The Role of IL-33 and ST2 in Innate and Adaptive Inflammation

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Abstract

Inflammation is the body's response to injury and infection, and is aimed at eradicating the threat and repairing the tissue through the activation of the innate and adaptive immune systems. The innate inflammatory response is triggered by a surveillance network of broadly specific receptors. It is immediate and pre-programmed, and involves the activation of phagocytic cells and the release of soluble anti-microbial mediators to eradicate infection as quickly as possible. It can also promote the subsequent adaptive response. The adaptive inflammatory response is regulated by antigen-specific T helper (Th) lymphocytes, and has memory to provide immunity against repeat infection. These two arms of the immune system are often activated concurrently and communicate through the release of cytokines and chemokines to co-ordinate and regulate an appropriate response.

Th cells differentiate into various effector cell types and co-ordinate different responses, often directing innate immune cells to carry out effector functions. Th1 cells produce IFNγ and direct responses against intracellular pathogens through macrophage activation. Th2 cells produce IL-4, IL-5 and IL-13 and are important in resistance to parasites through mast cell and eosinophil activation. Th17 cells produce IL-17 and are important in sepsis through neutrophil activation. Dysregulation of these responses often contributes to the development of disease. Autoimmune diseases are characterised by Th1 and/or Th17 inflammation, whilst dysregulated Th2 responses result in allergy and asthma. Inflammatory diseases are often chronic and debilitating, and current therapies are either ineffective or have significant side-effects, so novel inflammatory mechanisms and therapeutic targets are required.

ST2L is a member of the IL-1 receptor family that was identified as a surface marker for Th2 cells, and is associated with Th2 cell activation and functions, although its precise role is unknown. IL-33 is a novel member of the IL-1 cytokine family, and has recently been identified as a ligand for ST2L. It promotes Th2 cytokine release and eosinophil accumulation *in vivo*. sST2 is a soluble form of ST2L that has broad immunosuppressive activity, although the mechanism is unknown. Therefore IL-33 and ST2 are potentially important inflammatory mediators, and in this thesis I aimed to demonstrate the role of IL-33 and ST2 in

innate and adaptive inflammation, using murine models of allergic airways disease and autoimmune arthritis.

I have shown that IL-33 administered directly to the airways provokes an innate Th2 type response in the lung with many characteristics in common with allergic airways disease. There was increased production of Th2 cytokines, except IL-4, chemokines and eosinophilic inflammation. ST2 gene knockout ($ST2^{-/-}$) mice demonstrated this response was dependent on ST2, and SCID mice demonstrated that whilst non-lymphoid cells were sufficient to initiate a response, lymphoid cells enhanced it. IL-33 exacerbated the effector phase of Th2 allergic airways inflammation, with increased eosinophils, Th2 cytokines and chemokines in the airway, and increased lymph node responses. $ST2^{-/-}$ mice had reduced inflammation in the airway, despite normal lymph node responses, suggesting the role of IL-33 and ST2 is more important in the tissues. Pilot data also suggested that IL-33 exacerbates Th1/Th17 autoimmune collagen-induced arthritis (CIA), with increased disease severity and lymph node responses. $ST2^{-/-}$ mice had reduced disease severity, again despite normal lymph node responses.

In summary, I have demonstrated that in the airway IL-33 is sufficient to induce a Th2 type innate response with pathological features similar to asthma. I have also shown that IL-33 can exacerbate Th2 mediated airway inflammation, and in addition, Th1/Th17 mediated arthritis. Thus it has general pro-inflammatory actions, and warrants further investigation to elucidate the mechanisms involved and fulfil its potential as a target for future therapeutic intervention.

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Abbreviations

-/-	gene knockout mouse
+/+	wildtype mouse
+/-	heterozygous mouse
AHR	airway hyper-responsiveness
APC	antigen presenting cell
BAL	bronchoalveolar lavage
Bq	becquerel
CII	collagen type 2
CCP	cyclic citrullinated peptide
CCR	CC chemokine receptor
CFA	Complete Freund's Adjuvant
CIA	collagen-induced arthritis
CNS	central nervous system
COX-2	cyclo-oxygenase 2
CXCR	CXC chemokine receptor
DC	dendritic cell
(c)DNA	(complementary) DNA
DLN	draining lymph node
DMARD	disease modifying anti-rheumatoid drug
EAE	experimental autoimmune encephalitis
ELISA	enzyme linked immunosorbant assay
Fab	fraction antigen binding (of immunoglobulin)
Fc	fraction crystallisable (constant portion of immunoglobulin)
FGF	fibroblast growth factor
αGalCer	α galactose ceramide
GM-CSF	granulocyte-monocyte colony stimulating factor
H&E	haematoxylin and eosin
HEV(EC)	high endothelial venule (endothelial cell)
HLA	human leukocyte antigen
HRP	horseradish peroxidase
hrs	hours
ICOS	inducible costimulatory molecule
ID	intradermal
IFNγ	interferon γ
Ig	immunoglobulin
IL-	interleukin
IN	intranasal
iNOS	Inducible nitric oxide synthase
IP-10	IFNγ inducible protein 10
IP	intraperitoneal
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
KC	keratinocyte-derived chemokine
LPS	lipopolysaccharide
LRR	leucine rich repeat
LT	leukotriene
µl	microlitre
µg	microgramme
MAMP	microbe associated molecular pattern
MCP-1	monocyte chemotactic protein 1
MDC	macrophage-derived chemokine
mg	milligramme

MHC	major histocompatability complex
MIG	monokine induced by IFNγ
mins	minutes
MIP1α	macrophage inhibitory protein 1α
ml	millilitre
MMP	matrix metalloproteinase
mV	millivolt
ng	nanogramme
NALP	NOD-LRR and pyrin containing protein
NBF	neutral buffered formalin
NFκB	nuclear factor kB
NK cell	natural killer cell
(i)NKT cell	(invariant) natural killer T cell
NLR	NOD-like receptor
NO	nitric oxide
NOD	nuclear oligomerisation domain
NS	not statistically significant
NSAID	non-steroidal anti-inflammatory drug
OPG	osteoprotegerin
OVA	ovalbumin
PADI4	peptidyl arginine deiminase 4
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
(RT)PCR	(real time) polymerase chain reaction
pg	picogramme
PG	prostaglandin
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RANK(L)	receptor activator for NFκB (ligand)
RANTES	regulated on activation normal T cell expressed and secreted chemokine
RF	rheumatoid factor
rm/rhIL-33	recombinant murine/human interleukin 33
rpm	revolutions per minute
SAH	subarachnoid haemorrhage
SCID	severe combined immunodeficient mouse
secs	seconds
SEM	standard error of the mean
sST2	soluble form of ST2
ST2L	membrane found form of ST2
TARC	thymus and activation regulated chemokine
TMB	3,3,5,5-tetramethylbenzidine
TCA3	T cell activation protein 3
TGFβ	transforming growth factor β
Th	T helper cell
TIR	toll/IL-1 receptor domain
TNFα	tumour necrosis factor α
TLR	toll-like receptor
TSLP	thymic stromal lymphopoietin
VEGF	vascular endothelial growth factor
WT	wildtype

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Declaration

This thesis represents original work carried out by the author, and has not been submitted in any form to any other University. Where materials or practical support have been received, due acknowledgement has been made in the text.

