin as a loading control (Figure 4-5A). The data shown demonstrated that whilst IL-33 induced S6 phosphorylation, this effect was blocked by rapamycin. This experiment confirmed that IL-33 induces S6 phosphorylation in ILC in an mTOR-dependent manner.

As previously discussed (Chapter 1), the inhibitory effects of rapamycin on mTOR have been shown to be highly specific [264]. Nonetheless, the effect of rapamycin on IL-33-induced IκB-α degradation was ascertained, to ensure that any effects of rapamycin on IL-33-induced functions were direct effects of mTOR inhibition only. ILC were therefore pre-treated with rapamycin (100nM) for 30 minutes, as before, and stimulated with IL-33 for the indicated times. WB for IκB-α was performed on the cell lysates collected (Figure 4-5B). The blot was then stripped and re-blotted for actin. The data showed that rapamycin did not affect levels of IκB-α degradation indicating that whilst rapamycin inhibits IL-33-induced mTOR activation, it does not affect additional signalling pathways induced simultaneously by IL-33 in ILC.

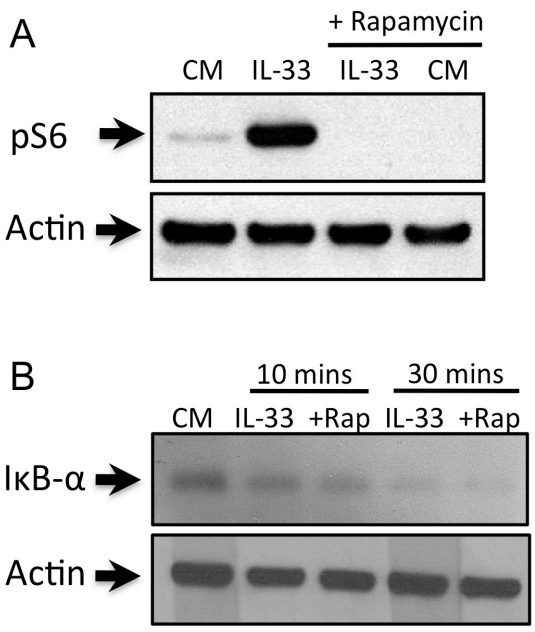

Figure 4-5 Rapamycin inhibits IL-33-induced S6 phosphorylation but not IκB-α degradation

Ex-vivo ILC (1 x10^5) were rested overnight and pre-treated with rapamycin (100nM) for 30 minutes prior to IL-33 stimulation (10ng/ml) for 60 minutes. Cell lysates were collected and WB for S6 (Ser 240-244) was performed, followed by re-blotting for actin (A). ILC were pre-treated with rapamycin as before and treated with IL-33 for the indicated time. Cell lysates were collected and WB performed. This was blotted for IκB-α and subsequently re-blotted for actin (B). Blots are representative of 3 (S6) and 2 (IκB-α) experiments. CM=complete medium.

4.2.4.2 Rapamycin inhibits IL-33-induced cytokine production in ILC

Whilst the above results confirmed that mTOR activation occurred in ILC in response to IL-33 and that rapamycin was able to inhibit S6 phosphorylation, the biological importance of mTOR activation by IL-33 in ILC functions was yet to be determined. In order to clarify this, *ex-vivo* ILC were stimulated with 10ng/ml IL-33 in the presence or the absence of 100nM rapamycin for 24 hours. Supernatants were collected and cytokine levels were measured by ELISA. As before, ILC produced large amounts of IL-5 and IL-13 (Figure 4-6A) in response to IL-33. Importantly, rapamycin significantly inhibited the amounts of IL-5 ($p=0.00074$) and IL-13 ($p=0.00069$) in the supernatants, reducing the levels of IL-5 and IL-13 by 40%, demonstrating a key role for mTOR in IL-33-induced cytokine production.

In addition to the well-described ability of ILC to produce type-2 cytokines, it has also been demonstrated that these cells are capable of producing IL-6 and granulocyte monocyte-colony stimulating factor (GM-CSF) [139]. Therefore, the effects of IL-33 on production of these cytokines was assessed. Not only was IL-33 able to drive ILC to produce IL-6 and GM-CSF, but rapamycin was shown to inhibit these processes in ILC (Figure 4-6B) with a reduction of 50% in IL-6 production and 40% GM-CSF production from the cells. However, the levels of both these factors were substantially lower than those measured for the type-2 cytokines.

These data conclusively demonstrate the vital role for mTOR signalling in ILC IL-33-induced cytokine production.

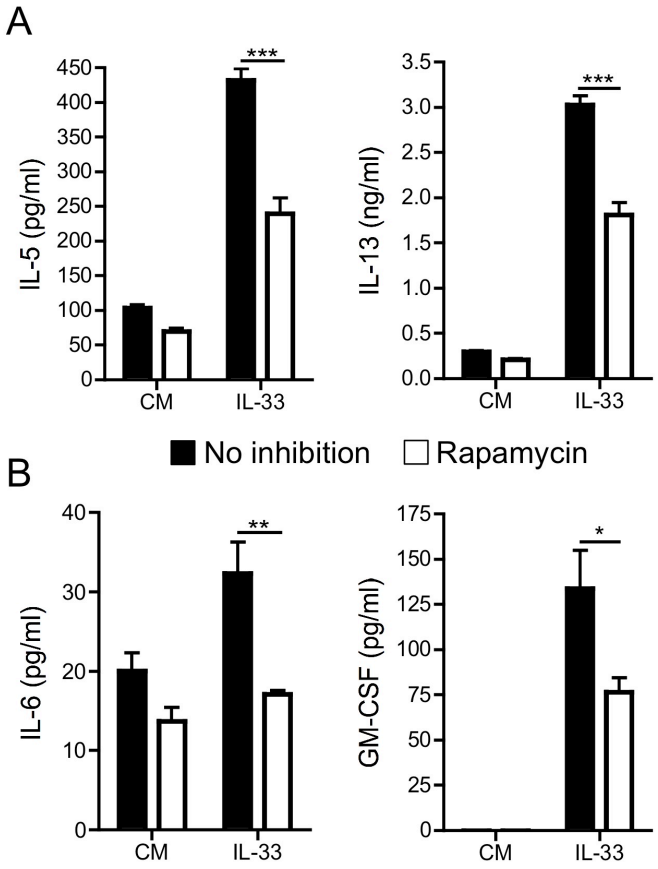

Figure 4-6 ILC produce cytokines in response to IL-33 in an mTOR-dependent manner

ILC were rested overnight and stimulated *in vitro* with IL-33 (10ng/ml) for 24 hours. Supernatants were collected and type 2 cytokines (A), IL-6 and GM-CSF (B) were measured by ELISA. Error bars represent SD from experimental triplicates. Data are representative of 3 separate experiments. *p<0.05, **p<0.01, ***p<0.001. CM=complete medium.

4.2.4.3 Rapamycin does not inhibit IL-33-induced cytokine message ribonucleic acid (mRNA) production

Since it was clear that rapamycin partially inhibited IL-33-induced cytokine production, the mechanism for this effect was sought. mTOR regulates protein synthesis via altering ribosomal biogenesis as well as by regulating rates of mRNA translation [351]. In order to determine whether rapamycin regulated type-2 cytokine mRNA levels in ILC, the levels of *Il5* (Figure 4-7A) and *Il13* (Figure 4-7B) were measured in ILC stimulated with 10ng/ml IL-33 for 4 hours. Cells were stimulated with IL-33 in the presence or absence of rapamycin and pelleted. The cell pellets were flash-frozen in liquid nitrogen followed by RNA extraction. Complementary deoxyribonucleic acid (cDNA) was prepared by reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR) was performed using specific Taqman probes for each cytokine. qPCR measurement of hypoxanthine phophoribosyltransferase (*Hprt*) levels was also performed to allow comparison of the cytokine mRNA levels with that of a stable 'house-keeping' gene. The data showed that rapamycin did not impact upon either basal or IL-33 induced levels of *Il5* or *Il13* mRNA. This suggests that mTOR primarily functions to regulate ILC cytokine expression at a post-transcriptional level.

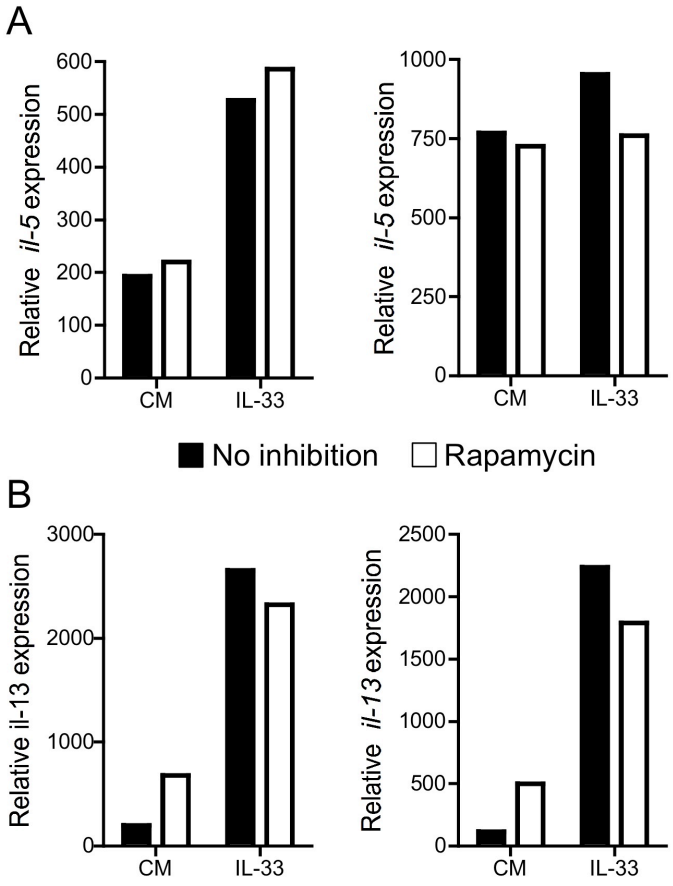

Figure 4-7 IL-33 induces *Il-5* and *Il-13 mRNA* production in an mTOR-independent manner.

ILC were stimulated with IL-33 for 4 hours. Cell pellets were obtained and flash frozen for *RNA* extraction as described before. cDNA was generated by reverse transcription polymerase chain reaction (RT-PCR) and levels of *Il5* (A) and *Il13* (B) were measured by quantitative PCR using Taqman probes and *Hprt* levels as a control. Data show 2 separate experiments with relative values calculated from technical triplicates of each sample. CM=complete medium.

4.2.4.4 IL-33 does not induce ILC proliferation in vitro

IL-33 vastly increased the number of ILC in the lung *in vivo* and rapamycin profoundly reduced their number when given concomitantly (Chapter 3). A possible reason for this was the possibility that IL-33 directly induced ILC proliferation in an mTOR-dependent manner. In order to test this possibility, ILC were cultured with IL-33 ± rapamycin for 72 hours. Proliferation was assessed by measuring incorporation of ^{3}H-Thymidine during the final 16 hours of culture. Whilst IL-33 appeared to enhance the amount of ^{3}H-Thymidine incorporation (Figure 4-8), this did not reach statistical significance. These data demonstrate that IL-33 did not induce ILC proliferation *in vitro*.

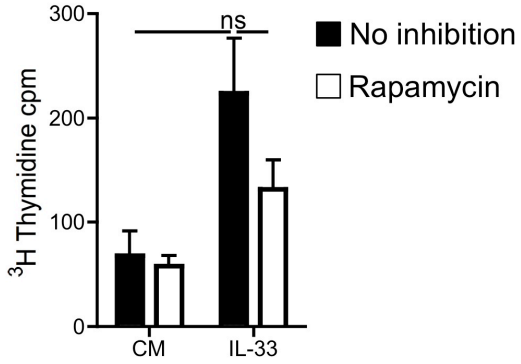

Figure 4-8 ILC do not proliferate in response to IL-33 *in vitro*

ILC were cultured with IL-33 in the presence or absence of rapamycin for 72 hours. They were pulsed with ^{3}H-Thymidine for the final 16 hours of culture. Thymidine incorporation was measured using a beta counter as described in Chapter 2. Error bars represent SD of experimental triplicates. Data are representative of 3 experiments. CM=complete medium.

4.2.4.5 IL-7 and Thymic stromal lymphopoietin (TSLP) synergise with IL-33 to induce ILC cytokine production

One of the first descriptions of ILC demonstrated that these cells expressed CD127 (IL-7Rα) [139] and this was also demonstrated in *ex-vivo* sorted ILC (Figure 4-9B). CD127 is important for biological responses to both IL-7 and TSLP [352]. Interestingly, whilst IL-7 has been shown to be important in lymphocyte development [353], TSLP has been shown by numerous groups to be involved in allergic processes [69, 72, 354], similarly to IL-33. Therefore, the effects of both IL-7 and TSLP on ILC function were determined. ILC were treated in vitro with combinations of IL-33 ± IL-7 or TSLP in the presence or absence of rapamycin. Whilst individually each of the cytokines induced type-2 cytokine production by ILC, the synergistic effect of IL-33 with either IL-7 or TSLP on IL-5 (Figure 4-9A) and IL-13 (Figure 4-9B) production was substantial. Interestingly, this effect was more marked with TSLP activation than with IL-7. Importantly, treatment of ILC with rapamycin strongly inhibited the induction of IL-5 and IL-13 production by IL-33, IL-7 and TSLP alone or by combinations of these cytokines (Figure 4-9). These data demonstrate the important role of mTOR signalling in cytokine production induced by other cytokine pathways in ILC as well as interesting synergistic effects of IL-33 with other innate cytokines.

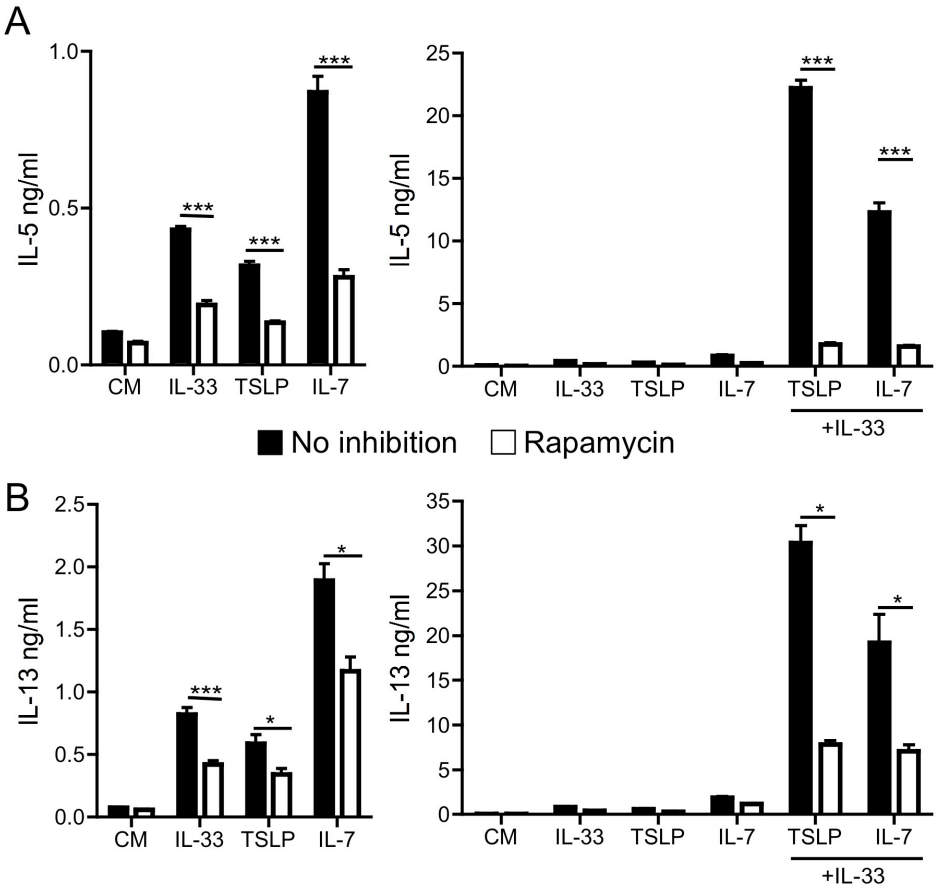

Figure 4-9 IL-33 synergises with TSLP and IL-7 to induce ILC type-2 cytokine production *in vitro*

Ex-vivo ILC were sorted as before and rested overnight. The cells were stimulated with the indicated cytokines (10ng/ml) in the presence or absence of rapamycin (100nM) and supernatants were collected. IL-5(A) and IL-13(B) were measured by ELISA. Error bars represent SD for experimental triplicates. Data are representative of 3 experiments. Left panels represent single cytokine stimulations. Data in right panels demonstrate both single (as per left panels) and combined cytokine stimulations (note different scales in left and right panels). *p<0.05, ***p<0.001. CM=complete medium.

4.2.4.6 IL-7 and TSLP induce ILC proliferation in an mTOR-dependent manner

Having determined the ability of IL-7 and TSLP to induce cytokine production, the effects of these cytokines on ILC proliferation were assessed. Whilst IL-33 did not induce proliferation of ILC *in vitro*, the effect of TSLP and IL-7 on ILC proliferation was clear (Figure 4-10A). TSLP, but not IL-7, in combination with IL-33, synergised to induce ILC proliferation *in vitro* (Figure 4-10B). Rapamycin inhibited levels of ^{3}H-thymidine incorporation induced by TSLP and IL-7 by 84%

and 46% respectively, indicating an important role for mTOR in ILC proliferation.

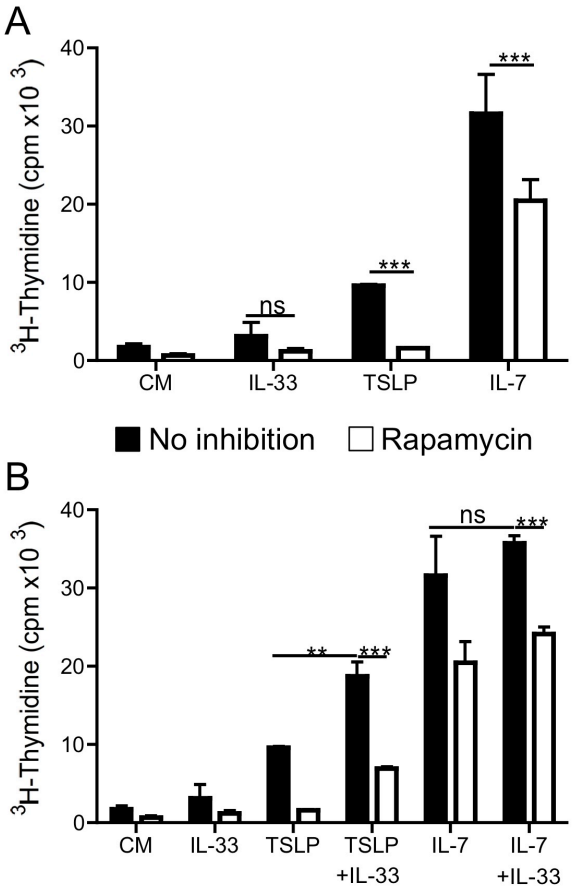

Figure 4-10 ILC proliferate *in vitro* in response to IL-7 and TSLP in an mTOR-dependent manner.

ILC were sorted and cultured as described previously. They were stimulated with the indicated cytokines at 10ng/ml for 72 hours with ^{3}H-Thymidine added in the final 16 hours of the culture period. Thymidine incorporation was measured using a beta-counter. Error bars represent SD from experimental triplicates. Data are representative of 3 experiments. **p<0.01, ***p<0.001, ns=not significant. CM=complete medium.

4.2.5 The role of ILC in IL-33-induced airway inflammation

Whilst others had demonstrated roles for ILC in gut immunity [139, 213, 214] and viral-mediated airway hyperresponsiveness [174], the importance of these cells in driving IL-33-induced airway inflammation was unknown. The following experiments were aimed at answering this important question.

4.2.5.1 The adoptive transfer model

Kondo and colleagues had demonstrated that intranasal IL-33 induced airway inflammation in the absence of the adaptive immune cells, as this model could

be replicated in recombination activating gene (Rag) $2^{-/-}$ mice [232]. This therefore suggested that this model was dependent on the innate immune response. Since ILC were the main source of type-2 cytokines in this model of airway inflammation (as discussed in Chapter 3), it seemed plausible that these cells could be sufficient to drive the inflammation induced by IL-33. As discussed previously, there are a variety of cells that express ST2 in the lung and are hence IL-33-responsive. It was therefore important to use an *in vivo* model in which the effects of IL-33 on ILC alone could be assessed. In order to accomplish this, an adoptive transfer model was used in which sorted WT ILC, induced by IL-33 as before, were intranasally transferred to $ST2^{-/-}$ mice (Figure 4-11). These mice were then challenged with IL-33 or PBS as a control. Since the transferred wild type (WT) ILC were the only cell-type in the recipient mice expressing ST2, these were the only cells that could respond directly to the IL-33 challenges. The effect of these challenges when compared to PBS-challenged mice would determine the contribution of IL-33-activated ILC to the airway inflammation model.