Peter Kewin

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Chapter 1

Introduction

1.1. Inflammation in health and disease

Inflammation is the response of the body tissues to any insult, such as infection, toxins, trauma, or ischaemia. It is a complex process involving activation of the resident tissue cells and recruitment of immune cells from the circulation, co-ordinated by the release of cytokines and chemokines, with resulting disruption to tissue structure and function. The process of inflammation is divided into distinct phases. The initial innate response is immediate and antigen non-specific, whilst the continued adaptive response is antigen-specific and has memory. Resolution of inflammation then aims to restore normal tissue structure and function. There is much overlap in these phases, and the same cells and mediators can have different roles in each phase. The innate and adaptive immune systems communicate throughout to direct the appropriate response to the insult.

The inflammatory process requires tight regulation to ensure an appropriate level of response, and then complete resolution. However, when the regulatory mechanisms break down chronic inflammation can arise, which leads to permanent tissue damage and dysfunction. Many human diseases are the result of chronic inflammation, contributing to much morbidity and mortality. Sensitisation to an exogenous antigen can result in allergic inflammation, typified by atopy and asthma. The immune system can also make inappropriate responses to self antigens, resulting in autoimmune disease, such as rheumatoid arthritis (RA). The underlying mechanisms of the dysregulation are not completely understood, and are likely to be multiple, which is reflected in the lack of effective treatments for many diseases. New mechanisms and targets for drug therapy are constantly being sought.

Interleukin 33 (IL-33) is a novel cytokine (1) with the potential to play an important role in inflammation. In this introduction I will discuss some of the key mechanisms of innate and adaptive inflammation, with particular reference to asthma and RA, and the possible role of IL-33 and its receptor, encoded by the *ST2* gene. This will identify this interaction as an important target for further study and potential new therapies.

1.2. Innate inflammation

The importance of the innate immune system is underlined by the fact that only vertebrates have adaptive immunity, so most organisms survive effectively with only innate immunity (2). The innate response is immediate and efficient, but has no facility for memory. It was originally thought to be non-specific, though it is now recognised that a network of broadly-specific receptors is in place to activate the inflammatory response. Once activated, the innate response aims to eradicate the stimulus (eg an invading organism) quickly and effectively, and if required activate and shape the adaptive immune system.

1.2.1. Epithelial defences

The epithelial surfaces of the skin, lung and gut are particularly vulnerable as they interface with a constantly changing environment. Epithelial cells are bound together by tight junctions to prevent micro-organisms passing between them, thus acting as a physical barrier. They are continually turning over, particularly in the skin and gut, and breaches are quickly repaired. In the lung, soluble microbicidal mediators, such as immunoglobulin A (IgA), enzymes, defensins, surfactant and complement are present in the mucus lining layer, which also serves to trap particles and micro-organisms and transport them out of the lung via mucociliary clearance. As the epithelial surfaces are constantly presented with potential threats, and therefore potential immune activation, the regulatory mechanisms are particularly tight, and often specific to the site. For instance, alveolar macrophages are constitutively suppressed by epithelial bound transforming growth factor β (TGFβ)(3), and the gut is able to distinguish between commensal and pathogenic bacteria, although the mechanisms for this are less clear.

1.2.2. Sensing and signalling danger

One of the crucial functions the innate immune system must perform is to determine when and how to initiate a response. The innate immune system must distinguish between self and non-self (4) and/or between danger and non-danger (5), through a ubiquitous network of receptors. Non-danger signals, such as self-antigens, and non-pathogenic commensal bacteria at epithelial surfaces, result in tolerance, whilst danger signals from invasion or tissue damage result in inflammation. Regulation is tight, particularly at epithelial surfaces, to prevent constant activation and tissue damage.

Sensing of pathogens relies on several principles. Microbes express a number of molecules and motifs which are highly conserved due to their critical roles in survival. These are termed pathogen associated molecular patterns (PAMPs), although they are not strictly limited to, nor do they define, pathogens. A complementary set of receptors known as pattern recognition receptors (PRRs) are encoded in the germline of organisms, which can bind to PAMPs and initiate immune responses. Their specificity is predetermined and often broad, and they do not undergo any gene rearrangement or somatic mutation. They fall broadly into two categories, those that are membrane associated, and those that are cytosolic.

1.2.2.1. Toll-like receptors - membrane associated PRRs

Toll is a protein involved in dorso-ventral organisation and defence against fungi in the fruit fly (*Drosophila melanogaster*). Homologous toll-like receptors (TLRs) are members of the pro-inflammatory Toll/IL-1R (TIR) family (6)(see section 1.6.2.), and have been identified as PRRs in many species, particularly humans and mice (7). Ligand binding initiates signalling leading to the activation of NFκB and pro-inflammatory gene expression, or type 1 interferon, regulated by TIR adaptor proteins (8). They are expressed on a wide variety of cells involved in both innate and adaptive immunity, and through modulation of cytokine, chemokine, and receptor expression can initiate innate responses (7) and modulate adaptive responses (9). Signalling through TLRs can also contribute to human disease (10). For instance, immunosuppression in sepsis can arise from TLR2 and TLR4 cross tolerance (11), and host DNA in immune complexes may be recognised by TLR9 and contribute to autoantibody production (12). TLR4 polymorphisms can result in hyporesponsiveness to lipopolysaccharide (LPS)(13), which increases susceptibility to Gram negative sepsis (14), but protects against atherosclerosis (15). Polymorphisms of TLR2 increases susceptibility to Gram positive sepsis (16).