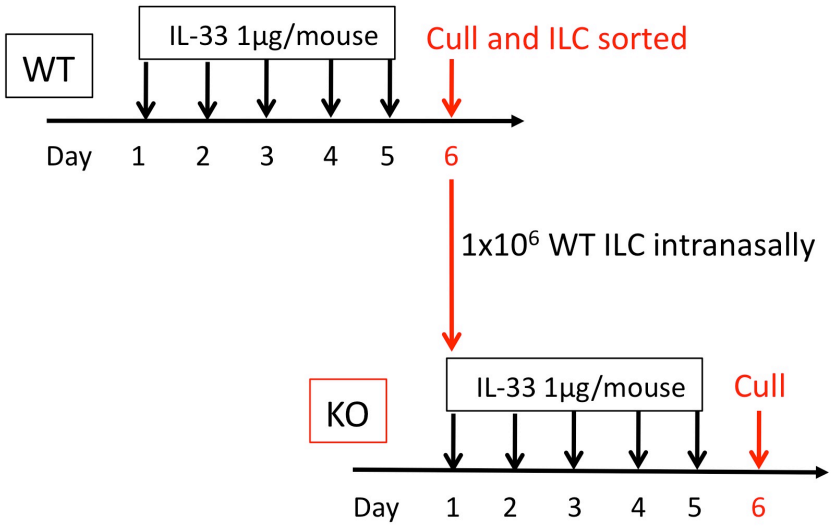

Figure 4-11 The adoptive transfer model

WT BALB/c mice were treated with intranasal IL-33 as before for 5 consecutive days. The mice were culled on day 6 and lungs were digested as before. Using a BD FACS Aria and the sorting strategy described (Fig 2-1A), WT lung ILC were isolated and rested overnight. They were then transferred intranasally in 30µL PBS into $ST2^{-/-}$ mice, followed by vehicle or 1µg IL-33 in 20µL PBS. The cytokine challenges were continued for a further 4 days and mice were killed 24 hours after the final challenge and samples were collected.

4.2.5.2 ILC are sufficient to induce IL-33-driven airway inflammation

Following ILC adoptive transfer and IL-33 or PBS treatment, $ST2^{-/-}$ mice were killed and BAL was performed following cardiac puncture under terminal anaesthesia. Total BAL cell counts were performed and a clear increase in the number of cells recruited in the lung was noted in mice transferred with ILC and treated with IL-33 (Figure 4-12A). This result stressed the importance of activating the ILC with IL-33 to induce the airway inflammation, as this effect was not seen in PBS-treated mice. In addition, lung digests were performed, as described previously, and total lung cell numbers were determined. The number of total lung cells was also increased in mice receiving IL-33 in keeping with the BAL results (Figure 4-12A).

The lung digest cells were stained for surface markers and FACS was performed to assess the cell types present. There was a clear increase in the number of granulocytes in the lung (*data not shown*) and further surface marker assessment demonstrated that the number of total eosinophils ($CD11b^{+}$, Siglec f^{+}, $Gr1^{int}$), macrophages ($CD11b^{+}$, Siglec f^{-}, $Gr1^{int}$) and neutrophils ($CD11b^{+}$, Siglec f^{-}, $Gr1^{hi}$) were significantly increased ($p=0.0016$, $p=0.0024$, $p=0.0072$, respectively) in IL-33-treated mice compared to mice receiving PBS (Figure 4-12B). Additionally, the number of ILC recovered from the lung was also increased in IL-33-treated mice (Figure 4-12B). These data demonstrated clearly that ILC were sufficient to mediate the lung inflammatory cell recruitment induced by IL-33.

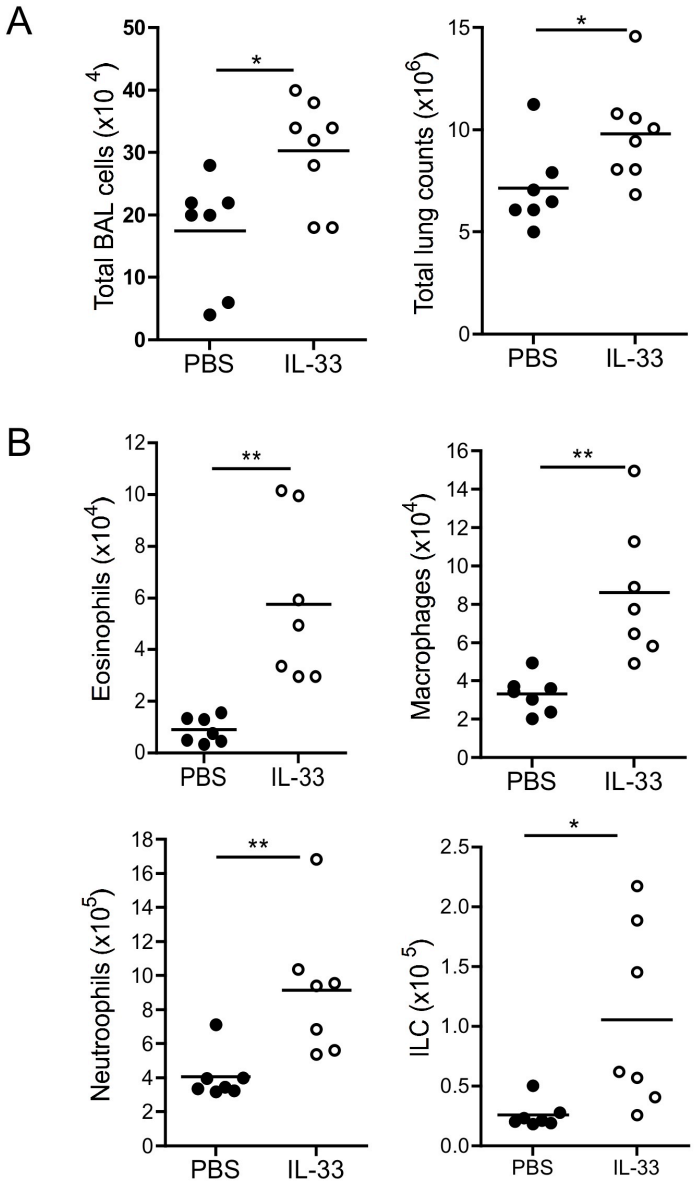

Figure 4-12 ILC are sufficient to drive IL-33-induced cellular infiltration in the lung

$ST2^{-/-}$ mice, which had received WT ILC, were treated with PBS or IL-33 for 5 days and killed on day 6. BAL were performed and total cell counts were determined (A). Left lungs were digested and total cells were counted (A). FACS staining of lung digests were performed and absolute numbers of each cell type was determined based on surface markers (B). Dots indicate individual mice and bars represent means. Data representative of 2 pooled experiments. *p<0.05, **p<0.01.

4.2.5.3 IL-33-activated ILC are sufficient to induce type-2 lung cytokine production

As discussed in chapter 3, ILC were found to be the main source of IL-5 and IL-13 induced by IL-33 in the lung. Consistent with these data, $ST2^{-/-}$ mice that received WT ILC and were treated with IL-33 had significantly higher (p=0.0007) amounts of IL-5 in their BAL fluid (Figure 4-13A) as well as enhanced IL-13 levels

(Figure 4-13B). Interestingly, transferring ILC alone was not sufficient to induce high levels of cytokine production in the lungs, underpinning the importance of IL-33 in driving ILC functions *in vivo*. Additionally, endogenous IL-33 was insufficient to stimulate the ILC in mice treated with PBS, suggesting that basal IL-33 levels are low in the resting mouse. Whilst it is likely that the ILC themselves are the main source of the IL-5 and IL-13 measured, the effect of ILC on the recruitment of other cells capable of producing type-2 cytokines, such as eosinophils [37], make it possible that these recruited cells might also contribute to the type-2 cytokine production.

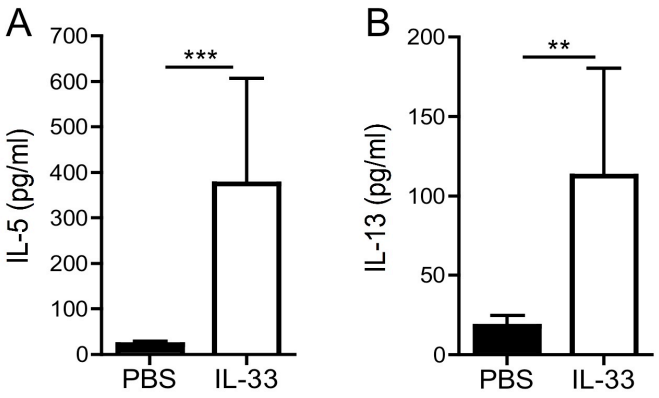

Figure 4-13 IL-33-stimulated ILC are able to increase IL-5 and IL-13 levels in the lung

BAL from ST2$^{-/-}$, treated with PBS or IL-33 following intranasal transfer of WT ILC, was collected and IL-5 (A) and IL-13 (B) were measured by ELISA. Error bars represent SD of measurements from individual mice (n=4-5/ group). **p<0.01, ***p<0.001. Data are representative of 3 experiments.

4.2.5.4 WT ILC are sufficient to increase cell recruitment and mucus production in IL-33-treated ST2-/- mice

As discussed previously, intranasal IL-33 induced histological changes in the lung. These had been shown to be dependent on the innate, rather than the adaptive immune system and were driven by IL-13 [232]. As shown above, IL-33-treated ILC had been shown to be sufficient to induce cell recruitment to the lungs and increase type-2 cytokine production in the lung. Therefore the ability of ILC to drive the histological changes seen in IL-33-induced airway inflammation was assessed.

Using the adoptive transfer model, the right lungs of recipient mice were fixed and heamatoxylin and eosin (HE) staining performed. In keeping with the results in WT mice (as discussed in chapter 3) and by Kondo *el al* [232], IL-33-treated ILC were able to recruit inflammatory cells to the peri-bronchial and peri-vascular areas of the lung. Additionally, some patchy changes were noted in the alveolar spaces (Figure 4-14A). Two blinded assessors quantified the changes in cell infiltration (Figure 4-14C) and lung architecture (Figure 4-14D) using an arbitrary scoring system. The lung sections were also stained with periodic-acid schiff (PAS) demonstrating that transferred WT ILC treated with IL-33 were sufficient to induce goblet cell hyperplasia and mucus hypersecretion in the lung of recipient $ST2^{-/-}$ (Figure 4-14B). The amount of mucus staining in the PAS-stained lung sections was quantified (Figure 4-15E) demonstrating a significant ($p=0.0141$) increase in mucus secretion when recipient mice were treated with IL-33 rather than PBS. These data demonstrate that, in the absence of additional IL-33-responsive cell populations, WT ILC are sufficient to induce all the key pathological changes associated with IL-33-driven airway inflammation.

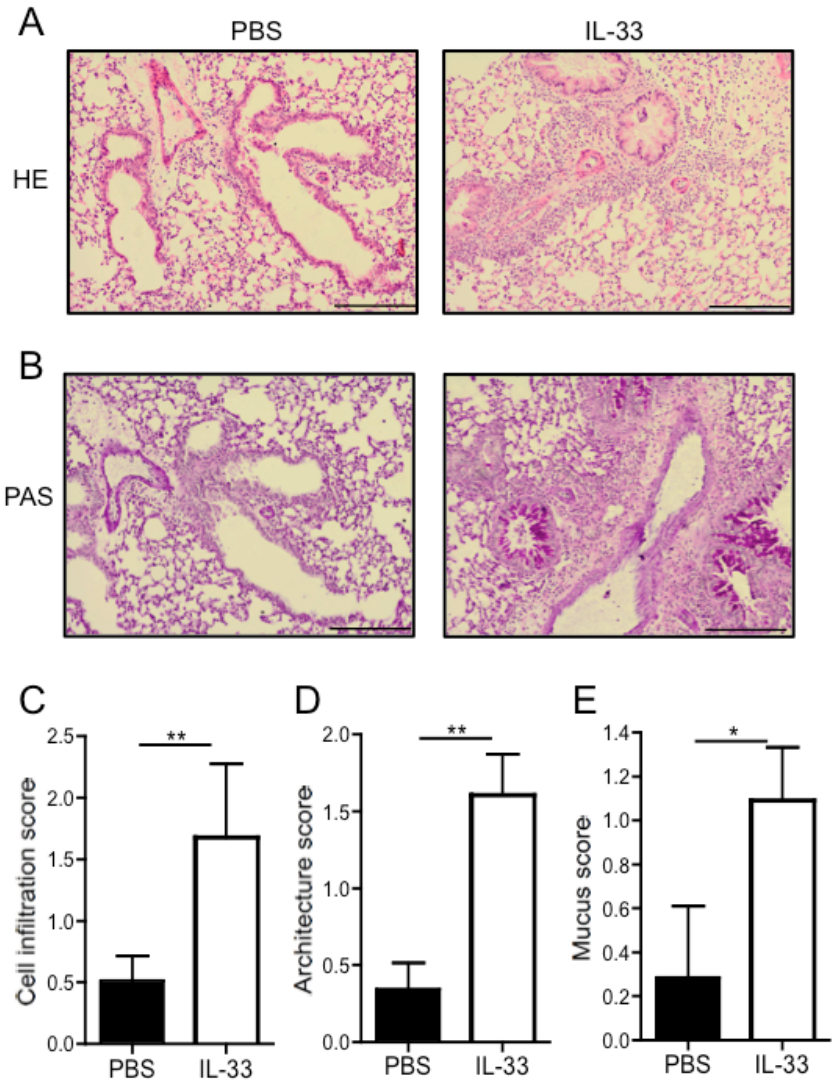

Figure 4-14 WT ILC are sufficient to induce lung histology changes in ST2$^{-/-}$ mice in response to IL-33

Lung sections from recipient ST2$^{-/-}$ treated with PBS and IL-33 were prepared and stained with HE (A) and PAS (B). Using an arbitrary score, 2 assessors quantified the differences in cell infiltration (C), lung architecture (D) and extent of mucus staining (E) blind. Sections were representative of the group. Bars on sections represent 200μm. Error bars represent SEM from individual mice (n=8-9/ group from two pooled experiments). *p<0.05, **p<0.01.

4.2.5.5 Rapamycin inhibits ILC-driven, IL-33-induced airway inflammation

Taken together, the data described above demonstrated a key contribution of ILC to IL-33-induced airway inflammation. In order to directly determine whether mTOR is important in IL-33-activated ILC function *in vivo* the adoptive transfer model was adapted to assess the effects of rapamycin by the inclusion of an IL-33+rapamycin group. The results demonstrated that ILC-administered ST2$^{-/-}$ recipient mice developed IL-33-induced airway inflammation that was

inhibited by rapamycin. Thus, the total lung cell numbers were reduced by concomitant rapamycin treatment (Figure 4-15A). The effect of mTOR inhibition was also noted in the reduction of the numbers of different granulocytes recruited to the lung (Figure 4-15B). Although there was a significant reduction in both neutrophil and eosinophil numbers in the lung, the reduction in macrophage numbers did not reach statistical significance. Nonetheless, there was a trend for a reduction in macrophage numbers with concomitant rapamycin treatment. Additionally, mTOR inhibition reduced the number of ILC recovered in the lungs of the recipient mice, in keeping with the results obtained from WT IL-33-treated mice (chapter 3). In addition, whilst IL-33 induced cytokine production in the lungs of ILC-recipient $ST2^{-/-}$ mice (Figure 4-15C), this effect was inhibited by rapamycin treatment. Finally, a further group of mice receiving a sham transfer and treated with IL-33 alone demonstrated that IL-33 had no effect on cell counts and cytokine levels measured in the lungs of $ST2^{-/-}$ mice (Figure 4-15). These data clearly demonstrate that mTOR is vital for IL-33-induced functions *in vivo* and that ILC are key to driving IL-33-induced airway inflammation.

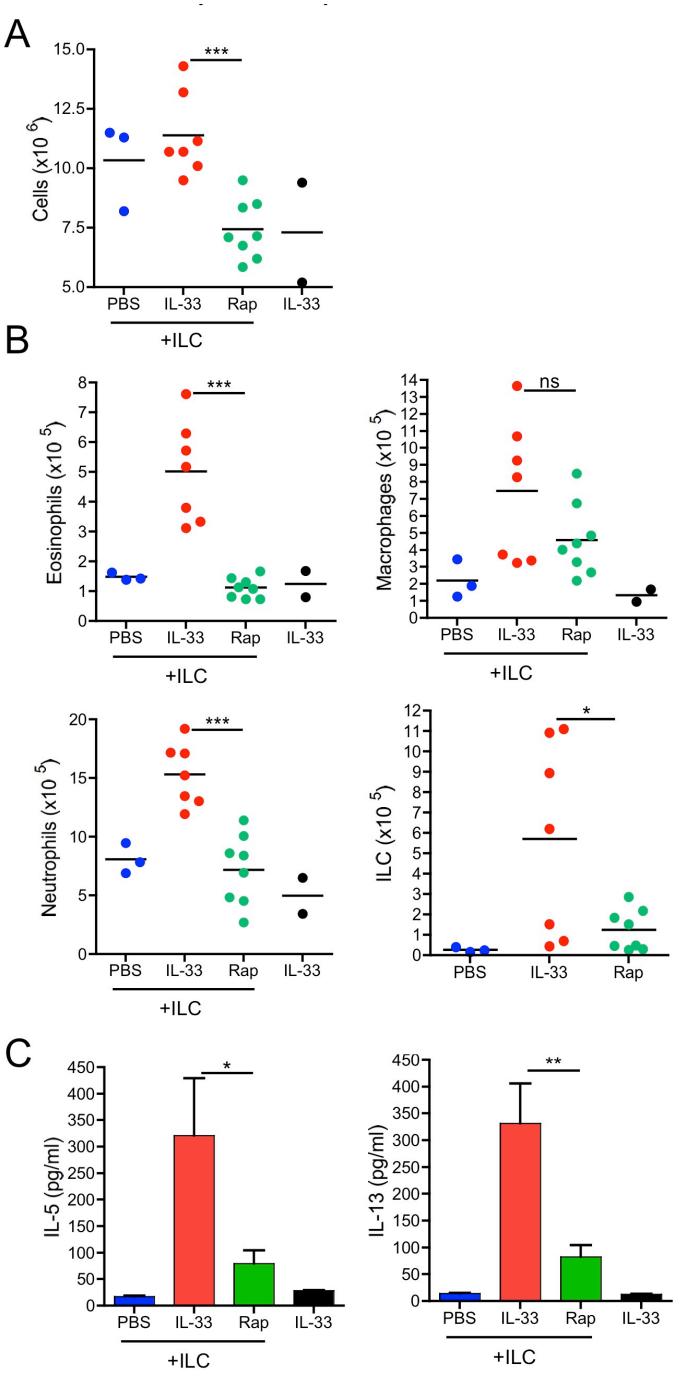

Figure 4-15 IL-33-activated ILC-induced airway inflammation is mTOR-dependent

ST2$^{-/-}$ mice received intranasal adoptive transfer of WT ILC or sham, were treated with intranasal PBS, IL-33 or IL-33+Rapamycin. The mice were killed and lung cells were counted as before (A). Lung digests, prepared as before and cells were stained as previously described. Absolute numbers of eosinophils (CD11b^{+}, Siglec f^{+}, Gr1^{int}), macrophages (CD11b^{+}, Siglec f^{-}, Gr1^{int}) and neutrophils (CD11b^{+}, Siglec f^{-}, Gr1^{hi}) were determined (B). Absolute numbers of ILC were assessed using the gating strategy described in Figure 4-1 (B). Cytokine levels were measured by ELISA. Dots represent individual mice with bars representing means for 2 pooled experiments. Error bars represent SD of ELISA measurements from individual mice (n=2-3/ group). *p<0.05, **p<0.01, ***p<0.001.

4.3 Conclusions

The experiments described in this chapter were designed to further characterise IL-33-induced responses in ILC and the importance of mTOR in these processes. Using FACS-sorted *ex vivo* ILC, the effects of IL-33 on ILC signalling pathways, cytokine production and proliferation were determined, increasing our understanding of this novel cell type. The results of these experiments prove that ILC are IL-33 targets and that it induces a canonical signalling pathway in these cells, as well as activating mTOR. In fact, mTOR activation is essential for optimal IL-33-driven ILC cytokine production *in vitro*. Since Kondo *et al*. had previously demonstrated that IL-33-driven airway inflammation was independent of the adaptive immunity [232], it was important to determine the contribution of ILC in this model of inflammation. The *in vivo* adoptive transfer model described in this chapter isolates ILC responses and demonstrates their contribution to IL-33-driven inflammation. The results from these experiments also demonstrate that, whilst ILC are sufficient to drive IL-33-induced airway inflammation, mTOR activation is vital for maximal IL-33 effect *in vivo*.

Firstly, it was essential to establish whether the ILC isolated from the lungs of IL-33-treated mice were phenotypically similar to those described previously by other groups investigating ILC in other organs. Using a similar gating strategy to that described by Neill and colleagues [139], it was clear that the lung ILC did not express a number of lineage markers (FcεRI, CD3, CD11b, B220) yet they did co-express CD45, ST2 and ICOS, demonstrating that these cells were indeed similar to those described by others [139, 174, 214].

Importantly, the absence of expression of other lineage markers such as TCRγδ, TCRβ, NK1.1 and CD11c was found in the lung ILC. This was also in keeping with the results published by others [139, 174, 214, 217, 355]. An important marker common to the first publications describing this novel population is the expression of c-kit [139, 174, 213, 214]. The extent of c-kit expression differed amongst the publications. However, this may simply be a reflection of the different models used to drive ILC numbers, which in turn may affect their activation status or phenotype. C-kit is a receptor tyrosine kinase that binds stem cell factor (SCF) and induces phosphoinositide 3-kinase (PI3K) phosphorylation. It is expressed in a number of structural cells as well as

haematopoetic precursor cells, stem cells [356], mast cells [357] as well as peripheral eosinophils and basophils [358]. Interestingly, ST2 expression occurs early in mast cell development [359] and IL-33-induced mast cell cytokine production is optimal with concomitant c-kit activation [192] [129]. The functional relevance of ILC c-kit expression is yet to be determined, however some preliminary experiments have shown that, similar to mast cells, co-stimulation of ILC with IL-33 and SCF had a synergistic effect on type-2 cytokine production (*data not shown*), suggesting that indeed, the presence of c-kit in ILC is functionally relevant.

As ILC expressed ST2, it was imperative to assess the direct effects of IL-33 on these cells. IL-33-induced MAP kinase and NF-κB canonical signalling pathways have been described in numerous cell types [82, 112, 117, 189, 190, 192, 350, 360], (discussed in chapter 1) and the activation of these pathways by IL-33 in ILC was tested. This was achieved by assessing the phosphorylation of p38 and degradation of IκBα, a marker of NF-κB activation [361]. Western blots performed on lysates of IL-33-activated ILC confirmed that these pathways were activated by IL-33.

In order to assess whether mTOR activation occurred in ILC as a result of IL-33 treatment, rapamycin was used and S6 phosphorylation measured. As discussed previously, mTORC1 activation leads to the phosphorylation of S6K, one substrate of which is the ribosomal protein S6. The phosphorylation of S6 is therefore a useful marker of mTOR activation. It is important to note, however, that S6 is also the substrate of the MAP kinase ribosomal S6 kinase (RSK), which phosphorylates S6 in an S6K-independent pathway [362]. S6 is phosphorylated at 4 of its sites, Ser235, Ser236, Ser240 and Ser244. Whilst phosphorylation at the Ser235 and Ser236 sites are partially rapamycin insensitive, phosphorylation of the Ser240 and Ser244 residues are not [362]. A further publication assessing the mechanisms of S6 phosphorylation downstream of TCR signalling in CD8 T cells demonstrated that, in fact, phosphorylation of S6 at the Ser235/236 residues involved redundant roles for RSK and S6K, and although RSK did contribute to phosphorylation of S6 at the Ser240/244 sites, S6K was the dominant kinase for those sites [363]. It therefore seemed sensible to assess S6 phosphorylation using an antibody directed against the Ser240/244 residues.

Western blots of lysates of IL-33-treated ILC demonstrated an increase in S6 phosphorylation levels, which was abolished by concurrent rapamycin treatment, conclusively demonstrating the role for mTOR activation in this model. Additionally, the lack of effect of rapamycin on p38 phosphorylation and IκB-α degradation demonstrate the specificity of rapamycin as an mTOR inhibitor [263].

The biological effect of mTOR inhibition in this *in vitro* system was determined by measuring the effects of rapamycin on IL-33-induced ILC cytokine production. Similarly to the *in vivo* experiments, rapamycin strongly inhibited IL-33 driven IL-5 and IL-13 production by ILC. Taken together, these results confirmed the vital role of mTOR signalling in IL-33-induced ILC cytokine production. It is important to note that rapamycin was able to significantly reduce, but not abolish, the effects of IL-33 on cytokine production. Experiments performed in our group, using Th2 cells and the mouse Th2 cell line, D10, demonstrated that IL-33 induced phosphorylation of both mTORC1 and mTORC2 substrates, S6 and Akt respectively. Akt is both upstream and downstream of mTOR activation. In the case of mTORC1, Akt activity is required to release mTORC1 from the inhibition of tuberous-sclerosis complex 2 (TSC2) [364]. mTORC2, however, phosphorylates Akt at the Ser473 residue [365], making it a downstream event in mTOR activation. As previously discussed rapamycin is known to be a relatively poor inhibitor of mTORC2 [263]. Furthermore, recent data suggest that inhibition of mTORC1 functions by rapamycin is incomplete [366]. It is therefore likely that not all IL-33-driven mTOR-dependent signals were inhibited by rapamycin in the current work. Further experiments using pan-mTOR inhibitors, capable of inhibiting both mTOR complexes, such as Torin-1 [264] would be informative. However, since rapamycin is already clinically available and in use, the experiments performed focused on the use of this commercially available, clinically relatively safe compound [367].

Interestingly, ILC have been shown to produce a variety of cytokines *in vivo* and *in vitro* [139, 214, 219]. Whilst neither IL-9 nor IL-4 was detected in this *in vitro* system (*data not shown*), this may be due to the fact that additional triggers are required to allow for these cytokines to be released. Certainly, the presence of IL-2 has been shown to be necessary to allow ILC IL-9 production *in vivo* [219]

suggesting that the cytokine milieu is important in defining the ILC cytokine profile. This issue is of particular interest since ILC are able to respond to a variety of cytokines that affect their own cytokine production. From the surface marker assessment made on sorted ILC, it was noted that they expressed CD127 (IL-7Rα), making these cells potentially responsive not only to IL-7 [368] but also TSLP [352]. TSLP requires the presence of the TSLP-specific receptor (TSLPR) as well as IL-7Rα to form a high-affinity binding site for TSLP [369]. Whilst the role of IL-7 in the development of numerous haemopoietic cell types has been described [353], TSLP appears to play a complex role in the immune system with an important role in supporting type-2 immune responses [354]. Experiments described in this chapter demonstrated synergistic effects of IL-33 with both IL-7 and TSLP on ILC cytokine production. These data suggest that the environments in which ILC are present will profoundly affect the effector function of these cells. The expression of TSLP is enhanced in airway epithelial cells stimulated with rhinovirus, a common respiratory pathogen [370] and TSLP is elevated in murine allergic airway inflammation models [73]. More so, mice with transgene-driven lung-specific *tslp* had evidence of spontaneous eosinophilic lung inflammation [73], confirming the role of TSLP as an important inducer of type-2 airway inflammation. Since ILC have been shown to play an important role in influenza-induced airway disease [174] and TSLP expression is increased by viral triggers in the lung [370], it seems plausible that ILC could respond to this cytokine during viral infection driving allergic airway inflammation. The most recent evidence that this may indeed be the case was recently reported by Shikotra *et al*. [72] who demonstrated increased TSLP expression in severe asthmatic patients' airways, compared to controls, as well as the presence of lineage negative, IL-13^{+} cells in these patients. These lineage negative cells could indeed be ILC, but further confirmation of this is required with additional studies.

Despite the synergy of either TSLP or IL-7 with IL-33 in ILC cytokine production, mTOR inhibition with rapamycin was still sufficient to significantly diminish this effect. Whilst IL-7 has been shown to activate mTOR [269], the effect of rapamycin on TSLP-driven effects has yet to be fully elucidated. From these data shown here, however, it would appear that TSLP-induced mTOR activation is important for ILC cytokine production.

Since mTOR was determined to be important in IL-33-induced ILC cytokine production, the mechanism for this effect was sought. *Il5* and *Il13* mRNA levels in ILC, activated with IL-33 in the presence or absence of rapamycin, were measured. Rapamycin did not appear to affect IL-33-induced mRNA levels of these cytokines, suggesting that mTOR was important in driving IL-33 effects at a post-transcriptional level. Importantly, mTOR is known to regulate gene expression at the level of translation by a number of mechanisms. Thus, the discovery that S6K modulation decreased the translation of a number of proteins vital for efficient ribosomal formation suggested that this could be a mechanism whereby this kinase affected gene translation [371]. S6K also phosphorylates eukaryotic initiator factor 4 (eIF4) B, which increases the RNA helicase activity of eIF4A, an important first step in translation initiation [258]. These 2 processes are believed to contribute to mTOR's ability to drive gene translation. Another key mTOR target is the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), a potent suppressor of translation initiation [372]. Whilst hypophosphorylated, 4E-BP1 binds eIF4E, preventing gene translation. Activated mTOR phosphorylates 4E-BP1, which in turn releases eIF4E, allowing translation of mRNA to proceed [372]. Therefore the effects of mTOR on 4E-BP1 phosphorylation and S6K may be responsible for driving IL-33-induced cytokine gene translation. Further experiments using commercially available S6K inhibitors [373] could prove helpful at pinpointing the exact mechanism for mTOR-dependant IL-33-induced cytokine production.

The effect of IL-33 on ILC proliferation was determined using ^{3}H-Thymidine studies. Interestingly, whilst IL-33 was a potent inducer of IL-5 and IL-13 production in ILC, it did not drive cell proliferation in this system. This result was unexpected since the number of ILC found in the IL-33-induced airway inflammation model (discussed in chapter 3) was greatly enhanced by IL-33 treatment. These data would therefore suggest that the increase in ILC numbers in the lung following IL-33 inoculation is driven by an indirect mechanism, either affecting proliferation of the cells or recruitment into the lung. Whilst IL-33 is able to affect the proliferation of other ST2-expressing cells, including basophils [128], eosinophils [44] and B1 cells [211], this effect is indirect via the stimulation of the production of additional growth factors and cytokines. Interestingly, the number of ILC recovered in the adoptive transfer model was

higher in IL-33-treated mice compared with PBS controls. Since ILC are the only cells in that system that were IL-33-responsive, this would suggest that IL-33 induced ILC to produce a factor that in turn could stimulate other cells to produce a pro-survival factor for ILC. Since ILC did proliferate in an mTOR-dependent manner in response to TSLP and IL-7 *in vitro*, it was possible that these cytokines mediated this effect on ILC numbers *in vivo*. TSLP levels in BAL fluid of WT IL-33-treated mice were measured to test this possibility. The levels of this cytokine in IL-33-treated mice were not elevated compared to PBS-treated mice (*data not shown*). IL-7 levels were not measured and performing this experiment could be informative. It is possible, however, that basal levels of TSLP or even IL-7 in the lung are sufficient to synergise with the exogenous IL-33 given, to drive ILC proliferation. Further experiments using *Tslpr*$^{-/-}$ or *Il-7r*$\alpha^{-/-}$ mice may help assess this question.

Interestingly, a synergistic effect on proliferation between IL-33 and TSLP was found, that was absent in IL-7+IL-33 cultures. This would suggest that both TSLP and IL-7 exert similar, but not identical effects on ILC. This is particularly interesting given the important role of both IL-33 and TSLP in allergic disease, suggesting that this synergy may be of relevance *in vivo*.

As discussed in chapter 1, there are a variety of cells in the lung that are IL-33-responsive (Table 1-1). Kondo *et al*. demonstrated that IL-33-induced airway inflammation[232] was independent of the adaptive immune system. From the data shown in Chapter 3 it was clear that ILC were an important source of IL-5 and IL-13 in IL-33-induced airway inflammation. It was essential, however, to determine how important these cells were in driving the model. In order to answer this question, an adoptive transfer model was designed, aimed at isolating IL-33-driven ILC responses in the mouse. These experiments demonstrated that, in an airway inflammation model, ST2-expressing ILC were sufficient to drive the inflammatory effects induced by IL-33 in the lung. These included not only the recruitment of cells to the lungs, but also the increased cytokine levels measures and key pathological changes which are characteristic of IL-33-induced airway inflammation. Importantly, they also demonstrated that transfer of ILC alone was insufficient to drive airway inflammation since they required IL-33 to induce this effect. Additionally, the data demonstrated that

rapamycin inhibited the IL-33-induced effects in the lung, proving that IL-33-induced airway inflammation is mTOR-dependent and can be driven by ILC. Unexpectedly, whilst rapamycin clearly reduced the recruitment of both neutrophils and eosinophils to the lung, it did not significantly diminish macrophage numbers. This effect may be simply due to the variability seen in the macrophage numbers, and increasing the mouse group size may make the trend for a reduction statistically significant.

The number of cells that could be sorted at one time limited the number of mice used in these experiments, hence restricting the numbers of mice per groups in each experiment. The decision to use rested cells for the transfers also had an impact on the final number of cells available, since some cells died during the overnight rest. However, it was felt that the overnight rest was necessary in order to be able to provide appropriate PBS-treated mice controls since freshly sorted cells had high basal production of IL-5 and IL-13.

Isolating the effects of ILC to determine their contribution in *in vivo* models has proven difficult and different groups have used a variety of methods, each with its own limitations. In a number of publications the contribution of type-2 cytokine-producing ILC in a parasite model [139] and an allergic airway inflammation model [217] was assessed by transferring WT ILC into *Il13*$^{-/-}$. Another group assessed the ability of ILC to drive parasite expulsion by transferring WT ILC into lymphocyte common gamma-chain ($\gamma c^{-/-}$)*x Rag2*$^{-/-}$ mice, devoid of both the adaptive immune system and cells reliant on common-γc cytokines [213, 214]. These mice were found to have no ILC. In these transfer models, ILC were sufficient to drive worm expulsion. Whilst demonstrating that ILC are sufficient to drive these responses in these models, proving that ILC are essential for an effect is trickier. In an attempt to determine whether ILC were essential to drive lung repair in a post-influenza model, *Rag 2*$^{-/-}$ were treated with CD90.2 antibody to remove all cells expressing this marker [141]. This included ILC as well as a small proportion of natural killer cells [374]. These mice were infected with influenza virus and CD90.1^{+}-sorted ILC were transferred intravenously into them. This model aimed to deplete the host ILC only and restore them with the transferred population, which differs from the other models discussed above. Whilst this is not a perfect experiment as ILC are not

the only lymphoid cell-type in *Rag 2*$^{-/-}$ that express CD90, it will remain difficult to specifically deplete ILC until a specific, unique marker is found for this cell-type.

Despite the caveats of the adoptive transfer experiments described in this chapter, these experiments were key to isolating the effects of IL-33-stimulated ILC *in vivo*. Contrary to a recent publication [375], no obvious inflammatory effects were noted by treating ST2$^{-/-}$ with IL-33 alone. Luzina *et al*. demonstrate that full-length IL-33 can induce non-Th2 inflammation in ST2$^{-/-}$ suggesting this is an ST2-independent effect [375]. Whilst the authors also noted a small lymphocytosis with full length and mature IL-33, this effect was not detected using the commercially available form of IL-33 used in the above experiments. The discrepancy between these results may result from the different delivery methods used to deliver the cytokine to the lung [375].

ILC are a novel cell type and little is known about their signalling pathways. Data shown in this chapter conclusively demonstrate that ILC are IL-33-responsive and that, in addition to the activation of the MAPK and NFκB pathways, IL-33 activates mTOR. Moreover, mTOR activation is essential for maximal IL-33-induced ILC functions, since concurrent treatment of ILC with rapamycin inhibits IL-33-driven cytokine production at a post-transcriptional level. In addition, these data reveal the key role played by ILC in IL-33-driven airway inflammation, as well as the importance of mTOR in these functions *in vivo*. Taken together however, the data in this chapter conclusively demonstrate that optimal IL-33-induced ILC responses require mTOR activation both *in vitro* and *in vivo*.

5 ILC and the adaptive immune response

5.1 Introduction

From the data described in chapters 3 and 4, it can be concluded that ILC, when activated with IL-33 are able to produce a variety of cytokines and induce an inflammatory response in the lung, characterised by an influx of granulocytes, including eosinophils. Previously, it had been demonstrated that IL-2 drives ILC proliferation [213] and IL-9 production *in vivo* [219], indicating that T cell-derived cytokines can influence ILC responses. Furthermore, Moro and colleagues showed that type 2 ILC could provide help for B1 cells and enhance immunoglobulin (Ig) A production [213]. These data indicate that type 2 ILC interact with and can influence the adaptive immune system. In this chapter, experiments to assess the effects of CD4 T cells on ILC function and the reciprocal effects of these innate cells on Th cells will be described.

5.2 Results

5.2.1 Adoptive transfer of ILC affects draining lymph node (LN) cells

In initial experiments, the effects of transferred wild type (WT) ILC on LN T and B cell numbers and phenotype were assessed. Using the adoptive transfer model described in chapter 4 (Figure 4-11), WT ILC induced by IL-33 were sorted using FACS and intranasally adoptively transferred into *St2*$^{-/-}$ mice. One group of *St2*$^{-/-}$ mice received a sham transfer only. These mice were then treated with intranasal PBS or IL-33 for 5 consecutive days. The mediastinal LN (mLN) were collected for assessment as described previously.

5.2.1.1 WT ILC are able to increase cell numbers in the mLN

Total mLN cell counts were performed (Figure 5-1A). The *St2*$^{-/-}$ mice that had received WT ILC had increased numbers of cells in their mLN compared to the sham-transferred mice. Interestingly, these differences achieved statistical significance ($p=0.004$) when the mice were treated with IL-33 intranasally. Further characterisation of the cell types in the mLN by FACS demonstrated an increase in the proportion of B220^{+} cells (Figure 5-1B) and the total number of B220^{+} cells (Figure 5-1C). B220 is a marker of B cells [376], as well as dendritic

cells (DC) precursors [377]. Since the B220$^+$ cells appeared lymphocytic, based on size and granularity (*data not shown*), these are more likely to represent B cells. These data indicate that IL-33-stimulated ILC are able to induce either cellular recruitment or proliferation of cells in the draining mLN.

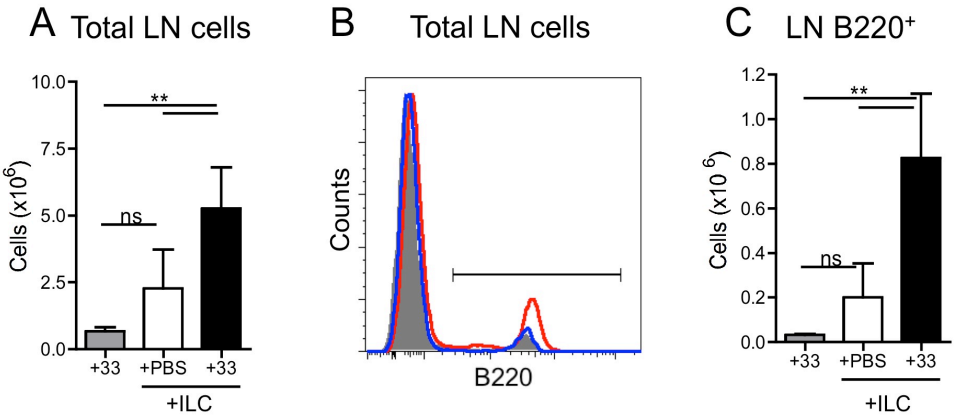

Figure 5-1 *St2*$^{-/-}$ recipient mice have increased cell numbers in their mLN following IL-33 treatment

WT ILC from IL-33-treated mice were sorted as described previously (Chapter 4) and transferred intranasally into *St2*$^{-/-}$ recipient mice. Following 5 days of either PBS or IL-33-treatment, the mice were killed and mLN were collected. Full mLN counts were performed (A). Using FACS, the percentage of mLN B220$^+$ cells were determined. Representative histogram of percentage of B220$^+$ cells (filled grey represents sham-transferred mice treated with IL-33 only, blue line represents mice receiving ILC+PBS and red line represent mice receiving ILC+IL-33) (B). mLN B220$^+$ cell numbers were quantified(C). +33 denotes mice receiving IL-33. Data are representative of 2 pooled experiments. Error bars represent SD measurements for individual mice (n=2-6/group). **p<0.01, ***p<0.001.

5.2.1.2 WT ILC affect mLN T cell numbers and activation profile in St2$^{-/-}$ *recipient mice*

The effects of transferred WT ILC on the number of and phenotype of host *St2*-/- CD4 cells were assessed (Figure 5-2A). Similar to the effects observed for total mLN cells, the number of CD4$^+$ cells was significantly increased when the mice receiving ILC were additionally treated with IL-33 (p=0.0055). Interestingly, the numbers of these cells that expressed high levels of CD44 (CD44hi) was increased in recipient mice treated with IL-33 (Figure 5-2B) suggesting an increased number of activated CD4 T cells in the mLN. Since this could merely reflect the increased number of cells in the mLN, the proportion of CD4 T cells expressing high levels of CD44 was determined (Figure 5-2C) compared to sham transferred mice. Additionally, other markers of CD4 T cell activation were measured, including CD25 and CD69 (Figure 5-2C). Quantification of the proportion of cells

expressing these activation markers demonstrated that the transfer of ILC with subsequent IL-33 treatment increased the proportions of CD4 T cells that expressed high levels of CD44, CD25 and CD69 (Figure 5-2D). Finally, the effect of ILC on CD8 T cells was determined. ILC transfer and activation by IL-33 increased the total number of CD8 T cells in the LN (Figure 5-3A). Whilst there was no increase in the proportion of CD44hi or the $CD25^{+}$ CD8 T cells in the LN (*data not shown*), there was a significant increase in the percentage of CD8 T cells expressing CD69 ($p=0.0371$, when compared to sham-transferred mice treated with IL-33) (Figure 5-3B and C). From this data, it appeared that WT ILC treated with IL-33 were sufficient to affect the activation status and numbers of CD4 and CD8 T cells in the draining LN.