1.2.2.2. NOD-like receptors - cytosolic PRRs

Many bacteria and viruses can invade the cytosol directly, thus bypassing detection by TLRs, and necessitating a different set of receptors to detect their presence and initiate responses. The NOD-like receptor (NLR) family includes over 20 members, and has some similarities with the TLR family (17). They all have an LRR domain for MAMP recognition, a nucleotide-binding oligomerisation domain (NOD), and a third, variable domain for signalling. Of this large family a

few have been found to have roles in specific diseases. NOD1 and NOD2 recognise components of peptidoglycan, and activate NFκB. Mutations in NOD1 are associated with asthma (18), and NOD1 and NOD2 mutations are associated with inflammatory bowel disease (19,20). NOD-LRR and pyrin containing proteins (NALPs) recognise a number of bacterial and viral PAMPs, and activate the inflammasome caspases 1 and 5 to increase processing and release of the pro-inflammatory cytokines IL-1β and IL-18. Mutations in NALP-3 result in IL-1-dependent auto-inflammatory disease, such as Muckle-Wells syndrome (21), characterised by recurrent systemic inflammation in the absence of infection.

1.2.3. Innate effector mechanisms

Virtually all cells express PRRs, in particular TLRs, and so may be directly activated by sensing pathogens or danger. Once danger has been recognised, cells of the innate immune system have a number of effector mechanisms designed to neutralise and eradicate toxins and micro-organisms, and to shape the subsequent adaptive immune response. Whilst a complete review is beyond the scope of this introduction, some of the key cells and functions are highlighted in table 1.1. In addition, non-immune cells, such as endothelial cells, epithelial cells, bronchial smooth muscle cells and fibroblasts express TLRs and cytokine receptors, and can be activated to release cytokines and chemokines. Endothelial cells can also upregulate adhesion molecules, and epithelial cells produce anti-microbial proteins such as defensins and surfactant in the lung.

CELL TYPE	MECHANISMS	EFFECTS
NEUTROPHIL	Phagocytosis Granule proteins and enzymes Reactive oxygen and nitrogen species	Microbial killing
	Granule proteins and enzymes	Host tissue damage
	Cytokines (especially IL-12)	Th1 cell differentiation
	Chemokines	Cell migration and activation
MACROPHAGE	Phagocytosis Reactive oxygen and nitrogen species	Microbial killing Removal of neutrophils
	Chemokines	Cell migration and activation
	Cytokines (TNFα, IL-1β and many others)	Cell activation T cell differentiation
	Antigen presentation (MHCII)	T cell activation
EOSINOPHIL	Granule proteins and enzymes	Parasite killing Host tissue damage
	Chemokines	Cell migration and activation
	Cytokines (especially IL-4)	Cell activation Th2 cell differentiation
	Antigen presentation	T cell activation
MAST CELL	Degranulation (FcεRI, FcγRIII, TLRs)	Rapid release of preformed mediators
	Histamine Lipid mediators	Increased blood flow and vascular permeability Increased adhesion molecules Th2 differentiation Bronchoconstriction Increased gut motility
	Proteases	Matrix degradation Host tissue damage
	Defensins	Microbial killing
	Chemokines	Cell migration and activation
	Cytokines (TNFα, IL-1β, IL-4 and others)	Cell activation T cell differentiation DC maturation
MYELOID DC	Phagocytosis	Antigen sensing and processing
	Antigen presentation (MHCII) Costimulatory molecules	T cell activation T cell differentiation
	Cytokines (IL-2, IL-4, IL-12 and others)	T cell differentiation NK cell activation
	Chemokines	Cell migration and activation
PLASMACYTOID DC	Type 1 interferons Cytokines and chemokines	Anti-viral response
NK CELLS	Perforin and granzyme "Missing self" (detects lack of MHCI)	Tumour lysis Infected cell lysis
	Cytokines (IFNγ, IL-4, IL-12 and others)	Macrophage activation T cell differentiation
NKT CELLS	Rapid early cytokine release	Cell activation T cell differentiation

Table 1.1 Innate effector mechanisms

1.3 Adaptive inflammation

In contrast to innate responses, adaptive responses are antigen-specific, and exhibit memory. Specificity is achieved by the random recombination of genes encoding T cell receptors (TCR) and antibodies, and somatic hypermutation of antibodies during a response. Memory arises from the sustained presence of antigen-specific cells capable of immediate and effective responses if the antigen is encountered in the future. There is huge complexity to the processes of T and B cell repertoire selection, tolerance to self antigens, antigen presentation, lymphocyte activation and maintenance of memory, and in the development of different T and B cell subsets. Here I will restrict discussion to the processes of $CD4^+$ helper T cell differentiation and effector function, the key regulatory cells in the adaptive response.

1.3.1. $CD4^+$ T helper cell differentiation

Naïve $CD4^+$ T cells are pluripotent progenitor cells (Thp cells), activated by recognising antigen presented on MHCII, and differentiating into a range of effector cells. The main antigen presenting cells are dendritic cells (DCs), although other cells can also play a role. DCs express co-stimulatory molecules and cytokines necessary for the complete activation, proliferation and differentiation of T cells. The nature of these signals depends on signals received by the DC, such as PRR stimulation (especially through TLRs), the type of peptide presented, the dose of antigen, and cytokines and chemokines from innate cells in the micro-environment.

Historically $CD4^+$ T cells were classified as Th1, releasing IFNγ, and Th2, releasing IL-4 (22). However in recent years it has become apparent that that this is an oversimplification, and other subsets of effector cells and regulatory cells have been identified in mice and rats. It is also clear that although it is often possible to clearly define terminally differentiated subsets in experimental model systems, the reality *in vivo* is that these cell types may co-exist in a response, and may be present at intermediate stages of differentiation. In addition, there is much less evidence that these subsets are so clearly defined in the human immune system. Although the cell fate pathways are not yet fully elucidated, some of the key effector cells and mediators are outlined in Figure 1.1. It is likely that further subsets of effector cells are yet to be identified.