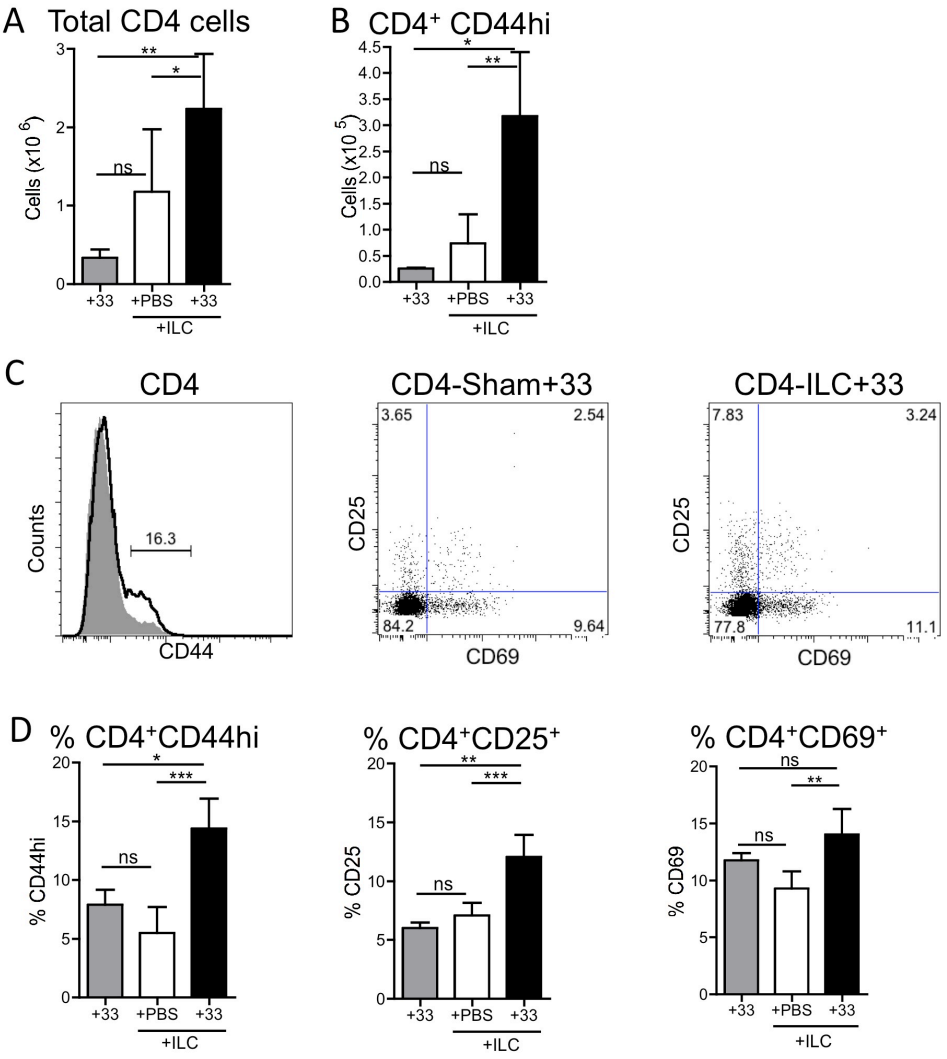

Figure 5-2 ILC affect mLN CD4 activation status *in vivo*

St2$^{-/-}$ recipient mice received adoptive transfers of ILC (or PBS only) intranasally and were subsequently treated with IL-33 or PBS. mLN were collected and total CD4 T cell numbers (A), as well as number of CD4 cells expressing high levels CD44, were assessed by FACS (B). Representative figures of CD4 T cell expression of CD44 (sham-transferred in filled grey histogram, ILC+IL-33 mice in solid line), CD25 and CD69 in sham-transferred and ILC-transferred mice, treated with IL-33 (C). Quantification of proportion of CD4^{+} cells expressing CD44, CD25 and CD69 (D). Data representative of 2 pooled experiments. Error bars represent SD measured for individual mice (n=2-6/group). *p<0.05, **p<0.01, ***p<0.001.

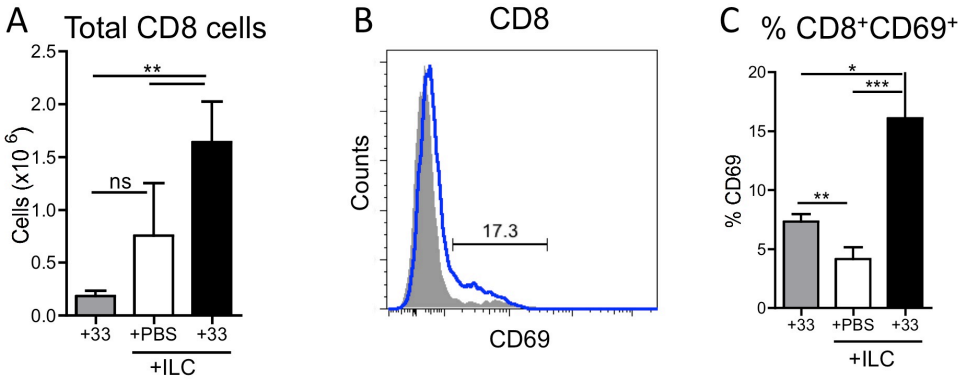

Figure 5-3 Adoptive transfer of ILC affects mLN CD8 T cell numbers and CD8 T cell activation

St2$^{-/-}$ recipient mice received adoptive transfers of ILC (or PBS only) intranasally and were subsequently treated with IL-33 or PBS. mLN were collected and total number of mLN CD8^{+} cells (A). Representative histogram of CD8 T cells CD69 expression levels (sham-transferred in filled grey histogram, ILC+IL-33 mice in solid line) (B) and percentages (C). Data representative of 2 pooled experiments. Error bars represent SD measured for individual mice (n=2-6/group). *p<0.05, **p<0.01, ***p<0.001.

5.2.2 CD4 T cell-ILC co-culture experiments - the effects of T helper (Th) cells on ILC function

Results from the adoptive transfer experiments suggested that IL-33 activated ILC were able to affect the phenotype and numbers of mLN CD4 cells. It was not possible however, from these data, to determine if these effects were a direct or indirect effect of the ILC. In order to determine whether ILC could have direct effects on CD4 cells and *vice versa*, *in vitro* experiments were performed.

5.2.2.1 ILC respond to IL-2 in vitro

It was imperative to first understand the effects of adaptive immune cell-derived cytokines on ILC function. In this regard, a role for IL-2 in ILC function *in vivo* has been reported by several groups [219]. Since ILC expressed CD25, the IL-2α-chain receptor, (Figure 5-4A and chapter 4) it was likely that they would be able to respond to IL-2. Therefore, the effect of IL-2 on ILC proliferation was determined by ^{3}H-Thymidine incorporation (Figure 5-4B). IL-2 induced ILC proliferation *in vitro* and was found to have greater mitogenic effects than IL-7. Additionally, the effect of IL-2 on ILC cytokine production was determined, by ELISA. When given alone, IL-2 substantially increased the amounts of IL-5 and IL-13 (Figure 5-4C) produced by ILC. Furthermore, there

was a powerful synergistic effect of IL-2 with IL-33, similar to that seen with IL-7 (Figure 5-4D). These data confirm that the T cell cytokine IL-2 potently stimulates ILC proliferation and cytokine production.

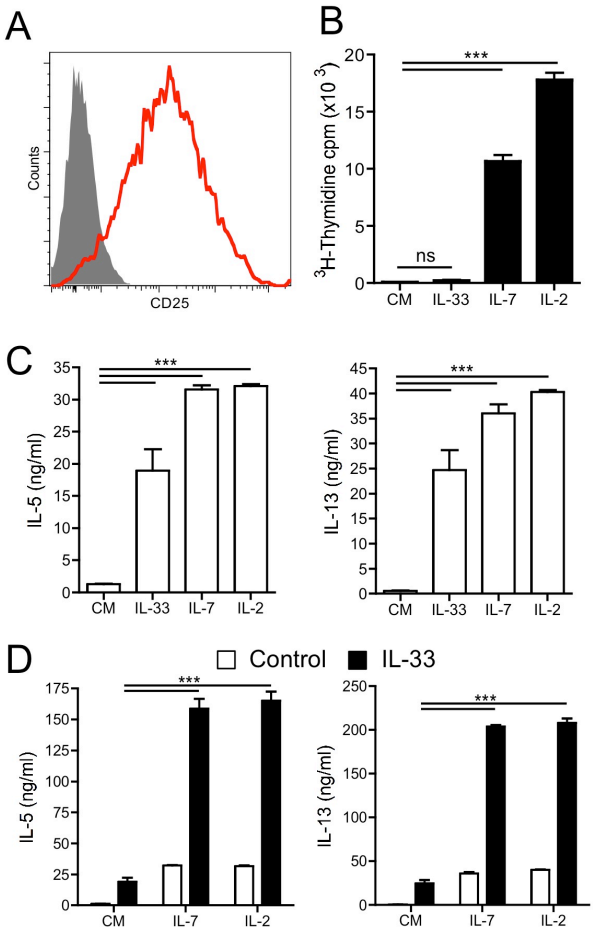

Figure 5-4 IL-2 stimulates ILC function *in vitro*

ILC were sorted as before and CD25 expression measured by FACS (A) (filled grey histogram represents Isotype control, solid red line represents CD25 staining). Rested ILC ($0.5x10^5$ cells/well) were cultured with CM (complete medium alone), IL-33, IL-7 or IL-2 (all 10ng/ml) for 72 hours and pulsed with ^{3}H-Thymidine for the final 16 hours of culture. Levels of ^{3}H-Thymidine incorporation were measured (B). Rested ILC ($1x10^5$ cells/well) were cultured for 72 hours with the indicated cytokines (10ng/ml) or CM and supernatants collected. Cytokine measurements were made by ELISA. ILC respond to IL-2 to produce IL-5 and IL-13 (C) and synergises with IL-33 to induce greater cytokine production (D). Error bars represent SD of experimental triplicates. Data are representative of 2 experiments. ***p<0.001.

5.2.2.2 IL-2 licences IL-4, an effect inhibited by IL-33

As IL-2 was clearly able to affect ILC production of IL-5 and IL-13, its effect on the production of other type-2 cytokines was sought. Since a role for IL-2 in ILC IL-9 expression *in vivo* had been reported [219], the effect of IL-2 on ILC IL-9

expression *in vitro* was determined. ILC were cultured for 72 hours with the indicated cytokines and were subsequently stimulated with phorbol myristate acetate and ionomycin (PMA-I), prior to intracellular cytokine staining and FACS analysis. Results indicated that only a small proportion of ILC expressed IL-9 at baseline (Figure 5-5A) and this was not modulated by IL-2 treatment (Figure 5-5B). Moreover, IL-33 appeared to inhibit IL-9 expression on ILC (Figure 5-5C) and this effect prevailed even with the addition of IL-2 (Figure 5-5D), although it must be noted that the basal levels of IL-9 were very low in the control group. Importantly, measurement of IL-9 in culture supernatants by ELISA failed to demonstrate measurable quantities of this protein. These data suggest that IL-2 is not sufficient to drive ILC IL-9 production *in vitro*.

Approximately 5% of ILC cultured in complete media alone expressed IL-4 following PMA-I re-stimulation (Figure 5-5A). Interestingly, the proportion of IL-4^+ cells in IL-2-stimulated ILC cultures was substantially higher (~26%) (Figure 5-5B). Intriguingly, IL-33-treated ILC failed to up-regulate IL-4 expression relative to control cells (Figure 5-5C). Furthermore, IL-33 appeared to inhibit the effects of IL-2 on IL-4 expression (Figure 5-5D). These data demonstrate that different cytokines have differing effects on ILC cytokine expression profiles and that IL-33 appears to oppose the effects of IL-2 on ILC IL-4 expression. Furthermore, whilst PMA-I re-stimulation resulted in IL-4 expression by IL-2-treated ILC, prior to PMA-I treatment, IL-4 levels in the supernatants of IL-2-treated cell cultures were below the level of detection by ELISA (*data not shown*). These data indicate that whilst IL-2-treated ILC are capable of IL-4 expression, additional stimuli are likely to be required to allow its secretion.

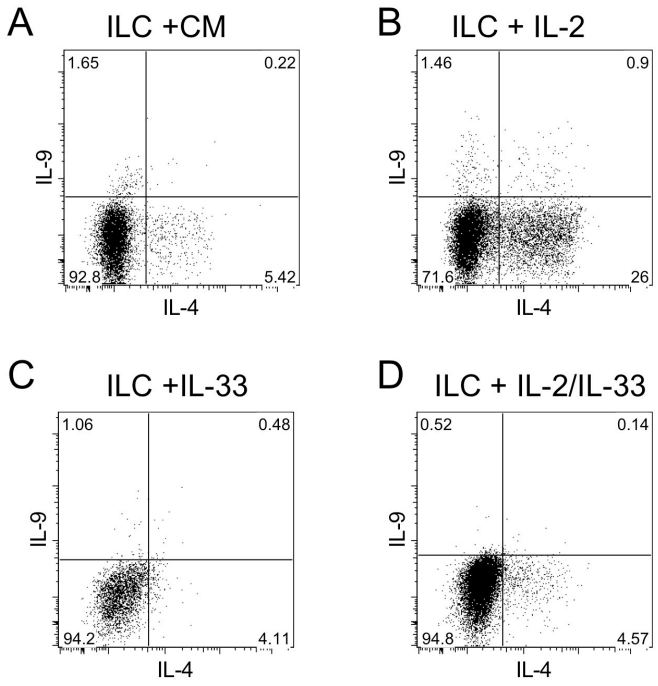

Figure 5-5 IL-2 increases ILC IL-4 expression *in vitro*, and this is inhibited by IL-33

FACS-sorted ILC were cultured with complete medium (CM) or the indicated cytokines (10ng/ml) for 72 hours prior to PMA-I and Golgi-stop (GS) for 4 hours. Cells were stained for intracellular cytokines as described previously and assessed by FACS. Levels of IL-9 and IL-4 expression were determined in cells cultured with CM alone (A), IL-2 (B), IL-33 (C), and both IL-2 and IL-33 (D). Data are representative of 3 separate experiments.

5.2.2.3 ILC proliferate in co-culture with CD4 T cells

The results described above indicated that the T cell cytokine IL-2 was a potent modulator of ILC responses *in vitro*. In order to directly ascertain the effects of CD4 T cells on ILC and *vice versa*, co-culture experiments were performed. ILC were sorted from IL-33-treated WT mice, as described previously. To allow assessment of the impact of ILC on T cell activation and differentiation, naïve CD4 T cells were sorted for co-culture, using levels of CD44 expression as a marker to distinguish naïve (CD44 low) from memory / effector CD4 T cells (CD44 hi) [378, 379]. Furthermore, in order to distinguish the effects of IL-33-driven ILC responses from direct effects of IL-33, the CD4 T cells were sorted from *St2*$^{-/-}$ mice.

The effects of CD4 T cells on ILC proliferation was assessed. ILC were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) prior to use and cultured either alone, with IL-7 as a positive control, or with naïve CD4 T cells.

In all cases, cells were cultured on anti-CD3+CD28-coated plates for 72 hours. Proliferation was assessed using FACS and CFSE dilution. ILC failed to proliferate when cultured with complete media (CM) alone (Figure 5-6A), in keeping with findings described in chapter 4, and showing that anti-CD3/28 did not impact directly upon their activation. As shown in Fig 1-4B and in chapter 4, IL-7 is a potent mitogen for ILC and in its presence, 54% of ILC divided during the culture period (Figure 5-6B). Finally, in co-culture with CD3/28-stimulated CD4 T cells, ILC also proliferated, with over 64% of cells demonstrating a dilution of CFSE fluorescence (Figure 5-6C). These data demonstrated that ILC were able to respond and proliferate when cultured in the presence of activated CD4 T cells *in vitro*.

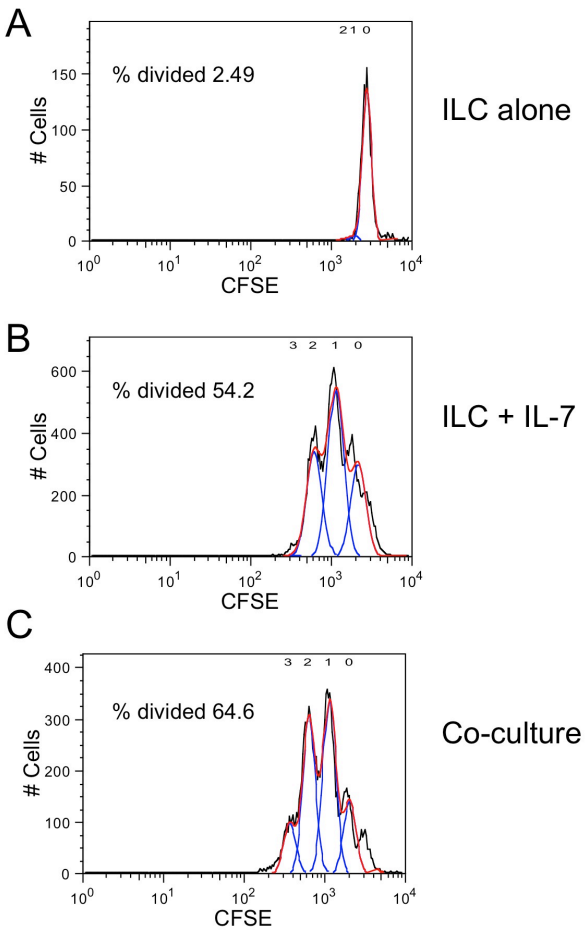

Figure 5-6 ILC proliferate in co-culture with CD4 T cells

Sorted ILC were rested overnight and stained with CFSE as described previously. Cells ($1x10^5$/ well) were cultured in flat-bottomed 96 well plates pre-coated with α-CD3 (1.5μg/ml) and α-CD28 (3μg/ml) with complete medium (CM), IL-7 (10ng/ml) or CD4 cells ($1x10^5$/ well) for 72 hours. CFSE dilution was measured by FACS. Percentage of divided cells was determined for ILC alone (CM), treated with IL-7 (B) or in co-culture with CD4 cells (C). Solid black line represents measured fluorescence with blue lines representing cell cycles as determined by the analysis software (FloJo 8.84) and solid red line representing contours imposed by the analysis software. Data are representative of 2 experiments.

5.2.2.4 ILC have increased IL-4 expression in co-culture which is inhibited by IL-33

In order to determine if CD4 T cells could affect ILC cytokine expression profile, following 72h of co-culture, cells were stimulated with PMA-I and GS to allow for intracellular assessment of individual cells in co-culture. Interestingly, ILC in culture with activated T cells expressed higher levels of IL-4 (Figure 5-7B) than ILC alone (Figure 5-7A). Furthermore, when ILC were treated with IL-33 in co-culture, this effect was abolished (Figure 5-7C). It must be re-iterated that the only IL-33-responsive cells in the culture were the ILC as the CD4 cells lacked

ST2. The percentage of IL-4$^+$ ILC was measured in experimental replicates (Figure 5-7D), showing that the effect of CD4 T cells on ILC IL-4 expression was increased compared to ILC cultured alone. Furthermore, the inhibitory effect of IL-33 on ILC IL-4 expression was also statistically significant, compared to co-culture alone (p=0.0162). These data demonstrate that CD4 T cells impact upon the cytokine profile of ILC *in vitro* and that IL-33 modulates this process.

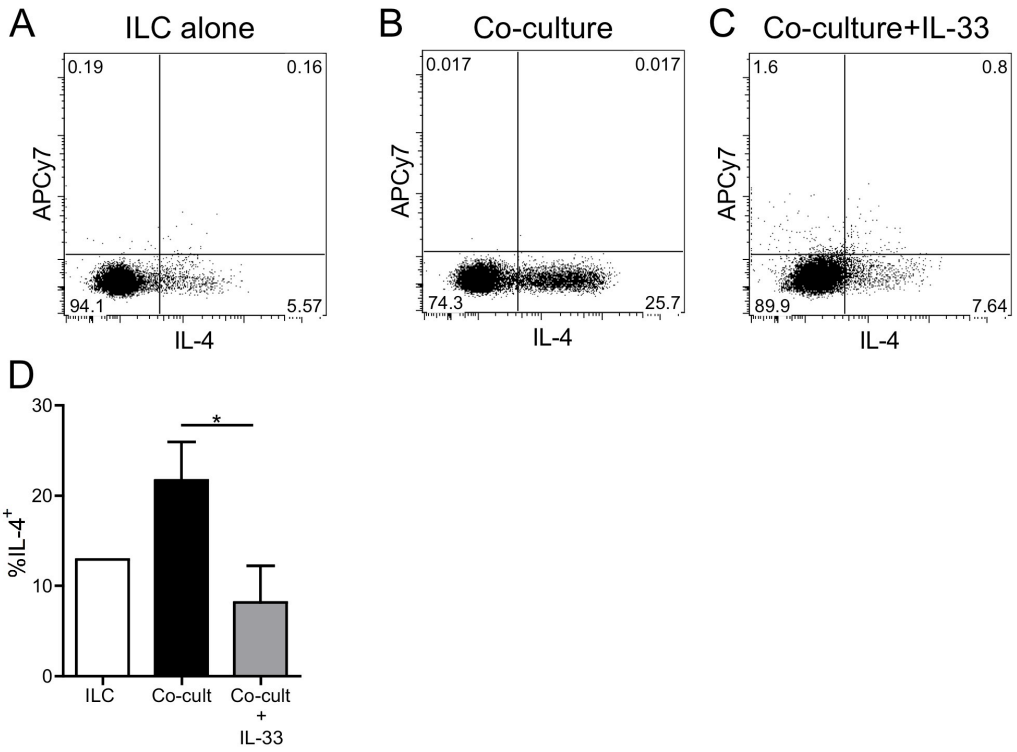

Figure 5-7 Co-culture in the presence of activated CD4 T cells augments ILC IL-4 expression and this effect is abrogated by IL-33

ILC and CD4 T cells were co-cultured (1x10^5 cells/ well each) in the presence or the absence of IL-33 (10ng/ml) on α-CD3/ α-CD28-coated 96 well plates. Cells were co-cultured for 72 hours followed by 4 hours of PMA-I and GS stimulation. The cells were then stained for surface and intracellular cytokines as described previously. ILC were distinguished from CD4 T cells by absence of CD4 marker and presence of ST2. FACS dot plots show ILC IL-4 expression in cells cultured in complete medium (CM) alone (A), co-cultured with CD4 T cells (B) or in co-culture plus IL-33 (C). Quantification of the proportion of IL-4$^+$ cells from experimental replicates for co-culture conditions (D). Error bars represent SD of experimental replicates (n=3 for all groups except ILC alone) and data are representative of 4 experiments. *p<0.05.

5.2.2.5 Effect of CD4 T cells on ILC is IL-2-dependent

The data shown in Figure 5-5 and Figure 5-6 demonstrate that co-culture with CD4 T cells or treatment with IL-2 have similar effects on ILC function, namely an increase in proliferation and enhanced IL-4 expression that is abolished by concurrent IL-33 treatment. In view of this, it was hypothesised that the effect

of CD4 T cells on ILC was IL-2-dependent. In order to answer this question, ILC and T cells were co-cultured as before, in the presence or absence of neutralising IL-2 antibody (Ab). As before, ILC cytokine profile was determined following 72 hours of co-culture and 4 hours of subsequent PMA-I and GS stimulation. The results of these experiments demonstrated that ILC IL-4 expression was enhanced by co-culture with CD4 T cells, as before, and this effect was abolished by concurrent anti-IL-2 treatment (Figure 5-8A). A significant reduction in proportions of IL-5^+ ILC was also apparent following anti-IL-2 treatment (Figure 5-8A). In order to rule out the possibility that anti-IL-2 treatment was directly toxic to ILC, the proportion of cells that were alive prior to intracellular staining was determined by FACS using a live/dead marker as described before. Not unexpectedly, the majority of the ILC cultured with complete medium alone were dead (Figure 5-8B). However, the proportion of dead cells in co-culture plus anti-IL-2, compared to that of cells in co-culture alone, was similar, suggesting that the effect of anti-IL-2 on ILC cytokine production was not via the decreased survival of these cells. These data confirm the hypothesis that ILC IL-4 expression was increased by CD4 T cell-derived IL-2.

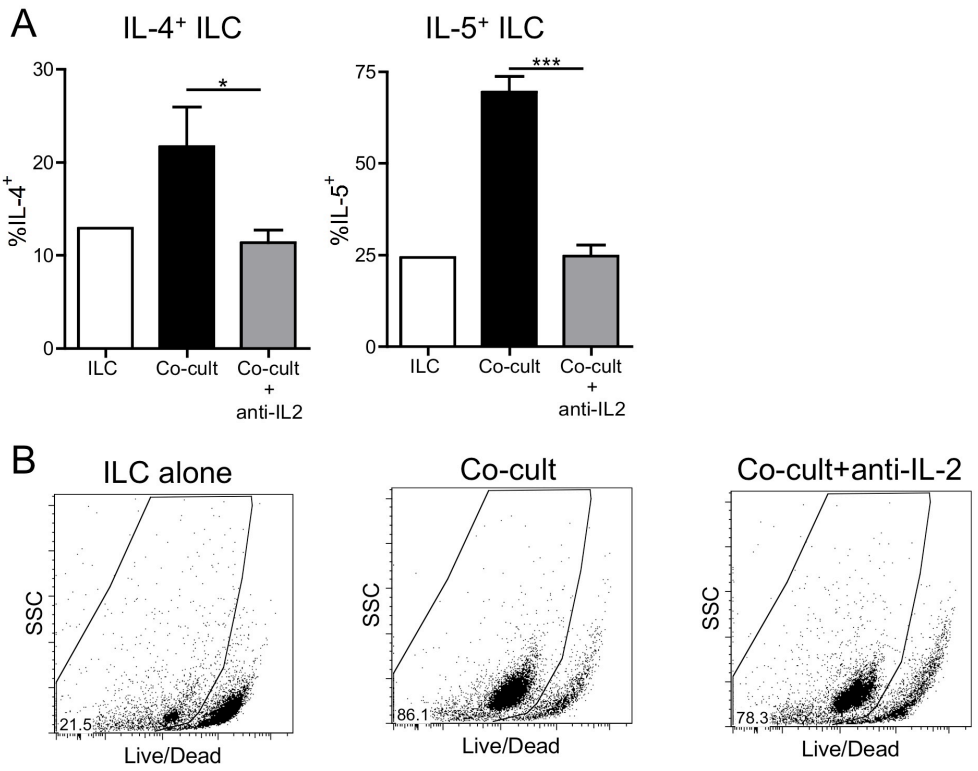

Figure 5-8 The effect of CD4 T cells on ILC IL-4 expression is IL-2-dependent

CD4 T cells ($1x10^5$ cells/ well) were cultured with ILC ($1x10^5$ cells/ well) as before, in the presence of plate-bound α-CD3/αCD28, with or without IL-2 neutralising antibody (anti-IL-2) (10μg/ml) for 72 hours, followed by 4-hour stimulation with PMA-I and GS. Intracellular cytokine levels were determined by FACS. ILC were differentiated from CD4 T cells on FACS plots by absence of CD4 marker and presence of ST2. Percentage of $IL\text{-}4^+$ and $IL\text{-}5^+$ ILC were determined (A). Percentage of live cells as determined by lack of fluorescence of live/dead marker was determined in each culture condition (B). Error bars represent SD of experimental triplicates (all groups except ILC alone). Data are representative of 2 experiments. *p<0.05, ***p<0.001.

5.2.3 CD4 T cell / ILC co-culture- effects on CD4 T cells

Whilst the data presented thus far confirms the effect of CD4 T cells on ILC, when in co-culture *in vitro*, the *in vivo* data supported the possibility of a role for ILC in CD4 T cell functions. The following experiments were designed to focus on the effects of ILC on CD4 T cell function.

5.2.3.1 CD4 cells do not proliferate with ILC unless activated

The effect of ILC on T cell proliferation was determined by analysis of dilution of Alexa fluor 647 cell proliferation dye by FACS (Figure 5-9). Un-stimulated CD4 T cells did not proliferate, irrespective of the presence (Fig 1-9B) or the absence (Figure 5-9A) of ILC in the culture. CD4 T cells were, however, stimulated to proliferate by the presence of CD3 and CD28 antibodies (Figure 5-9C) and this

did not appear to be greatly affected by ILC (Figure 5-9D). These data suggest that, whilst ILC proliferate in response to CD4 T cell-derived cytokines in co-culture, ILC alone are unable to induce CD4 T cell activation.

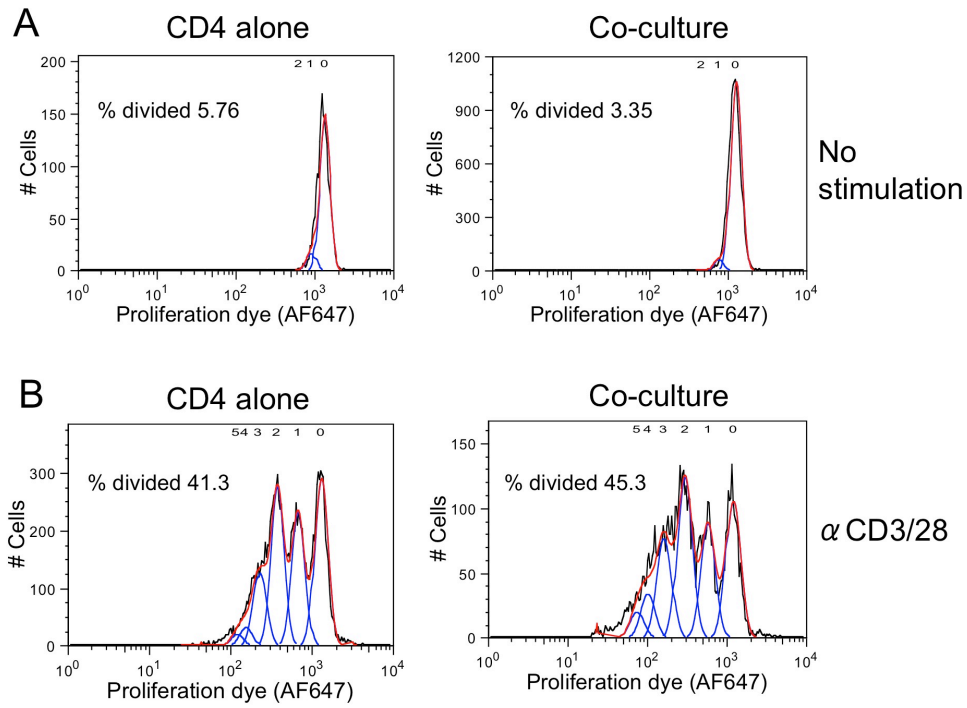

Figure 5-9 CD4 T cells fail to proliferate unless stimulated in co-culture

Sorted CD4 T cells were stained with an alexa-fluor 647 (AF647) cell proliferation dye and cells were cultured ($1x10^5$ cells/ well) alone or in co-culture with ILC ($1x10^5$ cells/ well) in the absence (A) or the presence (B) of α-CD3/αCD28, as before. The cells were cultured for 72 hours and CD4 cell proliferation was determined by assessing fluorescence dilution by FACS. Solid black line represents measured fluorescence with blue lines representing cell cycles as determined by the analysis software (FloJo 8.84) and solid red line representing contours imposed by the analysis software. Data are representative of 3 separate experiments.

5.2.3.2 CD4 T cells express type-2 cytokines when co-cultured with ILC

Whilst it appeared that ILC were unable to affect CD4 T cell proliferation *in vitro* either in the presence or absence of T cell receptor (TCR) stimulation, the effect of ILC on the cytokine profile of CD4 T cells was determined. Interestingly, co-culture with ILC appeared to induce a Th2-like phenotype on the naïve CD4 T cells in co-culture, which was absent when the cells were cultured with TCR-stimulation alone (Figure 5-10A). Importantly, the presence of ILC resulted in IL-4, IL-5 and IL-13 expression in the CD4 T cells (Figure 5-10B). In fact, the proportion of IL-4$^+$ CD4 T cells increased 3 fold, when cultured with ILC, whereas, the increase in IL-5$^+$ CD4 T cells was 30 fold. Finally, the increase in IL-13$^+$ CD4 T cells in co-culture with ILC was 20 fold.

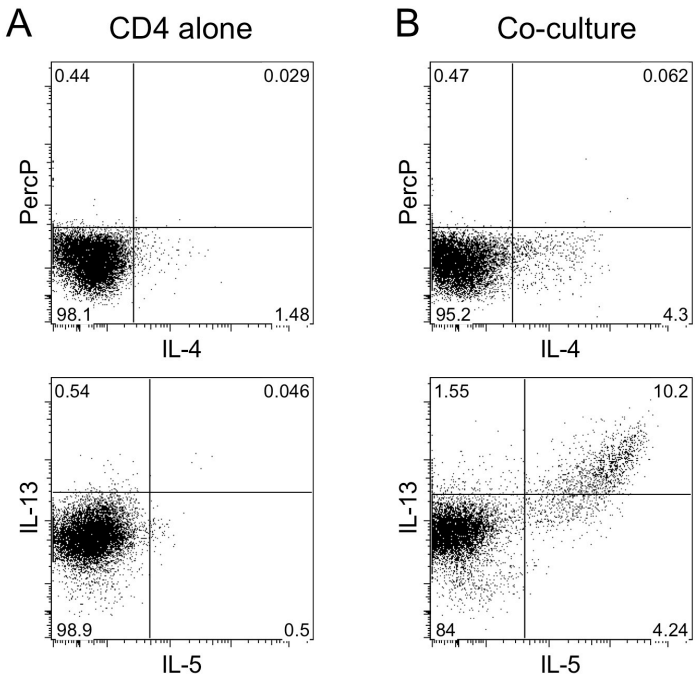

Figure 5-10 CD4 T cells express type-2 cytokines when co-cultured with ILC

CD4 T cells were stimulated as before (with platebound α-CD3/αCD28) and cultured alone or in combination with ILC. Following 72 hours of culture the cells were re-stimulated with PMA-I and GS and subsequently stained for surface markers (CD4 and ST2) as well as intracellular cytokines as above. Dot plots demonstrate IL-4-, IL-5- and IL-13-expressing CD4 T cells (gated as CD4$^+$ST2$^-$) when cultured alone (A) or in co-culture with ILC (B). Panels are representative of experimental triplicates of 3 separate experiments.

5.2.3.3 The effect of ILC on CD4 T cell type-2 cytokine expression is contact-dependent

In order to determine how ILC were able to influence the CD4 T cell cytokine signature, co-culture experiments using a semi-permeable membrane/ transwell (TW) were performed. These were done to ascertain if the co-culture effect was through a soluble mediator or via a contact-dependent mechanism. From these experiments it was clear that the ILC had an effect on CD4 T cell cytokine expression in a contact-dependent manner since separating the cells with a TW abolished the induction of type-2 cytokines completely (Figure 5-11).

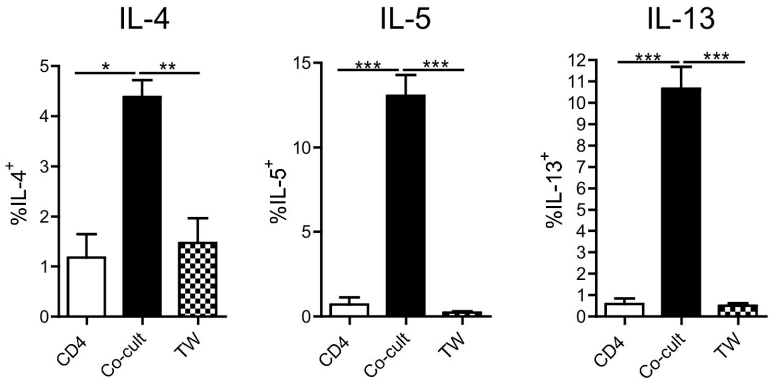

Figure 5-11 The effect of ILC on CD4 T cell cytokine expression is contact dependent

CD4 T cells ($1x10^5$ cells/ per well) were cultured in the presence or the absence of ILC ($1x10^5$ cells/ per well) for 72 hours. The plate was coated with α-CD3/αCD28 as before. Whenever the transwell (TW) was used to separate the two cell types, the CD4 T cells were plated in the bottom compartment to ensure contact with the plate-bound antigens. Following 4 hours of PMA-I and GS stimulation, the cells were stained with surface and intracellular antibodies. CD4 T cells were identified as $CD4^+ST2^-$ cells and percentage of $IL\text{-}4^+$, $IL\text{-}5^+$ and $IL\text{-}13^+$ cells were quantified. Data are representative of 5 experiments. Error bars represent SD for experimental triplicates. *p<0.05, **p<0.01, ***p<0.001.

5.2.3.4 ILC inhibit CD4 T cell interferon-γ (IFNγ) in a contact dependent manner

Whilst it was interesting that ILC were able to induce CD4 T cell production of IL-4, IL-5, and IL-13, it was possible that the effects of ILC were not limited to Th2 cytokine production. In order to clarify this possibility, the expression of the Th1 signature cytokine, IFNγ was measured in co-cultured CD4 T cells. Intriguingly, co-culture with ILC reduced the proportion of $IFN\gamma^+$ CD4 T cells compared to CD4 T cells cultured alone from 3.5% to 1.65% (Figure 5-12A). This inhibitory effect was lost upon separation of the cells by a TW membrane. In

fact, analysis of replicate cultures demonstrated that differences in levels of IFNγ production were statistically significant when comparing cells in co-culture to CD4 T cells alone or cells separated by TW ($p=0.0219$ and 0.0035, respectively) (Figure 5-12C).

To ascertain if ILC also had an inhibitory effect on CD4 T IFNγ production when the T cells were deliberately polarised to a Th1 phenotype, the cells were cultured in the presence of IL-12 (Figure 5-12B). The results of these experiments demonstrated that the inhibitory ILC effect was still present when cells were treated with IL-12 (Figure 5-12D). As expected, IL-12 enhanced the proportion of $IFN\gamma^+$ CD4 T cells with over 30% of CD4 T cells expressing this cytokine (Figure 5-12B and D). Co-culture with ILC resulted in a ~70% decrease in the proportion of $IFN\gamma^+$ CD4 T cells. However, this inhibitory effect was lost when the cells were cultured separated by a semi-permeable membrane (TW) (Figure 5-12B and D). ILC did not express IFNγ in either culture condition (*data not shown*).

Interestingly, whilst ILC inhibited CD4 T cells' expression of IFNγ in cultures supplemented with the IL-12, they were still able to enhance the T cells' expression of type-2 cytokines IL-4 (Figure 5-13A) and IL-5 (Figure 5-13B) in a contact-dependent fashion. These results demonstrate that ILC drive a Th2 cytokine profile on CD4 T cells in co-culture and simultaneously inhibit the development of a Th1 phenotype in a contact-dependent manner. Furthermore, the effect of ILC on CD4 T cell expression of IL-4 and IL-5 is still evident when the cells are cultured in a Th1-polarising milieu.

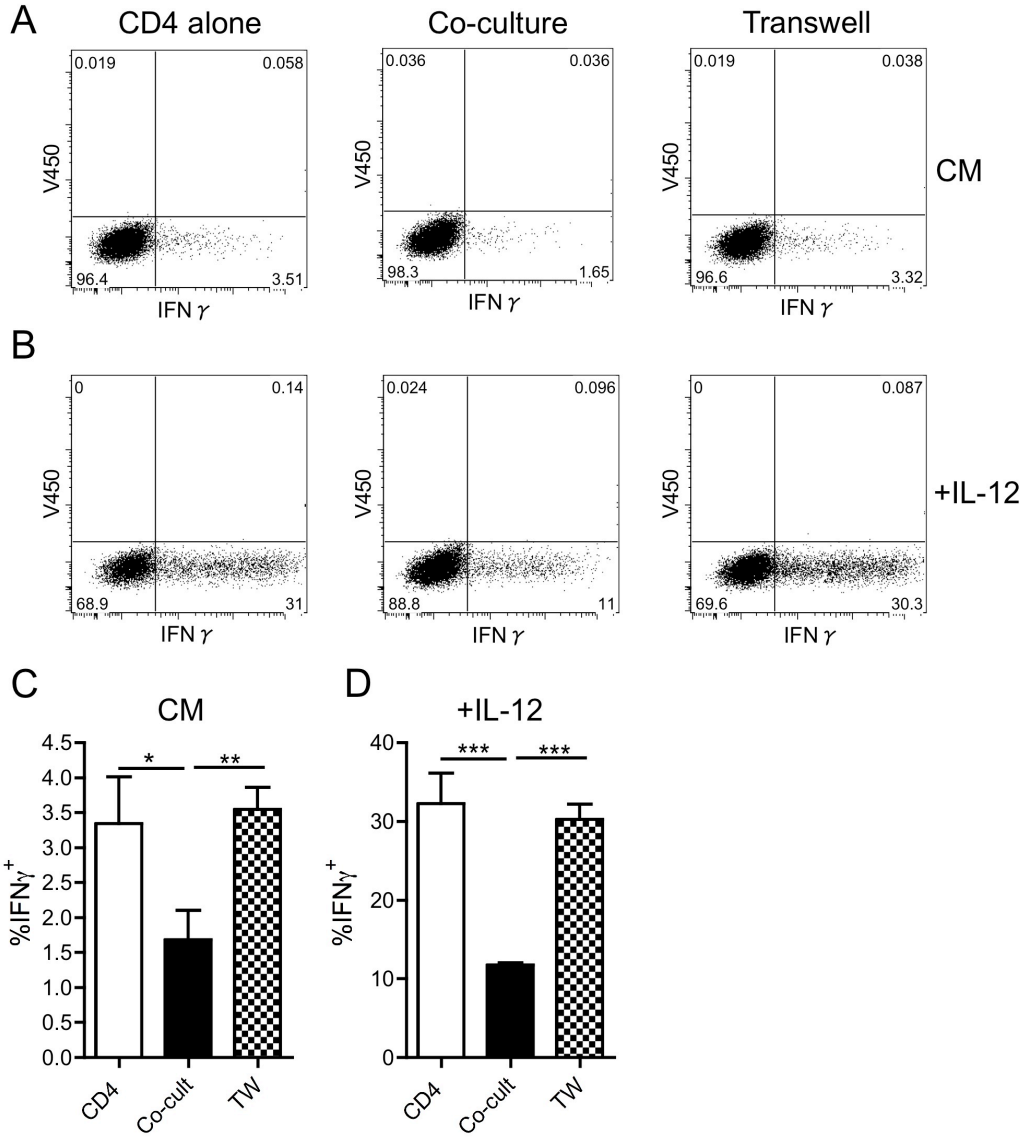

Figure 5-12 ILC inhibit CD4 T cell IFNγ production in a contact-dependent manner

Cells were co-cultured as before, on α-CD3/αCD28-coated plates for 72 hours, followed by PMA-I and GS treatment. The cells were stained for surface and intracellular markers and CD4 T cells were gated ($CD4^+ST2^-$). The proportions of $IFN\gamma^+$ cells were quantified in cells cultured in complete medium (CM) (A) or in the presence of IL-12 (10ng/ml) (B). Panels are representative of experimental triplicates for each condition. The percentage of $IFN\gamma^+$ cells in the experimental triplicates of CM-cultured (C) and IL-12-treated (D) was quantified. Error bars represent SD of experimental triplicates, representative of 3 experiments. *p<0.05, **p<0.01, ***p<0.001.