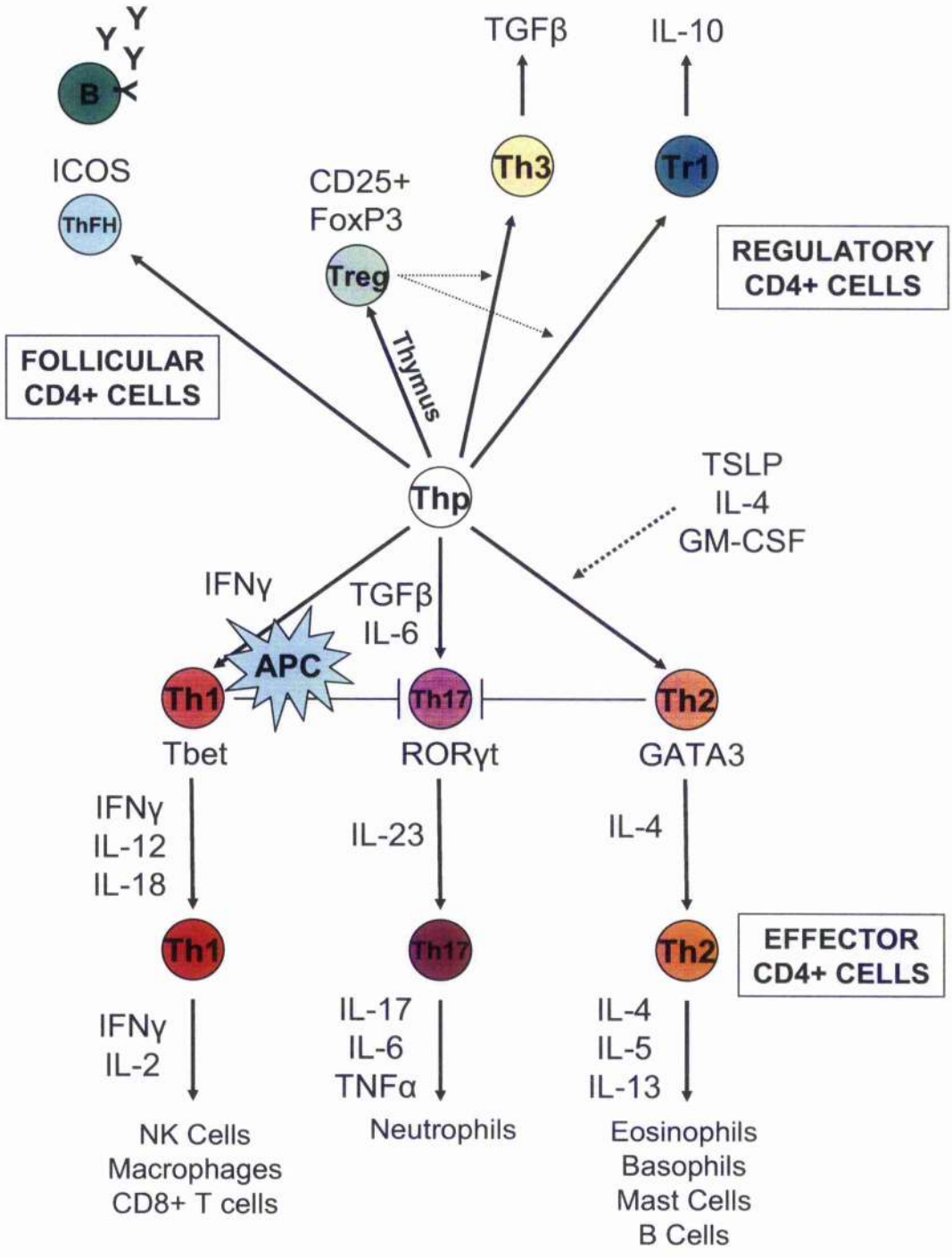

Figure 1.1 CD4⁺ T effector cell differentiation

Naïve pluripotent CD4⁺ progenitor T cells (Thp) can develop into a number of functionally distinct T effector cells following stimulation by antigen-presenting cells (APCs). T helper 1 (Th1) cell differentiation is initiated by interferon γ (IFNγ), and maintained by IFNγ, interleukin (IL)-12 and IL-18. Differentiated Th1 cells produce the effector cytokines IFNγ and IL-2, which promote natural killer

(NK) cell, macrophage and $CD8^-$ cytotoxic T cell activity against intracellular organisms. Th17 cell development is initiated by transforming growth factor β (TGFβ) and IL-6, and maintained by IL-23. They produce IL-17, IL-6 and tumour necrosis factor α (TNFα) and promote neutrophil responses. The essential factors initiating Th2 cell development are less well defined, but include thymic stromal lymphopoietin (TSLP), IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-4 also maintains the Th2 phenotype and is an important Th2 effector cytokine, along with IL-5 and IL-13. Th2 cells promote immunoglobulin E (IgE) and cellular (eosinophils, mast cells, basophils) responses against extracellular parasites. Th differentiation is a complex multi-step process that is not yet fully understood, expression of the lineage specific cross-regulatory transcription factors Tbet, RORγt and GATA3 is an irreversible step after which cells cannot be redirected to a different fate. In addition to these effector T cell subsets there are also a number of regulatory T cell subsets, the differentiation of which is less well understood. A constitutive population of $CD25^+$ regulatory T cells (Tregs) matures in the thymus with expression of the transcription factor FoxP3 essential for regulatory function. They suppress autoreactive T cell responses through a cell-contact dependent mechanism and prevent autoimmunity. Other regulatory T cells can develop in the periphery during immune responses under the influence of Tregs. These include Th3 cells and Tr1 cells which act through the release of TGFβ and IL-10 respectively. Follicular T helper cells (ThFH) are a novel subset which promote B cell antibody production through an inducible costimulatory molecule (ICOS) dependent mechanism.

1.3.1.1. Th1 differentiation

Th1 cells secrete IFNγ, and mediate cellular immunity against intracellular infection and tumours through activation of macrophages, NK cells and $CD8^+$ T cells. Dysregulation of Th1 responses can lead to autoimmunity, and destructive responses against self antigens, typified by type 1 diabetes mellitus. They may also contribute to the pathology of rheumatoid arthritis.

IFNγ from antigen presenting DCs and macrophages initiates Th1 differentiation by inducing the expression of the transcription factor Tbet, which is specifically expressed in Th1 and not Th2 cells and correlates with IFNγ expression (23). Mice deficient in Tbet spontaneously develop Th2 inflammation in the airways (24), and have severely impaired IFNγ expression and Th1 responses (25). Tbet induces the expression of IL-12Rβ (26), exposes the IFNγ gene through chromatin remodelling (27), and represses Th2 cytokine production (23). As well as being the key effector cytokine, IFNγ is also important in maintaining the Th1 phenotype.

This is illustrated by the observation that *in vitro* polarised Th1 cells from IFNγ deficient mice can be redirected to produce IL-4, whereas wild type Th1 cells cannot (28).

IL-12 is important in IFNγ production in Th1 cells. It induces the expression of the IL-18R (29), and IL-18 augments IFNγ production, and induces further expression of the IL-12R (30). Thus IL-12 and IL-18 together are potent Th1 differentiation factors. IL-12 and IL-12R deficient mice have weak, but not absent, IFNγ production and Th1 responses (31,32). Thus IL-12 is not an absolute requirement, and other factors can replace it. IL-27 and IL-23, other members of the IL-12 family, can induce proliferation and IFNγ production from naïve and memory CD4+ cells respectively (33,34), and type 1 IFNs can also induce IFNγ expression during viral infection (35).