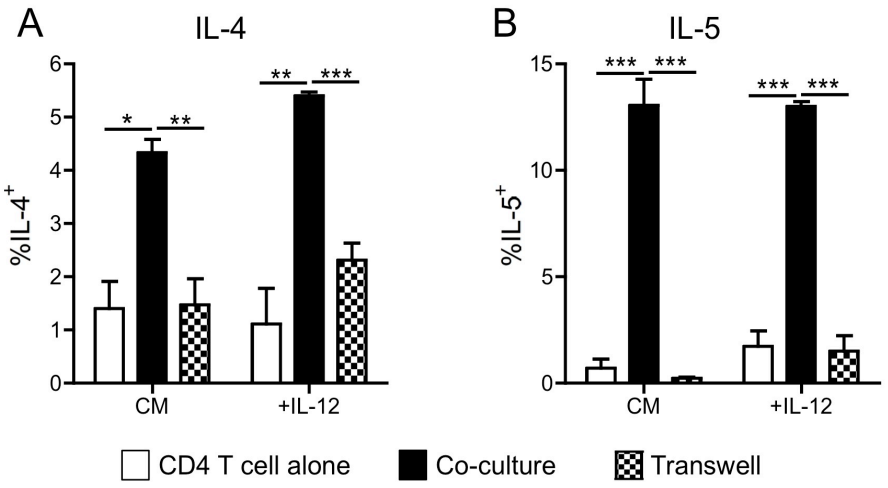

Figure 5-13 ILC are able to induce CD4 T cell expression of type 2 cytokines in Th1 polarising conditions

Cells were co-cultured as before, on α-CD3/CD28-coated plates for 72 hours, followed by PMA-I and GS treatment. The cells were stained for surface and intracellular markers and CD4 T cells were gated ($CD4^+ST2^-$). The proportions of $IL\text{-}4^+$ (A) and $IL\text{-}5^+$ (B) cells were quantified in cells cultured in complete medium (CM) or in the presence of IL-12 (10ng/ml) alone, in co-culture or separated by a semi-permeable membrane (transwell). Error bars represent SD of experimental triplicates, representative of 3 experiments. *p<0.05, **p<0.01, ***p<0.001.

5.2.3.5 Blocking ICAM-1 partially inhibits ILC-induced, contact-dependent CD4 type-2 cytokine expression

Since the effects of ILC on CD4 T cell cytokine production were mediated in a contact-dependent manner, it was likely that a surface receptor interaction was important. Whilst they have been shown to lack numerous common lineage markers, ILC have been shown to express a small number of cell surface receptors [139]. A search of the published literature suggested that ILC express intercellular adhesion molecule 1 (ICAM-1), as identified by microarray analysis [139]. Salomon *et al*. had demonstrated that neutralising Ab for ICAM-1 and ICAM-2 altered CD4 T cells' ability to produce type-2 cytokines *in vitro* [380]. The ligand for ICAM-1 is lymphocyte function-associated antigen-1 (LFA-1, also CD11a). It was therefore hypothesised that ILC-CD4 T cell ICAM-1-LFA-1 interactions could be important in ILC regulation of CD4 T cell type-2 cytokine production. In order to test this hypothesis, the levels of expression of LFA-1 (Figure 5-14A) and ICAM-1 (Figure 5-14B) by ILC were measured. FACS analysis demonstrated that ILC expressed high levels of both LFA-1 and ICAM-1 both when cultured in the presence or absence of T cells. Interestingly, the levels of ICAM-

1 expression were lower in the ILC cultured in the TW, when compared to ILC in co-culture (Figure 5-14C), suggesting that ILC ICAM-1 expression may be modulated in a contact-dependent manner. The expression of ICAM-1 was also measured in CD4 T cells (Figure 5-14D), yet no difference in ICAM-1 expression was found in the CD4 T cells in the different culture conditions.

In order to determine if ICAM-1 played a role in the ILC-CD4 interaction, the levels of type-2 cytokines expressed by CD4 T cells were determined following co-culture with ILC in the presence or absence of an ICAM-1 blocking antibody. Interestingly, whilst control IgG did not have significant effects on either IL-4 or IL-5 expression by CD4 T cell, blocking ICAM-1 led to a small but significant reduction in the proportions of IL-4- and IL-5-expressing CD4 T cells in co culture (Figure 5-14E) ($p=0.0053$ and $p=0.0289$, respectively). These data suggests that ICAM-1-LFA-1 interactions are important in ILC-driven induction of CD4 T cell expression of type-2 cytokines *in vitro*.

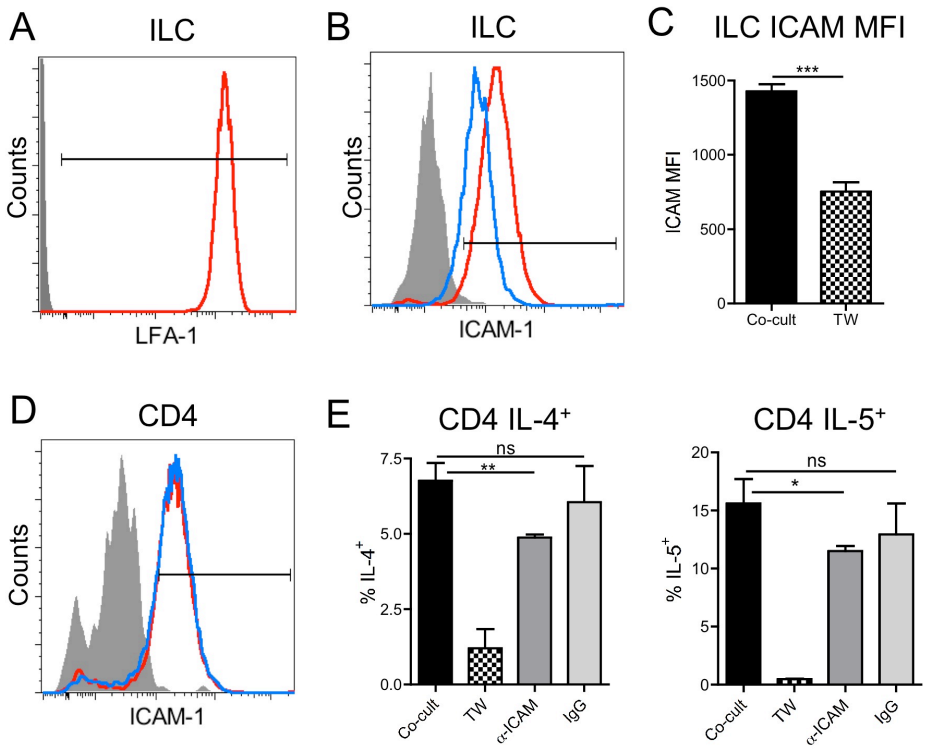

Figure 5-14 ICAM-1 is important in ILC-CD4 T cell interactions

ILC were freshly sorted and expression of LFA-1 was measured by FACS (filled gray= Isotype control, solid black line= ICAM-1) (A). ILC and CD4 T cell were cultured as before and ILC expression of ICAM-1 was determined following culture in co-culture (red solid line) or in transwell (TW) (blue solid line) (B). Mean fluorescence index was calculated for ILC ICAM-1 expression in experimental triplicates (C). ICAM-1 expression in CD4 T cells in co-culture (red solid line) and TW (blue solid line). Isotypes in filled in gray. ILC and CD4 T cells were cultured as before, for 72 hours, in the presence or the absence of ICAM-1 blocking antibody (10μg/ml) or control IgG (10μg/ml). Expression of IL-4 and IL-5 was measured in $CD4^{+}ST2^{-}$ cells following PMA-I and GS stimulation followed by intracellular staining (E). Error bars represent SD of experimental triplicates. *p<0.05, **p<0.01, ***p<0.001.

5.2.4 Impact of ILC-CD4 T cell interactions in vivo

Having shown that ILC could modulate T cell cytokine production *in vitro*, it was important to ascertain the impact of ILC on CD4 T cell polarisation and activation *in vivo*. The experiments detailed below were designed to answer this question.

5.2.4.1 Double adoptive transfer model

In order to test the influence of ILC on CD4 T cell activation *in vivo* an adoptive transfer model was designed. DO11.10 TCR transgenic mice, that express a major histocompatibility (MHC) Class II restricted TCR specific for a peptide

derived from ovalbumin (ova), were used as the source of CD4 T cells for the transfer. DO11.10 CD4 T cell can be tracked using a mAb specific for the clonotypic TCR (KJ-126). Since, in previous experiments (Figure 5-1, Figure 5-2), ILC activation with IL-33 appeared to be important *in vivo*, the decision to use *St2*$^{-/-}$ mice as recipients, was made in order to avoid effects of IL-33 on host cells. The model is described in Figure 5-15, but briefly: 2 groups of *St2*$^{-/-}$ mice received ova-specific KJ-126^{+} DO11.10 CD4 T cells intravenously and an intranasal challenge of 1μg IL-33+ 100μg ova. Only one group received WT ILC concurrently intravenously, alongside the CD4 T cells. Mice were killed after 5 days. Lungs and mLN were collected for total cell counts, CD4 counts, DO11.10 T cell counts and lung CD4 intracellular cytokine measurements, by FACS. Additionally, both total lung and mLN cell preparations were used for antigen (Ag)-recall responses *in vitro*, followed by cytokine measurement from the supernatants. The model therefore tested not only the ability of ILC to interact and enhance CD4 T cell activation *in vivo*, but also their ability to induce the recipient CD4 T cells to produce cytokines and the strength of Ag recall responses.

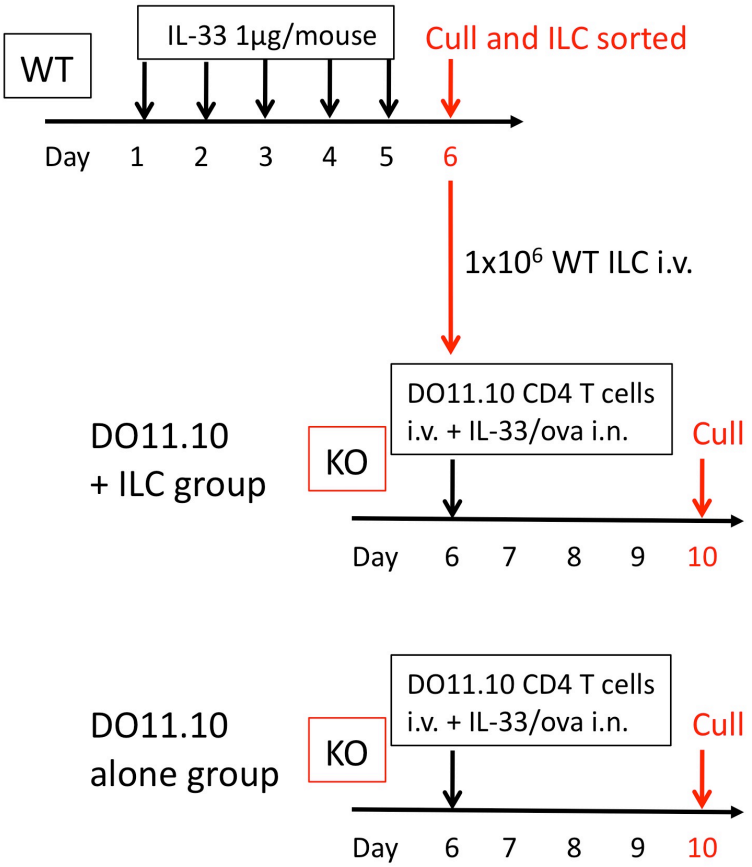

Figure 5-15 Double adoptive transfer model

WT ILC were sorted as described in Chapter 4. The ILC ($1x10^6$) were adoptively transferred into *St2*$^{-/-}$ mice intravenously (i.v.) alongside magnetic-bead-sorted DO11.10 CD4 T ($0.5x10^6$ cells/ mouse). A further group of *St2*$^{-/-}$ mice received only DO11.10 CD4 T cells. Both groups were treated intranasally (i.n.) with 1µg IL-33 and 100µg ova. The mice were killed as described previously after 5 days. Lungs and mLN were harvested.

5.2.4.2 ILC increase recipient CD4 T cell activation and cytokine production as well as DO11.10 T cell recovery in the lung

Mice in the DO11.10 CD4 T cells + ILC group had increased total lung cell counts (Figure 5-16A) compared to mice receiving DO11.10 T cells alone. Despite this increase in total lung cells, the number of recipient mouse CD4 T cells in the lung was not increased (Figure 5-16B). Interestingly, however, the proportion of host CD4 T cells expressing IL-4, IL-5 and IFNγ in the lung were all significantly increased in the lungs of recipient mice in the DO11.10 T cells+ILC group (Figure 5-16C) (p=0.0152, 0.0129 and 0.0263, respectively). Additionally, when the host CD4 T cells were assessed for their expression of CD44, the mice that had received DO11.10 T cells+ILC had increased proportions of activated CD44^{hi} CD4

T cells (Figure 5-16D). This increase was statistically significant (p=0.0109) compared to mice that received DO11.10 CD4 T cells alone (Figure 5-16E).

Importantly, following i.v. transfer, ILC were found to migrate to the lungs of recipient mice (Figure 5-16F). Notably, the proportion of KJ-126^{+} DO11.10 T cells in the lung of mice in the DO11.10 T cells+ILC group was increased 10-fold compared to control mice (DO11.10 CD4 T cells alone group) (Figure 5-16G). Furthermore, the increase in the number of KJ-126^{+} cells in the lung was highly statistically significant (0.0003) (Figure 5-16H). Taken together, these data indicate that transfer of ILC enhances both the host CD4 T cell and co-transferred DO11.10 T cell response to intranasal Ag.

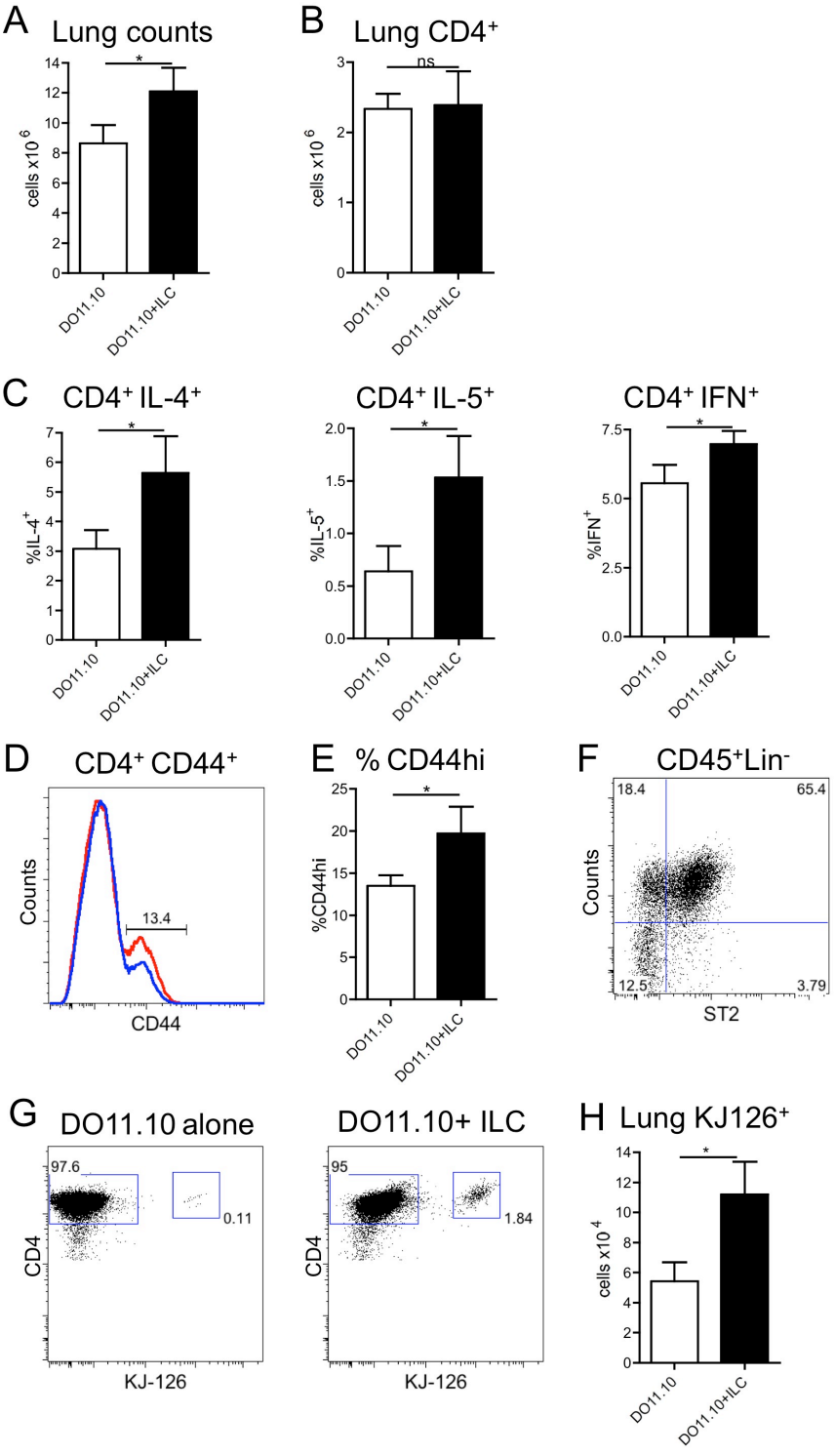

Figure 5-16 Co-transfer of ILC increases host and DO11.10 CD4 T cell responses to Ag *in vivo*

DO11.10 CD4 T cells ± WT ILC were transferred i.v. to *St2*$^{-/-}$ mice, followed by i.n. administration of IL-33 and ova. The mice were killed after 5 days and total lung cells counted (A). Cells were stimulated with PMA-I and GS for 4 hours followed by staining for surface and intracellular markers. The number of CD4 T cells were determined by FACS (B). Intracellular expression of IL-4, IL-5 and IFNγ (C) in recipient mice CD4 T cells were determined by FACS. Representative panel of levels of CD44 expression in recipient lung CD4 T cells (D) (mice that received DO11.10 cells in solid blue, mice that received ILC+DO11.10 cells in solid red). Proportions of CD44hi CD4 T cells in the lung (E). Homing of ILC to the lung (F) (lung cells gated as described in Chapter 4).

Increased numbers of DO11.10 CD4 T cells in the lung of concurrently transferred ILC (G). Representative panels for each group shown. Quantification of DO11.10 T cells in the lung of each group. Error bars represent SD for individual mice (n=4/ group). Data are representative of 3 similar experiments. ns= not significant, *p<0.05, ***p<0.001.

5.2.4.3 Increased mLN cell counts and CD4 T cell numbers in mice receiving ILC+DO11.10 CD4 T cells

The mLN were also assessed to determine the effect of ILC on T cell responses in lung draining lymph nodes. Interestingly, the total numbers of cells in the mLN were increased in mice receiving both ILC and DO11.10 T cells (Figure 5-17A), as was the number of host (KJ-126$^-$) CD4 T cells (Figure 5-17B). There was also a trend for an increase in the proportion of activated CD4 T cells, denoted by their expression of high levels of CD44, however, this difference did not achieve statistical significance (Figure 5-17C). Whilst the proportion of mLN KJ-126$^+$ T cells was similar in both groups (Figure 5-17B), the absolute number of DO11.10 cells recovered from the mLN of mice that received ILC was significantly increased ($p=0.0155$), similar to the increased number of KJ-126$^+$ cells present in the lung (Figure 5-17D).

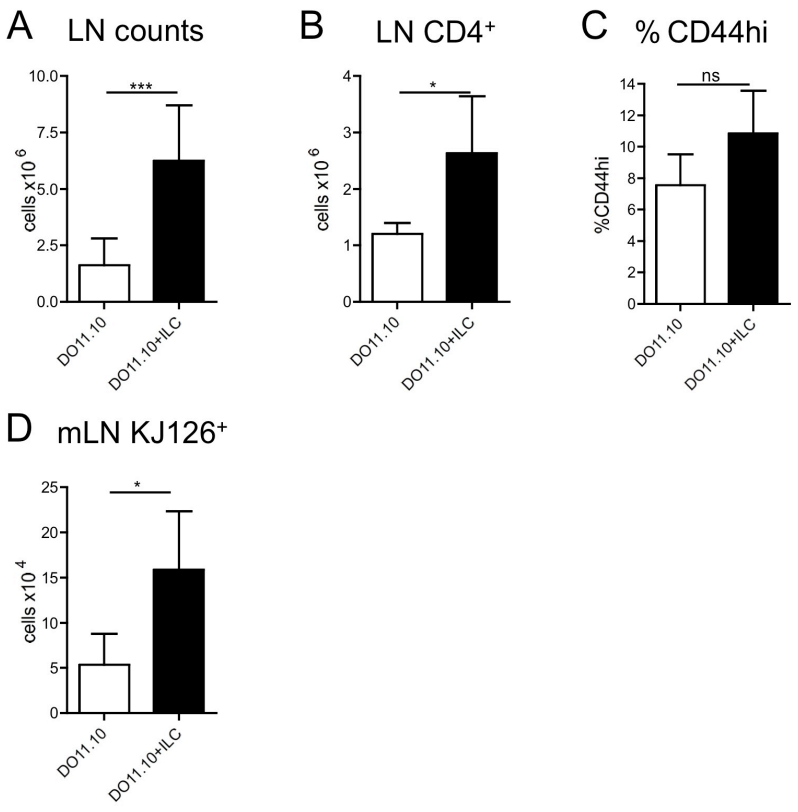

Figure 5-17 Increased cell counts and CD4 T cells in mLN of *St2*$^{-/-}$ mice that received DO11.10 cells +ILC

St2$^{-/-}$ mice underwent the model as described above. mLN of recipient mice were dissociated mechanically and total cell counts assessed (A). Total cells were stained with surface markers as before and total CD4 T cells measured (B). DO11.10 T cells +ILC cells had increased numbers of CD4 T cells in mLN. Levels of CD44 expression in recipient CD4 T cells quantified (C) and number of KJ-126^{+} cells determined (D). Error bars represent SD from individual mice (n=4-5/ group). Data are representative of 2 pooled experiments. ns= not significant, *p<0.05, ***p<0.001.

5.2.4.4 Enhanced cytokine production in Ag-recall assays of lung and mLN of mice receiving ILC+DO11.10 T cell transfers

In order to measure the magnitude of the DO11.10 CD4 T cell recall response to their cognate Ag, cells from both lung and mLN were cultured with CM alone or varying concentrations of ova peptide 323-339, the cognate antigen for the DO11.10 TCR. When equal numbers of total cells were used in recall assays, Ag-induced cytokine production was elevated in the lung cell cultures derived from mice that received ILC. In particular, there were increased levels of IL-2 (Figure 5-18E) and IFNγ (Figure 5-18F) measured in the supernatants of the lung cultures of mice that received both ILC and DO11.10 T cells. It was important to ascertain what proportion of the cells in the starting cultures were stained by the KJ-126 mAb and would therefore be able to respond to the Ag. Importantly,

the proportion of KJ-126$^+$ cells was significantly increased (p=0.0064) in the lungs of mice that received ILC (Figure 5-18A). Therefore, the elevated levels of cytokine production by cells from these mice may simply reflect differences in the numbers of KJ-126$^+$ cells within the respective cultures. By contrast, the proportion of KJ-126$^+$ cells in the mLN of both groups were not significantly different and hence differences in the levels of cytokine production between groups were likely to reflect altered activation or differentiation rather than differences in the numbers of responding cells. In this regard, increased levels of IL-2 and IFNγ were measured in mLN cultures from the mice that also received ILC (Figure 5-18G and H). The baseline IL-2 levels were not significantly different in both groups, however the levels of this cytokine increased in a dose-dependent manner to a greater extent in the cultures from mice that received both DO11.10+ILC, than in the control group. This increase was 3.5 fold higher in mice that received ILC (Figure 5-18G). In a similar fashion, the amounts of IFNγ measured in cultures from mice that received ILC was the same at baseline to the control group. However, these increased upon Ag-stimulation and were significantly higher at the highest Ag dose, when compared to control mice levels (Figure 5-18H). Type-2 cytokines were also measured in these cultures, however no IL-4 was detected in them. Additionally, the levels of both IL-5 (*data not shown*) and IL-13 in both the lung (Figure 5-18C) and mLN (Figure 5-18D) cultures were substantially higher in the group of mice that had ILC adoptively transferred alongside DO11.10 CD4 T cells. However, since both ILC and CD4 T cells are able to produce these cytokines it is not at this stage possible to determine which cells in the cultures are responsible for the measured levels IL-5 and IL-13.

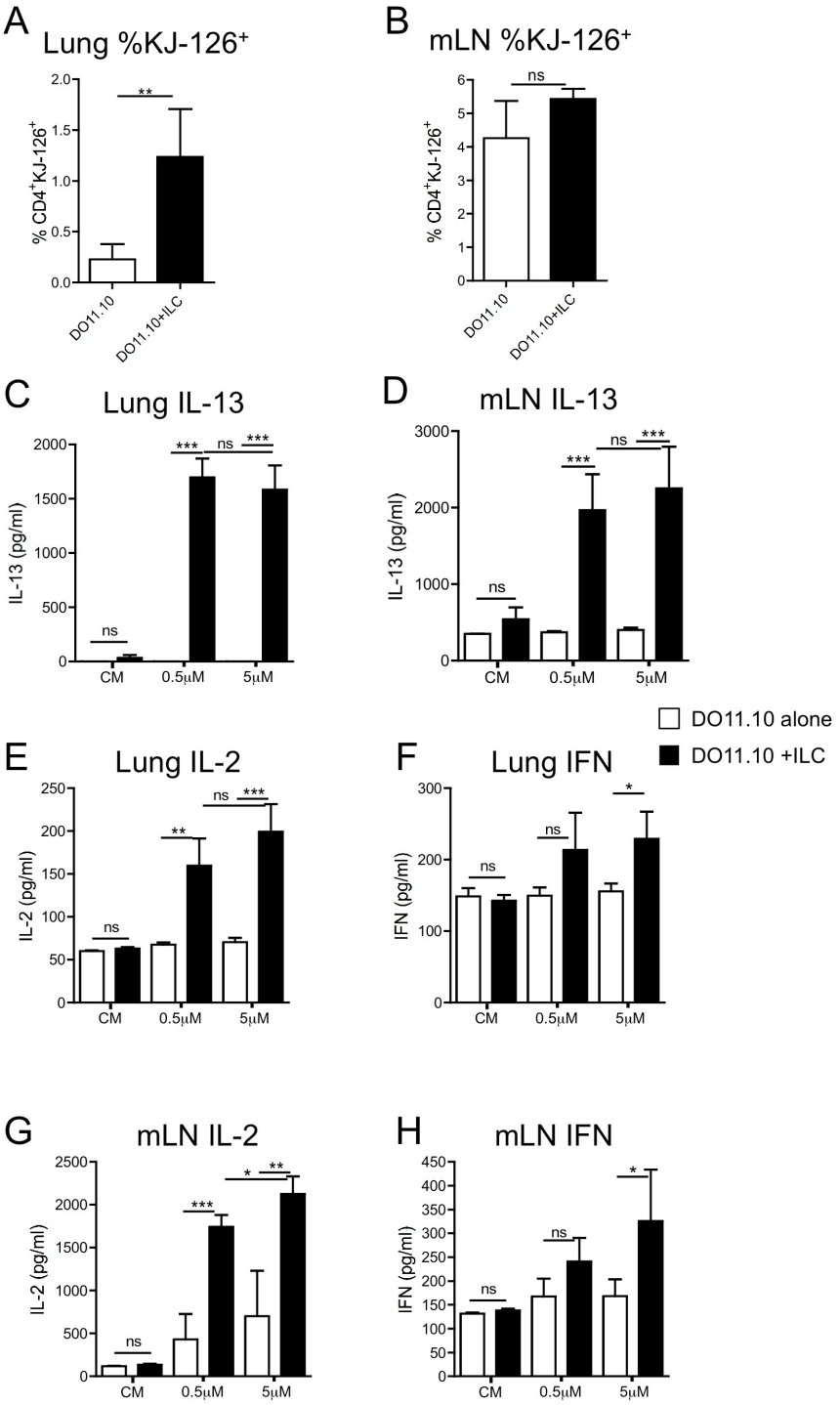

Figure 5-18 Elevated Ag recall responses of DO11.10 T cells from ILC-transfer mice

St2$^{-/-}$ recipient mice adoptively transferred with DO11.10 CD4 T cells± ILC were killed and mLN and lungs collected. Proportions of DO11.10 cells in lung (A) and mLN (B) were determined by FACS (KJ-126^{+} CD4^{+} cells). 1.5x10^{5} cells of either lung cells or mLN cells were cultured with complete medium alone (CM), 0.5μM or 5μM ova 323-339 for 72 hours and cytokines were measured by ELISA on culture supernatants. Lung (C) and mLN (D) culture supernatant levels of IL-13. IL-2 (E) and IFN (F) levels were measured in lung culture supernatants. IL-2 (G) and IFN (H) levels were measured in culture supernatants of mLN. Error bars represent SD of individual mice (A and B) and experimental culture triplicates for individual mice (C-H). Data are representative of 2 experiments. ns=not significant, *p<0.05, **p<0.01, ***p<0.001.

In summary, the results from these *in vivo* adoptive transfer experiments demonstrate a role for ILC in CD4 T cell activation in the lung and draining LN. More so, despite being transferred systemically, ILC migrate to the lung and enhance the numbers of Ag-specific CD4 T cells within this organ, suggesting that ILC may be important in regulating the pulmonary recruitment and/or expansion of adaptive immune cells. ILC also appear to be able to induce a milieu in which Ag-specific T cell responses are enhanced in the mLN, suggesting a supportive role for ILC in Ag-specific immune responses.

5.3 Conclusions

In this chapter the interactions between ILC and CD4 T cells were assessed. The experiments were prompted by the initial finding that intranasal transfer of ILC resulted in an increase in mLN cell numbers and an increase in the proportion of activated T cells in the draining lymph node. Whilst an increase in cell numbers was not entirely unexpected, since in Chapter 4 ILC were shown to be sufficient to drive IL-33-induced airway inflammation, the finding that ILC were able to affect CD4 T cell activation was interesting and unforeseen. A variety of markers for CD4 T cell activation were used to assess this effect, including CD44, CD25 and CD69. Whilst CD25 expression is up regulated in regulatory CD4 T cells [381] it can also be present in effector CD4 T cells [382], hence making CD25 a less reliable marker of activation. In a similar manner, CD69 expression is increased upon cellular activation. However, this is only transient [383] and it is therefore not the best marker for CD4 T activation. CD44 is a glycopeptide involved in cell trafficking and adhesion as well as regulation of apoptosis [384] that is stably up regulated in activated T cells [378, 379]. All T cells express this marker. However, naïve T cells express lower levels whereas activated cells express this receptor at higher levels [385]. Since the up regulation of this marker is permanent, it was a reasonable choice for assessing both CD4 T cell activation and as a tool to sort naïve CD4 T cells by FACS.

ILC are responsive to a variety of innate-derived cytokines, including IL-33, IL-25, TSLP and IL-7 [74, 139, 174, 214, 219]. The finding that they are also responsive to IL-2, links their function to the adaptive immune response. Whilst Wilhelm and colleagues demonstrated that IL-2 *in vivo* could alter the biological function of ILC [219], the results described above demonstrated that the

cytokines ILC express are dependent on the cytokine milieu. This may explain why some groups have reported that ILC produce the type-2 cytokine IL-4, whilst others have failed to do so [139, 214]. Interestingly, IL-2 and IL-33 synergise to induce IL-5 and IL-13 production by ILC, in a similar fashion to the effects of IL-7 and TSLP with IL-33, described in chapter 4. Furthermore, synergistic effects of IL-2, IL-7 and TSLP with IL-33 on Th2 cell cytokine production have previously been reported [214]. The importance of this synergy *in vivo* has yet to be elucidated. However, it would suggest that ILC are an important bridge between the innate and the adaptive immune responses. It is therefore likely that, whilst ILC can provide an innate immune response as a first line defence against organisms [139, 213, 214] or in allergic inflammation [74, 217, 219], this response is further enhanced and modulated by the recruitment of adaptive immune cells. More so, DC have also been shown to be a source of IL-2 early during microbial infections [386] and therefore it is possible that the synergy observed between IL-33 and IL-2 allows an escalation of the ILC immune response even in the absence of activated T cells.

Culture in the presence of IL-2 followed by PMA-I re-stimulation induced IL-4 expression by ILC, yet this was inhibited by IL-33 suggesting that ILC IL-4 expression is under tight control. This is further supported by the fact that *in vitro* stimulation of ILC with IL-2, without PMA-I re-stimulation did not result in detectable levels of IL-4 being secreted into culture supernatants. Therefore, whilst IL-2 was able to polarise the ILC into an IL-4-'competent' phenotype, it was not sufficient to drive IL-4 protein production. It is likely that an additional stimulus is therefore required to allow its release from the cells.

The Stockinger lab had shown that IL-2 was essential for ILC-derived IL-9 production *in vivo* [219], yet the levels of IL-9-expression in ILC cultured with IL-2 were very low. This difference may simply be due to the lack of the additional stimuli that were available *in vivo*. An additional difference lies on the type of mice used by Wilhelm and colleagues in their experiments. The group used an IL-9 fate-reporter mouse and hence, by definition, ILC would be marked by expression of enhanced yellow fluorescent protein (eYFP) at any stage after the IL-9 gene had been expressed, irrespective of the cell's current IL-9-production status. More so, they demonstrated that ILC-derived IL-9 was an early event during the onset of inflammation and that their IL-9 production gradually

diminished over time, to be replaced by IL-5 and IL-13 secretion [219]. It is possible that the ILC in the experiments described in this chapter expressed IL-9 early on but that expression was lost in favour of other type-2 cytokine such as IL-4, IL-5 and IL-13. Importantly, the ILC sorted for these experiments had received IL-33 for 5 days *in vivo*, and therefore, these cells may have expressed IL-9 prior to sorting. Further experiments assessing different culture time points, sorting ILC from WT, untreated mice or even using IL-9-fate reporter-derived ILC would be informative.

Since the biological effects of IL-2 on ILC had been assessed *in vitro*, it was exciting to confirm the similar effects that co-culturing ILC with CD4 T cells had on their function. The results of the co-culture experiments confirmed that CD4 T cells were able to support ILC proliferation and IL-4 expression via the production of IL-2. The use of an IL-2 neutralising antibody was useful. However, a more definitive method to confirm these results would involve using ILC derived from mice lacking IL-2Rα [387], which would make them unresponsive to the IL-2 produced by activated CD4 T cells. These mice were not available, however.

Whereas CD4 T cells were able to induce ILC proliferation, the converse was not observed. The CD4 T cells proliferated in response to α-CD3/α-CD28 treatment and co-culturing them with ILC did not enhance their level of proliferation. It must be noted, however, that the mitotic stimulus conveyed by the TCR-stimulation used was substantial and therefore it could potentially mask any effect induced by the ILC. Additional experiments titrating the level of TCR activation could clarify this point.

One of the most interesting effects of co-culturing ILC with naïve CD4 T cells was their ability to enhance the T cells' expression of type-2 cytokines. Since ILC were able to express IL-4 in co-culture with CD4 T cells, it was possible that the effect of the ILC on T-cells was a result of ILC IL-4 production. Whilst this was the initial hypothesis, it became clear that this was unlikely to be the mechanism whereby ILC exerted their influence on the T cells. Firstly, as aforementioned, no IL-4 was detected in the culture supernatants of stimulated ILC nor in co-culture with CD4 T cells, suggesting that, whilst the ILC were capable of IL-4 production, they did not release it in these culture conditions.

Secondly, the finding that separating the cells with a semi-permeable membrane completely abrogated this effect, confirmed that a soluble factor, such as IL-4, was not sufficient for this phenomenon. Notably, whilst promoting a Th2 cytokine profile by a contact-mediated mechanism, ILC also inhibited the expression of the Th1-cytokine, IFNγ. This effect was also abrogated upon cell separation by TW in culture, further substantiating the importance of cell-to-cell contact. More so, ILC also impacted upon CD4 T cell cytokine production when cells were cultured in a Th1 milieu, with the addition of IL-12 to the culture media, i.e. a contact-mediated reduction in IFNγ expression and concomitant increase in Th2 cytokine expression.

Using microarray data published by others [139, 214], the decision to test the possibility that ICAM-1 could mediate the ILC-CD4 interactions was made. ICAM-1 is an adhesion molecule which can be expressed by a variety of cell types including T cells, B cells, eosinophils, macrophages, DC as well as stromal cells including endothelial and epithelial cells [388]. ICAM-1 ligands are integrins that allow cell-to-cell contact and include LFA-1 and macrophage antigen (Mac)-1 [388]. Investigators have demonstrated increased expression of ICAM-1 in the airways of asthma patients [389, 390] and on peripheral blood CD4 T cells, following allergen exposure [391]. Interestingly, allergen exposure via bronchoscopy, significantly increases local cellular recruitment (eosinophils, neutrophils, $CD3^+$ cells) as well as ICAM-1 expression in the treated area, within 6 hours of exposure, compared with control areas (treated with saline only) in the lungs of the same asthma patient [390]. This is mirrored by an up regulation of LFA-1 expression in stromal cells [390] suggesting that ICAM-1/LFA-1 interactions are enhanced in allergy [390, 391]. Interestingly, the Bluestone laboratory had demonstrated that blockade of ICAM-1 led to reduced Th2-type cytokine production *in vitro*, when co-cultured with DC [380]. However, others have shown the opposite [392]. In fact, ICAM-1 blockade in *in vivo* models of allergic airway inflammation have been shown to be therapeutic [393]. Rhinovirus (RV) infections have been linked to asthma development in childhood [394] and are a common cause for asthma exacerbations [215]. RV can bind ICAM [388] and hence its role in asthma pathogenesis has been linked to the expression of this molecule [394]. T cells have been shown to express both LFA-1 and ICAM-1 [388] and ILC also express both these molecules on their surface,

as shown above. This would suggest that the cross-talk induced by these molecules could be bi-directional in the co-culture. Blocking the ICAM-1/LFA-1 interactions in the co-culture significantly reduced the effects of ILC on CD4 T cell cytokine expression. This reduction was only partial, however, suggesting that there may be other cell surface molecules contributing to these effects. It is interesting that the effects of ILC on the CD4 T cells was contact-dependent, whereas, the effects of the T cells on the ILC was not since the presence of the TW membrane did not affect T cell-induced ILC IL-4 expression (*data not shown*). More so, the effect of T cells on ILC was blocked by neutralising the soluble factor, IL-2, demonstrating the variety of mechanisms in play mediating the ILC-CD4 T cell cross-talk.

It was interesting to observe that ILC were able to impact upon the adaptive CD4 T cell response. There are other important examples of such interactions in asthma. In particular, there has been much interest in the way DC can help define adaptive immune responses depending on their priming milieu [70, 395, 396]. The interactions between DC and T cells are not solely Ag-driven, but also rely on DC expression of co-receptors that induce CD4 T cell activation, including OX40L (CD134) [70] or RELM-α (also known as found in inflammatory zone 1) [395]. The interactions of DC expressing these receptors with their ligands on CD4 T cells have demonstrated that CD4 cells develop into particular phenotypes following the cues offered by the innate immune system. Interestingly, OX40L is up regulated by DC following treatment with TSLP *in vitro* [70] and *in vivo* [397], and this co-receptor allows priming of T cells to a Th2 phenotype that drive airway inflammation [397]. Neill and colleagues had demonstrated that gut ILC did not express OX40L [139]. However, it was possible that lung ILC could express this protein or up-regulate its expression in co-culture. This possibility was tested, however, experiments performed demonstrated that freshly sorted lung ILC did not express OX40L and co-culture did not induce its expression (*data not shown*). More so, using a blocking antibody for OX40L during co-culture, there was no reduction in the ILC-induced T cell type-2 expression levels (*data not shown*). These data show that, unlike DC, ILC do not enhance CD4 Th2 responses through OX40L-OX40 interactions.