1.3.1.2. Th2 differentiation

Th2 cells mediate humoral immunity against extracellular parasites, in particular helminths, through the production of IgE from B cells, and the release of toxic mediators from mast cells, eosinophils and basophils. Thus an environment hostile to parasite survival is created, and an inflammatory reaction occurs to limit the spread or expel the organism. Dysregulation of Th2 responses results in allergic inflammation, such as atopic dermatitis and asthma.

The initiation of Th2 differentiation is less well understood than Th1. It is clear that IL-4 is important in commitment to the Th2 lineage, but may come from eosinophils, mast cells, NKT cells, or early Th2 cells themselves, rather than from antigen presenting cells. Recently it was shown that thymic stromal lymphopoietin (TSLP) can induce Th2 cytokine release from mast cells (36), condition DCs to differentiate Th2 cells (37,38), and can induce IL-4 expression in $CD4^+$ cells to promote Th2 differentiation (39). IL-4 induces the transcription factor GATA-3, which is present in Th2 cells and not Th1 (40). GATA-3 upregulates the expression of IL-4, IL-5 and IL-13 as well as itself (41,42), setting up a positive feedback loop. It also suppresses the expression of IFNγ and IL-12Rβ (43). Mice deficient in IL-4 or IL-4R can still produce Th2 cells, albeit at a lower frequency (41,44), and can mount Th2 responses (45-47) so there are other pathways by which Th2 commitment can occur. For instance, granulocyte-monocyte colony-stimulating factor (GM-CSF) can induce Th2 responses in the absence of IL-4 (48). The role of IL-33/ST2 in Th2 differentiation is discussed in section 1.6.8.

1.3.1.3. Th17 cells

Th17 cells increase neutrophil synthesis in bone marrow through GM-CSF, and specifically recruit and activate neutrophils through the expression of IL-17, IL-6, and TNFα, and so have an important role in the response to extracellular bacteria and in sepsis (49). The transcription factor RORγt is specific to Th17 cells and is not expressed in Th1 or Th2 cells (50). They have also been shown to play pathological roles in experimental models of autoimmunity, including experimental auto-immune encephalitis (EAE) (51), and collagen-induced arthritis (CIA), a model of rheumatoid arthritis (52). They appear to be a separate lineage of T cells, as they can develop when IL-4 and IFNγ are blocked and do not express Tbet or GATA3 (53,54). TGFβ and IL-6 are critical to early commitment to the Th17 lineage, enhanced by TNFα and IL-1β, whereas IL-23 is important in maintaining differentiated cells (55,56).

1.3.1.4. Follicular T helper cells

Follicular Th cells (ThFH) are a novel subset of $CD4^+$ cells which migrate to the B cell area of lymph nodes after stimulation and play a role in supplying costimulation to B cells for antibody production through inducible costimulatory molecule (ICOS). High levels of ICOS leads to elevated numbers of ThFH, increased germinal centre formation, and resulted in autoimmunity (57).

1.3.2. Regulatory T cells

In addition to pro-inflammatory effector cells, a number of $CD4^+$ cells with suppressive functions have been identified. Approximately 5-10% of circulating $CD4^+$ cells are naturally occurring Tregs with constitutive expression of CD25 (IL-2Rα). They do not proliferate after antigen-specific TCR activation, but can suppress the activity of polyclonally stimulated effector T cells (58), as well as $CD8^+$ T cells, B cells and DCs, in a cell-contact dependent manner. Although the exact mechanisms are not fully elucidated, they may involve induction of apoptosis, cell lysis, and TGFβ and IL-10 production (59). They play a vital role in peripheral tolerance and the prevention of autoimmunity, and are increasingly implicated in autoimmune disease and allergy (60,61). Expression of the transcription factor forkhead box P3 (FOXP3) is the critical event in the development of natural Tregs (62), and in humans, FOXP3 mutation leads to the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans, characterised by autoimmunity (63).

Naturally occurring Tregs can also induce the development of other regulatory T cells in the periphery during a response, such as TGFβ producing Th3 cells (64), and IL-10 producing Tr1 cells (65). Both of these cell types have been shown to suppress both Th1 and Th2 responses *in vivo* (66,67).

1.4 Asthma

Asthma is a common condition (68), and the incidence has been increasing (69). The clinical syndrome of asthma includes episodic wheeze, cough and shortness of breath, with increased mucus expectoration. These are the result of largely reversible narrowing of the airways caused by inflammation, goblet cell hyperplasia, airway hyper-responsiveness (AHR) and remodelling of the airway structure (70). Extrinsic (allergic or atopic) asthma, characterised by high serum IgE levels, accounts for the majority of patients, and intrinsic (non-atopic) asthma, with normal serum IgE, accounts for the rest (71). The pathology seen in both extrinsic and intrinsic asthma is similar, and is the result of an endogenous inflammatory process with superimposed exacerbations triggered by exogenous factors. It is characterised by a Th2 type inflammatory infiltrate consisting of lymphocytes, eosinophils, mast cells and sometimes neutrophils. The epithelium is denuded in some areas, and shows goblet cell hyperplasia with mucus hypersecretion. The airway exhibits remodelling, with subepithelial fibrosis and further thickening of the airway wall by smooth muscle hypertrophy (72). Some of these changes have been shown to be present even before the onset of symptoms (73), and may contribute to susceptibility to airway inflammation.

The innate and adaptive immune systems are both important in the initiation and persistence of inflammation in asthma (see figure 1.2). In addition, structural elements of the airway are taking a much more prominent role in initiating and establishing inflammation. In this section I will outline some of the key mediators in asthma.

1.4.4. T lymphocytes and asthma

1.4.4.1. Th2 cells and cytokines – key regulators of inflammation and AHR

Elevated numbers of $CD3^+CD4^+$ cells producing IL-4 and IL-5 have been found in BAL and bronchial biopsies from asthmatics (74). These have been presumed to be T helper (Th) 2 cells, but recent studies have now identified invariant natural killer T (iNKT) cells, which also express CD3 and CD4 and produce Th2 cytokines, in the airway, so there is now some controversy over the exact contribution of each. The level of iNKT cells in the normal airway has generally been found to be less than 1% of T cells, and this is increased in the asthmatic airway in children (75) and in adults with moderate to severe asthma, with levels of 10% (76,77) up

to 60% (78) found in BAL. A further study, however, seems to show little difference in iNKT levels in mild to moderate asthma (79). This may reflect the method of detection, and the asthmatic phenotype of the patients studied. There is mounting evidence, then, that both Th2 cells and iNKT cells feature in the asthmatic airway, and the relative function of each may help to determine the phenotype seen. Functional studies in mice have demonstrated differential roles for both iNKT cells (predominant in airway hyper-responsiveness (AHR); see section 1.4.6.2.) and Th2 cells (central regulator of inflammation; see below).