The importance of direct ILC-CD4 T cell interactions *in vivo* were difficult to measure, since isolating the effects of ILC on only one population of cells is impossible *in vivo*. The double adoptive transfer model was designed to be able to assess the effect of ILC on the response of CD4 T cells to Ag, hence the use of DO11.10 CD4 T cells. Preliminary *in vivo* experiments performed assessed effects of ILC on CD4 cells in the spleen, following i.v. cell transfers and intraperitoneal (i.p.) Ag and IL-33 injection. Interestingly however, no ILC were found in either the spleen or the mesenteric LN. These cells were found in the lung, suggesting that lung-derived ILC will preferentially home back to the lung when transferred systemically. As a result of these observations, the model was modified to include an i.n. challenge following i.v. cell transfer. The results of this model demonstrated that ILC were not only able to home the lung, but also increase the number of Ag-specific CD4 T cells in the lung. It is difficult to ascertain if the increased numbers of KJ^+ cells found in the lungs of mice receiving ILC was due to these cells being recruited in the lung, or due to increased local proliferation and / or cell survival as any of these processes alone or in combination could explain this effect. Further experiments transferring CFSE-labelled cells could be informative, however, the model would have to be shortened as the dilution of this marker after 5 days is likely to be too great to allow accurate assessment of proliferation.

Interestingly, ILC were unable to drive CD4 T cell proliferation *in vitro,* yet elevated numbers of DO11.10 CD4 T cells were found in the lungs and mLN of mice that also received ILC. It is likely that the reductionist *in vitro* experiments did not reflect the *in vivo* milieu. The presence of additional cytokines and cell types in the *in vivo* experiments is likely to explain the differences in the ability of ILC to support T cell expansion *in vitro* and *in vivo*. Furthermore, ILC have been shown to express low levels of major MHCII [139], suggesting that these cells may be able to present Ag. Improved Ag-presentation by lung ILC could enhance DO11.10 CD4 T cell proliferation and additional experiments to assess this possibility would be informative.

Another exciting finding of the *in vivo* model was the increased cytokine levels measured in the recipient CD4 cells in the lung. Whilst this is likely to represent an ability of ILC to enhance the host T cell response to ova, since ILC produce

large amounts of type-2 cytokines when stimulated with IL-33, it is possible that this contributed to the activation of lung CD4 T cells in a direct manner.

The elevated levels of cytokine production measured in mLN cell Ag-recall assays demonstrated the ability of ILC to drive CD4 T cell activation. In this regard, it was interesting to see that mice that had received ILC had greater Ag-induced recall responses than mice that only received KJ^+ cells. Unfortunately, measurement of intracellular cytokine expression by mLN cells using FACS was not possible due to limited numbers of cells and hence, whether ILC were able to induce a Th2 or Th1 response in T cells was difficult to confirm. This is due to the high levels of type-2 cytokines measured in the cultures of mice that received ILC. It is possible that the ILC are an important source of the IL-13 measured since, whilst the baseline levels were not very different in both groups, the levels of IL-13 measured in the cultures containing Ag were over 24 fold higher in the mice that received ILC, in the lung, and 2.8 fold higher in the mLN cultures. Whilst the increases in IL-13 measured in these cultures did not increase in an Ag dose-dependent manner, the levels were increased, from baseline in the cultures containing Ag. ILC had been found, following i.v. transfer, in the lung of recipient mice and since IL-2 was shown to enhance their proliferation and cytokine production, it was possible that CD4 T cell-derived, Ag-induced IL-2 was sufficient to drive ILC IL-13 production in the lung cultures. ILC were not identified by FACS in the mLN (*data not shown*) however, so the effect of IL-2 on ILC-induced cytokine production does not completely explain the phenomenon seen in the mLN. Since ILC do not appear to be able to produce IFNγ or IL-2 (*data not shown* and [139]) it is clear from the ELISA results that the activation of the Ag-specific CD4 T cells were responsible for the cytokine levels measured. The measured type-2 cytokines appeared to increase in response to Ag, however the precise cells responsible for that effect would require single cell-type assessment and intracellular cytokine expression measurement by FACS.

Whilst ILC were able to inhibit CD4 T cell expression of IFNγ in the *in vitro* co-culture experiments, they enhanced their production of this cytokine in the *in vitro* Ag-recall assays as well as by intracellular staining in recipient lung CD4 cells of the *in vivo* model. Whilst the *in vitro* environment is very controlled,

there will be numerous cell interactions occurring in the lung during the *in vivo* model. Indeed, as described in chapter 4, ILC were sufficient to drive IL-33-induced airway inflammation, driving the activation and recruitment of numerous cell types that could affect the lung CD4 T cell responses. In this respect, the ILC were able to influence the proportion of lung CD4 T cells expressing type-2 cytokines, more so than IFNγ levels.

In the double cell transfer experiment, IL-33 was included in the model since this appeared to be important in the effects of ILC seen *in vivo*. In the *in vitro* experiments, IL-33 was not used since preliminary results showed no improvements in the effects of ILC on CD4 T cells in the co-culture models (*data not shown*). The DO11.10 CD4 cells transferred in the double adoptive transfer model were $ST2^{+/+}$, and hence capable of responding to IL-33. Whilst both groups received the same IL-33-treatment, ideally, the *in vivo* transfer experiments should be conducted using Ag-specific CD4 T cells derived from $St2^{-/-}$ mice to eliminate this variable. Additional experiments could also be performed *in vitro* using both ILC and CD4 cells from WT mice, treated with IL-33, to ascertain if the interactions between the two cell types differed when both were able to respond to this cytokine.

In conclusion, the results described in this chapter demonstrate that CD4 T cells influence ILC function and cytokine production via IL-2. Additionally, ILC are able to induce a Th2 phenotype, whilst inhibiting a Th1 phenotype, on CD4 T cells in a contact-dependent manner *in vitro*. This process is partially dependent on ICAM-1/LFA-1 interactions between the cells. Finally, ILC enhance the cytokine production and activation status of CD4 T cells in the lung and mLN *in vivo*.

6 Final discussion

6.1 mTOR activation in IL-33 signalling

The results shown in this thesis demonstrate the importance of mTOR activation in IL-33 biology. The finding that IL-33-induced airway inflammation can be inhibited by rapamycin demonstrates that the cells in the lung responding to IL-33 require mTOR activation to drive inflammation. It was interesting to discover that the main cells producing type-2 cytokines in the lung in response to IL-33 were the newly described cell type, type-2 innate lymphoid cells (ILC) and additionally, these cells were sensitive to rapamycin-induced mTOR inhibition *in vivo*. The *in vitro* experiments described in chapter 4 demonstrate that ILC are able to respond to IL-33 stimulation directly. Furthermore, in keeping with the *in vivo* findings, their ability to produce type-2 cytokines in response to IL-33 *in vitro* is diminished by mTOR inhibition. This inhibitory effect appears to be at a post-transcriptional level, since IL-33-induced transcripts of *Il-13* are unaffected by rapamycin. These data therefore, in combination, demonstrate a previously unknown role for mTOR in IL-33 signalling in lung ILC. Based on the results in this thesis and additional experiments performed by others in the lab group [75], an additional signalling pathway induced by IL-33 is therefore described in Figure 6-1.

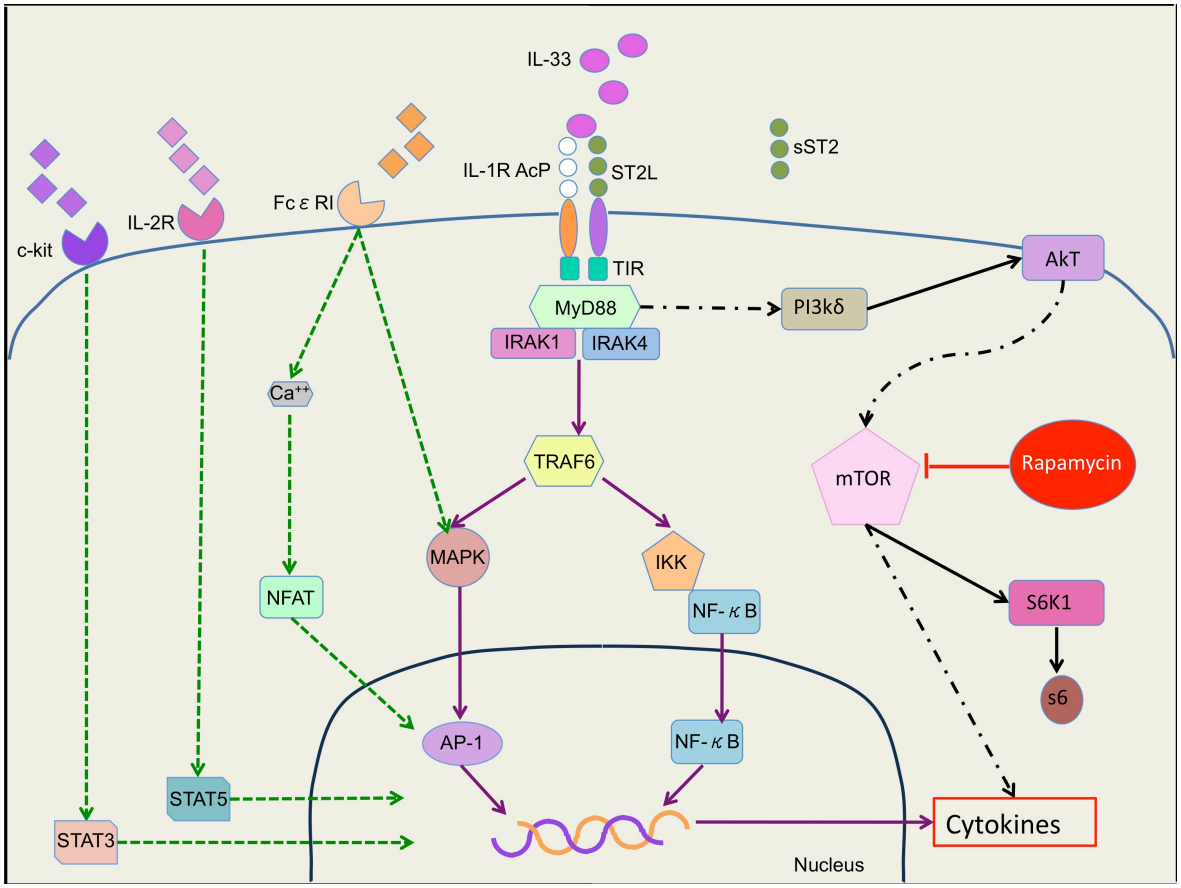

Figure 6-1 Summary of revised IL-33 signalling pathway including mTOR activation

The IL-33 signalling pathways as described previously are shown with the addition of the novel mTOR pathway in IL-33 signalling, as supported by data shown in this thesis, in solid and dashed black line.

6.2 IL-33 and ILC in asthma

Since its discovery, our understanding of the physiological roles of IL-33 has developed from the initial description as a type-2 cytokine [82] to include numerous other functions including its roles as an alarmin (reviewed in [159]), as well as a nuclear factor affecting gene transcription [196]. IL-33 has been implicated in numerous diseases, with both beneficial and detrimental effects, demonstrating the complexity of the interactions induced by this molecule. Its roles in allergy and asthma have been the subject of much research and the recent discovery of its importance in a genome-wide association study (GWAS) as a key gene associated with the risk of developing asthma [228] has placed IL-33 at the forefront of research in these disease processes. Additionally, the recent addition of a novel subset of innate helper cells [139, 213, 214] to the growing list of IL-33-responsive cells suggests that much is still to be learnt about the functions of IL-33 in health and disease. More so, ILC appear to have important

roles in the lung, both in the provision of homeostasis and in disease [74, 141, 174, 216-219, 398]. Furthermore, the possibility that their presence is associated with more severe forms of asthma [72] offers the exciting possibility of a previously overlooked pathogenic population of cells that could be targeted with better treatments.

As discussed previously, asthma is a heterogeneous disease and whilst the incidence and prevalence of this condition continue to increase, our ability to provide effective therapies to those who endure the most severe forms of this condition remains poor. These patients constitute a large socioeconomic burden but more importantly, they continue to be failed by the medical profession due to the lack of adequate, safe and effective interventions to treat them. Whilst corticosteroids (CS) remain the mainstay treatment for asthma, increasing understanding of the condition suggests that targeted immunomodulation may be required for those who fail to improve with maximal CS treatment.

6.3 mTOR inhibition in asthma

Rapamycin is a highly effective immunosuppressant in clinical use [326]. The growing interest and the increasing number of applications for mTOR inhibitors in clinical medicine makes the findings described in this thesis timely. Whilst the importance of mTOR in a variety of diseases is increasingly understood, the field of oncology is pioneering the use of drugs targeting the mTOR pathway in cancer (reviewed in [302]). These are important seminal studies addressing not only clinical effectiveness of the drugs but also safety, an important aspect given the significant rate of side effects associated with use of rapamycin and its derivatives.

Smoking is associated with significant co-morbidity in numerous diseases including asthma and chronic obstructive airways disease (reviewed in [13]). Within the population of asthma sufferers, one in four patients smoke [13]. Cigarette use has been shown to worsen lung function decline in these patients and hinder the therapeutic effects of the treatment regimens used in asthma [12]. The finding that mTOR inhibition, induced by cigarette smoke, is a key mechanism preventing lung repair demonstrates the importance of mTOR pathways in lung homeostasis [307]. These data additionally highlight the

limited understanding of the roles of the mTOR pathways in normal lung physiology. A further avenue to be explored in this subgroup of asthma patients is the role of ILC in smoking-induced lung damage. These cells have been shown to contribute to lung homeostasis and epithelial repair [141], yet their role, if any, in smoke-induced lung damage is unknown. More so, the role of mTOR in ILC-driven lung repair and homestasis is also yet to be determined.

The therapeutic use of mTOR inhibitors in allergy and asthma may be a possibility in the future. Certainly the data shown in this thesis and by others would support the use of mTOR modulation in allergic airways disease [303-305], however, better understanding of the roles of IL-33, mTOR and ILC in health and disease is still necessary. An important question raised by the experiments described in this thesis is the role of rapamycin in airway hyperresponsiveness (AHR). Whilst others have clearly demonstrated the therapeutic effects of systemically delivered rapamycin in mouse models of airway inflammation, the findings described in Chapter 3 suggest that mTOR inhibition could exacerbate AHR. There were limitations in these experiments due to the methods used to measure AHR, however, before mTOR modulation can be used in clinical practice, better understanding of the role of mTOR in AHR is required. IL-13 has been shown by numerous publications to be a key cytokine driving mouse AHR, and whilst rapamycin treatment reduced the levels of this cytokine in the lungs of IL-33-treated mice, there was no inhibition of AHR, highlighting the fact that the mechanisms driving AHR in mice are incompletely understood.

6.4 Important outstanding questions regarding IL-33-induced ILC in the lung

Whereas the discovery of a role for mTOR activation in IL-33-induced functions is novel, these data have also led to further important questions that need to be answered to drive the understanding of this field further. Whilst intranasal administration of IL-33 results in an increase in the number of ILC in the lung, these experiments and those performed by others have not addressed the question of the origin of these cells [74, 141, 174, 216, 217, 219, 398]. ILC have been shown to be of lymphoid origin [222], and they are systemically dispersed when mice are treated with systemic IL-25 or IL-33 [214]. Further experiments to ascertain whether ILC proliferate locally or are recruited from the bone

marrow or peripheral lymphoid organs would enhance our understanding of these cells.

An additional question that arises from IL-33-induced ILC induction in the lung is within what compartment of the lung are these cells found? This may be important since it may suggest the possible interactions with adjacent cells. Additionally, macrophages within different compartments in the lung have differing roles and phenotypes (reviewed in [399]), important to their function and this may be true for ILC too. Since the ILC in the experiments described in this thesis were assessed by FACS analysis of whole lung digests, it was impossible to pinpoint the location of ILC within the lung. Analysis of the BAL of IL-33-treated mice did show small numbers of ILC in this fluid, however ILC were not detected in the BAL of untreated control mice (*data not shown*). Following the findings described in chapter 5, it seemed that ILC preferentially relocated to the lung following i.v. adoptive transfer. It would therefore be informative to perform immunohistochemical (IHC) analysis of the lungs of mice receiving ILC to determine their location following the transfer. Since ILC do not express any lineage markers and the surface markers they do express are shared with other cell types, the cells would have to be labelled prior to transfer in order to be able to localise them within the lung.

The lack of a specific cell surface marker for ILC is an important hurdle when assessing their role in human disease. This is exacerbated by the difficulty in obtaining human lung tissue from both healthy controls and patients with asthma. ILC are found in very small numbers in untreated control mice and it is likely that this is also the case in humans. The difficulty in conclusively demonstrating the presence of ILC in human lung tissue was demonstrated by Shikotra and colleagues [72]. They showed the presence of IL-13^{+} cells in lung biopsies of patients with asthma and they suggested that they could be ILC. They demonstrated that these cells did not express markers for T cells, mast cells or macrophages, yet they were unable to show that these cells expressed any ILC-associated markers [72]. Much of the cell type assessment in human tissue is performed by IHC methods due to the limited tissue availability, however this restricts the number of markers that can be excluded from a cell type at one time. The Artis laboratory overcame this particular problem by using whole lungs that had been deemed unsuitable for organ donation to

establish the presence of ILC in human lung by FACS [141]. This method is not sustainable to assess the role of ILC in pulmonary disease and better markers to identify these cells are required.

As discussed above, ILC have been shown in the lung of non-asthma patients [141] and they have also been identified in the nasal polyps of allergic rhinitis patients [140]. These findings suggest a role for ILC in human asthma and allergy, however further research in this field is warranted to determine whether ILC are simply innocent bystanders or whether they have pathogenic roles in these conditions.

One of the important roles of ILC is their function in lung repair and homeostasis [141]. The immune system has evolved as a protective mechanism, however, its roles in dampening inflammation and driving restoration of normality following an insult are important too. Type-2 immune responses have been shown to be key mediators of this function (reviewed in [220]), and therefore the finding that ILC are able to aid epithelial growth following viral damage is interesting yet not entirely unexpected [141]. ILC are highly responsive to stromal-cell derived factors such as IL-33, IL-25 and thymic stromal lymphopoeitin (TSLP) and epithelial cells are able to produce these three factors [400] suggesting an important reciprocal relationship may exist between ILC and epithelial cells. This possible relationship has not been explored extensively and further studies to assess ILC-epithelial cell interactions could be informative, since epithelial cell dysfunction is a common finding in asthma [401].

6.5 ILC and CD4 T cell interactions *in vitro* and *in vivo*

The interactions shown in chapter 5 between ILC and CD4 T cells have demonstrated the important cellular cross-talk that is present in the lung in both health and disease. The demonstrated effects of CD4 T cells on ILC functions are consistent with previous work showing that ILC are responsive to IL-2 and their cytokine profile can be modulated by this cytokine [219]. It was rather more unanticipated to see the effects of ILC on the T cells. Interestingly, ILC are able to induce type-2 cytokine production in naïve CD4 T cells in a contact-dependent manner. This is partly mediated by ICAM-1-LFA-1 interactions as a blocking antibody for ICAM-1 significantly reduced this effect. Furthermore, ILC

are not only able to induce type-2 cytokine production, but also inhibit expression of the type-1 cytokine, IFNγ by CD4 T cells. The importance of Th2 cells in asthma and allergy has been extensively investigated and hence, the fact that IL-33-induced ILC are able to drive this adaptive immune response is interesting. This finding further supports an important role for IL-33 in asthma and offers a novel pathway whereby IL-33 can drive innate and adaptive immune responses in the lung. Whilst the effects of ILC on T cells were shown to be partially dependent on ICAM-1, the interactions of other co-receptors are also likely to be in play. The nature of these additional stimuli are unknown, however, further experiments to determine what other receptors are important in these interactions would enhance the understanding of this field. Furthermore, experiments to assess whether ILC can impact upon human CD4 T cell activation are also necessary.

The effects induced by ILC on CD4 T cells required stimulation of the TCR, but the effect of ILC on the response of effector CD4 T cell to cognate antigen was not assessed. ILC have been shown to express major histocompatibility complex (MHC) Class II [139] and preliminary experiments performed on IL-33-induced ILC demonstrated that they were able to present ova peptide to CD4 T cells from DO11.10 mice (that express ova-specific TCR) (*data not shown*). This finding raises the possibility of a further mechanism for ILC to modify the adaptive immune responses in the lung and future work specifically addressing the question of the antigen presentation capacity of ILC is fundamental.

6.6 Final comments

Taken together, the data presented in this thesis provide a contribution to the greater understanding of IL-33. Importantly, the results shown demonstrate a novel signalling pathway contributing to IL-33 functions *in vivo* and *in vitro*, as well as the role of mTOR in ILC cytokine-induced functions. These findings provide a possible avenue for future work, which may provide ideas for potential therapies for asthma. In addition, a novel mechanism for ILC-driven, contact-mediated induction of CD4 T cell type-2 immune responses has been shown, demonstrating the complex interactions of the innate and the adaptive immunity in the lung.

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