Elevated levels of Th2 transcription factors, such as GATA3 (80), have also been found in the airway of asthmatic patients, indicating Th2 cells play an important role. Adoptive transfer of antigen-specific Th2 cells is sufficient to cause allergic airways inflammation and AHR in naïve mice subsequently challenged with antigen (81), and absence of T cells abrogates these responses (82). Th2 cytokines play a key role in airways inflammation and AHR, and often have overlapping functions. Whilst Th2 cells probably constitute an important source of these, it must be remembered that other sources, such as eosinophils, mast cells and NKT cells also contribute, and may particularly play a role in the early stages.

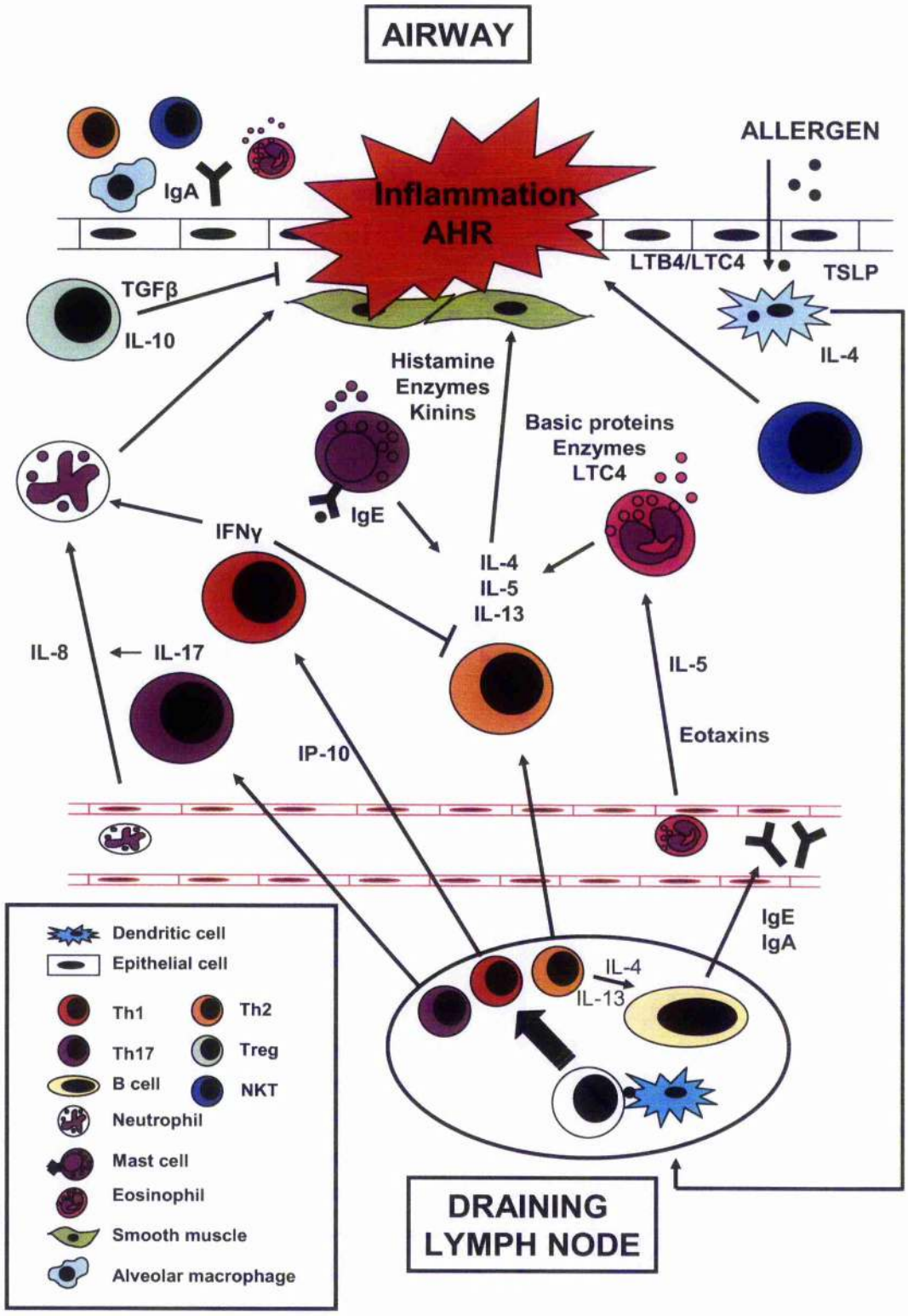

Figure 1.2 The pathogenesis of asthma

Allergen is taken up by dendritic cells (DCs) in the lung, which mature into Th2 differentiating DCs under the influence of thymic stromal lymphopoietin (TSLP) released from the epithelium in response to toll-like receptor (TLR) stimulation. Epithelial cells also produce pro-inflammatory lipid mediators

such as leukotriene (LT) B4 and LTC4. After uptake of allergen the DCs migrate to the local draining lymph nodes where they initiate T cell differentiation. Th2 cells migrate via the blood to the lung and produce interleukin (IL)-4, IL-5 and IL-13, the key regulators of airway inflammation. Natural killer T (NKT) cells may also play a role early in the response by producing Th2 cytokines. Th2 cytokines promote airway hyper-responsiveness (AHR) and remodelling, and inflammation through eosinophil accumulation and production of immunoglobulin (Ig) E and IgA. Eosinophils produce a large number of pro-inflammatory cytokines, chemokines and other mediators in the lung, and also contribute to tissue damage through the release of proteolytic enzymes. Subsequent inflammation can be triggered through allergen binding to IgE on the surface of mast cells, causing degranulation and *de novo* synthesis of mediators. This releases pro-inflammatory cytokines and chemokines, vasoactive mediators such as histamine and kinins, and proteolytic enzymes. In addition to Th2 cells, Th1 and Th17 cells may play a role in asthma through the induction of neutrophilic inflammation. This is though to be particularly important in virally-induced exacerbations and in steroid-resistant asthma. Regulatory T cells (Tregs) are able to suppress the inflammation seen in asthma through cell-contact, transforming growth factor β (TGFβ) and IL-10 dependent mechanisms. Many of the cells and mediators involved in the inflammatory response are not limited to the lung tissue itself, but migrate or diffuse to the airway surface where they can contribute to epithelial damage.

IL-4 is one of the central mediators of airway inflammation through its Th2 differentiating properties, and promoting IgE production. IL-4 producing $CD4^+$ cells, probably a mix of Th2 and NKT cells, have been found in BAL, bronchial biopsies and blood of patients with established asthma (83,84). IL-$4^{-/-}$ mice have reduced airways inflammation and AHR compared to wildtype (85), but whilst adoptive transfer of Th2 cells from IL-$4^{-/-}$ mice resulted in less severe airway inflammation than Th2 cells from wildtype mice (86), AHR was unaffected (87). Neutralisation of IL-4 during sensitisation did reduce AHR (88), and IL-4 administered to the airways directly can induce airway inflammation and AHR in mice, even in $Rag^{-/-}$ lymphocyte deficient mice, and this effect is absent in IL-4Rα$^{-/-}$ mice (89). However, IL-4 shares IL-4Rα with IL-13, so this effect may be indirect. Overall, IL-4 has a clear pro-inflammatory role in asthma, but its role in AHR may be indirect or dispensable.

IL-5 has been found in BAL fluid and serum from asthmatic patients, and its levels correlate with $CD4^+$ T cell activation (90,91). The major source of IL-5 in established disease is likely to be $CD4^+$ Th2 and NKT cells (74,78). Its key role is

in the maturation, recruitment and differentiation of eosinophils (92). In mice, the enforced expression of transgenic IL-5 resulted in eosinophilic airways inflammation (93), and neutralising IL-5 inhibited both eosinophilia and AHR (94). However, although clinical trials of anti-IL-5 showed a marked reduction of eosinophils in sputum, there was no improvement in symptoms or AHR (95). In another model of transgenic IL-5 expression, this time restricted to the bronchial epithelium, AHR was actually reduced, and this effect abolished by anti-IL-5 antibody (96). Thus the role of IL-5 and/or eosinophils in AHR remains controversial. However, IL-5 does appear to play a crucial role in airway remodelling, with anti-IL-5 antibodies having a beneficial effect in both mice and humans (97,98).

IL-13 shares a receptor chain with IL-4, but has independent and fundamental roles in the effector phase of inflammation through modulation of epithelial cells and smooth muscle. IL-13 is found in sputum and biopsies from human asthmatics (99,100), and is overexpressed in both atopic and non-atopic disease (101). There is also a strong link between IL-13 gene polymorphism and asthma (102). Administration of IL-13 alone to mice is sufficient to induce eosinophilia, AHR and mucus hypersecretion (89), and blocking IL-13 can reduce these features (103). The effects of IL-13 on eosinophil recruitment may be mediated by IL-5 and eotaxin, or independently of these (104,105). IL-13$^{-/-}$ mice fail to develop AHR or mucus hypersecretion in an allergic airways disease model, despite the presence of IL-4, IL-5 and eosinophilic inflammation (106). This perhaps demonstrates most clearly the dichotomy between inflammation and AHR, and the importance of IL-13 in the latter.

Other Th2 cytokines such as IL-9, IL-25 and GM-CSF are also expressed in the asthmatic airway, and have been shown to promote either Th2 differentiation or effector mechanisms in airways inflammation (107). IL-25 in particular has recently been shown to have a potentially important role in asthma. Both IL-25 and its receptor, IL-17RB, are upregulated in atopic asthmatic patients (108). It is particularly produced by epithelial cells after allergen stimulation (109) and is also produced by eosinophils and basophils in the lung (108), and may have a role in the initial phases of Th2 polarisation. In naïve mice IL-25 instilled into the airway induces a profound increase in lung and BAL eosinophils, and increases in AHR (109,110). In addition, it is able to polarise naïve T cells towards the Th2

phenotype *in vitro* through a GATA-3 mediated increase in early IL-4 production (109). Recent data has also shown that memory Th2 cells express IL-17RB, and IL-25 increases their proliferation and cytokine production after they are stimulated by anti-CD3 or by TSLP-treated DCs (108), thus demonstrating IL-25 may also play a role in promoting established inflammation. A recent study showing that a blocking anti-IL-25 antibody can substantially reduce eosinophilic airway inflammation in mice, and prevent the development of AHR, indicates it is a potentially exciting therapeutic target (111).

1.4.4.2. Th1 cells and cytokines – a biphasic effect mediated by TLRs

Th1 cells and cytokines are generally regarded as counter-regulatory to Th2 responses. Certainly, IFNγ or IL-12 given to mice during sensitisation prevents or reduces airway inflammation and AHR (112,113), and $Tbet^{-/-}$ mice develop spontaneous Th2 airway inflammation (24). IL-12 can also reduce inflammation and AHR when given during antigen challenge, probably by inducing IFNγ production from pre-existing Th1, $CD8^+$ and NK cells (113,114). However, Th1 cells and cytokines can also promote established Th2 airways inflammation. $CD4^+$ cells producing IFNγ have been found at the same time as $CD4^+$ cells producing IL-4 and IL-5 in human asthmatic airways (115), and transfer of Th1 cells exacerbated established airway inflammation, probably by TNFα release and recruitment of neutrophils (81).

The ability of TLRs to translate innate signals into immunomodulatory signals allows an explanation for why the timing of Th1 type responses may be important. Increased exposure to LPS or viral illness early in life protects against atopy and asthma, presumably by signalling through TLR4 (116). In established asthma, viral infections are the most common cause of an exacerbation, and LPS is present in many other triggers of asthma exacerbations, including house dust and cigarette smoke. In fact, levels of LPS in house dust correlate better with poor asthma control than DERp1, the house dust mite allergen, which does not correlate at all (117). These observations are borne out experimentally, as LPS administration during antigen sensitisation reduced airway inflammation (118), whereas low levels of LPS during antigen challenge increased it (119). Furthermore, inhalation of LPS in asthmatic patients caused exaggerated AHR compared to normal subjects (120). Thus, LPS appears to prevent sensitisation by biasing towards a

Th1 response early on, but once Th2 inflammation and AHR are established, the Th1/pro-inflammatory response exacerbates them.

Whilst IL-18$^{-/-}$ mice have enhanced airway inflammation (121), the role of IL-18 in asthma can be modulated by other cytokines present in the micro-environment. Co-administration with IL-12 reduced airway inflammation and AHR in mice (122), but administration of antigen with IL-18 alone induced severe eosinophilic airway inflammation after transfer of Th1 cells *in vivo*, possibly dependent on Th1 production of IL-9 and IL-13, and increased production of IFNγ and TNFα (123). IL-18 can also induce expression of Th2 cytokines from T cells, NK cells and mast cells.

1.4.4.3. Th17 cells and neutrophilic inflammation

A role for Th17 cells is emerging in mediating steroid resistant asthma, which is characterised by predominantly neutrophilic inflammation. CD4$^+$ cells from mice lacking Tbet preferentially differentiate into Th17 cells, and mediate neutrophilic airway inflammation (124). In keeping with this, IL-17 mRNA was elevated in sputum from asthmatic patients, and correlated with IL-8 and neutrophil levels, despite treatment with steroids (125).

1.4.4.4. Regulatory T cells and asthma

The role of regulatory T cells in preventing auto-immune diseases is well established, and evidence is accumulating that defective CD4$^+$CD25$^+$ Treg expansion may also play a role in allergy (61). Children with asthma or atopy have fewer circulating CD4$^+$CD25$^+$ Tregs than normal controls, although interestingly those with persistent symptoms had higher levels than those who were well controlled, which suggests a possible functional upregulation of Treg function (126). CD4$^+$CD25$^+$ Tregs can suppress airways inflammation and AHR in mice, through cell-contact or IL-10 dependent mechanisms (127-129).

1.4.5. B cells and Immunoglobulin E

The main role for B cells in asthma is through the production of IgE. An increase in total and antigen-specific IgE is one of the hallmarks of atopic asthma, but is not essential for AHR or eosinophilic inflammation. IgE production is influenced by Th2 cytokines, in particular IL-4 and IL-13, although they are not essential (130). Most IgE is attached to mast cells in the tissues through the high affinity IgE receptor

FcεRI. Antigen crosslinking of this cell-surface IgE results in degranulation with release of mediators of hypersensitivity. The importance of IgE *in vivo* is illustrated by the successful use of omalizumab, a monoclonal antibody against IgE, in improving control of asthma symptoms in those with persistently elevated IgE levels (131). Subsequent studies have shown this is not just through a reduction of total IgE, but also a reduction in $FcεRI^+$ cells, and reduced infiltration of eosinophils and T and B cells (132).

1.4.6. Innate cells and asthma

Innate cells often play a dual role in asthma. They are involved in the initial response to allergens/pathogens, and thus set the scene for the subsequent response, but they are also co-opted by T cells to carry out the effector functions of inflammation. Some of these functions will be summarised below.

1.4.6.1. Dendritic cells

Dendritic cells (DCs) form a network under the epithelium and constantly ingest and process inhaled antigen, then mature and migrate to local lymph nodes to present it to T cells (133,134). Two main populations of dendritic cell exist within the lung, and the activation status and phenotype of the DC will influence whether the end result is tolerance or inflammation (135).

Myeloid (m)DCs, expressing MHCI and II and CD11c, generally promote inflammation by activating T cells to become effector cells. The presence of a danger signal (eg exogenous PAMPs or endogenous mediators) in addition to allergen triggers full maturation and migration to the lymph node. A high level of IL-6 production allows them to overcome naturally occurring $CD4^+CD25^+$ Treg suppression of immune activation (136). TSLP is produced by epithelial cells, is increased in the airways of asthmatics, and correlates with disease severity (137). It conditions mDCs to promote Th2 differentiation through expression of the co-stimulatory molecule OX40 ligand (138). T-bet and IL-12 expression in mDCs can promote Th1 responses in the lung (139,140), which may have some regulatory role in Th2 inflammation. The importance of mDCs *in vivo* is underscored by the observations that transfer of antigen primed mDCs into the airways of naïve mice leads to Th2 inflammation after antigen challenge (141), and absence of mDCs prevents airways inflammation (142). In addition to their key role in the initiation of Th2 responses, there is some evidence that they may play a role in secondary

tissue responses by producing CCL17 (143), a powerful Th2 cell chemoattractant, and presenting antigen to T cells in the lung (144).

In the absence of a danger signal allergens do not induce inflammation, they induce tolerance instead. Ovalbumin (OVA) is an experimental allergen which induces tolerance to subsequent immunisation if administered alone to the airway, but allergic inflammation if administered in the presence of a low dose of LPS (119) or an adjuvant such a alum (145). Many clinically relevant allergens, such as Der p 1 from house dust mites, are proteolytic enzymes that can directly activate DCs to mature (146), whereas others probably become allergens due to the presence of exogenous microbial contamination (eg LPS). There is evidence that in the absence of danger mDCs do not fully mature and induce Tregs and tolerance (147). However, the second main DC subset in the lung, the plasmacytoid (p)DC, is probably more important in tolerance to allergens. They share expression of MHCI and II and CD11c with mDCs, but also specifically express 120G8 and PDCA-1. They produce large quantities of anti-viral type 1 interferons after viral infection, but have also been shown to mature into antigen-presenting cells after activation of TLRs on their surface. It is though that the balance between mDC and pDC function is important in asthma. In keeping with this, induction of pDC differentiation *in vivo* by Flt-3 ligand reduced allergic airway inflammation (148), whereas depletion of pDCs from mice breaks inhalational tolerance to OVA and allows mDCs to be more immunogenic and induce allergic airway inflammation (149). The precise mechanisms are unknown but may be related to the immature phenotype, and involve the expression of programmed death ligand 1 delivering a negative signal to naïve T cells (149), or pDC production of indoleamine-2,3-deoxygenase, which inhibits T cell proliferation and airway inflammation (150,151).

1.4.6.2. NKT cells

NKT cells are a novel subset of lymphocytes expressing an invariant T cell receptor which recognises glycolipid antigen in the context of CD1d on presenting cells, such as epithelial cells. As discussed above, some of the $CD4^+$ cells producing Th2 cytokines in the airway in asthma are likely to be iNKT cells. There is some argument over the numbers of iNKT cells seen in asthma, but this may not be crucial as iNKT cells are rapid and efficient producers of many cytokines, including IL-4, IL-13 and IFNγ. Although iNKT cells produce a variety of cytokines

after activation, repeated activation leads to a predominance of Th2 cytokine production, suggesting a possible role in allergic disease (152,153). Mice deficient in iNKT cells demonstrated their crucial role in the development of AHR and inflammation through the production of IL-4 and IL-13, independent of conventional T cells (154,155). In patients with moderate to severe asthma (atopic and non-atopic) up to 60% of $CD3^+CD4^+$ cells in BAL, and almost all the $CD3^+CD4^+$ cells in bronchial biopsies, were iNKT cells rather than conventional Th2 cells (78). They produced large amounts of IL-4 and IL-13, but little IFNγ. $CCR9^+CD4^+$ iNKT cells have also been seen in the blood of asthmatics, and numbers decreased after steroid treatment, and in well controlled patients. They were found to induce Th2 polarisation of naïve T cells through CCR9 and CD226 dependent cell contact mechanisms (77). Thus iNKT cells may play both direct and indirect roles in IL-4 and IL-13 production, and the asthma phenotype.

1.4.6.3. Eosinophils

Eosinophils are another hallmark of atopy and allergy, and there is a large body evidence that not only are they present in the inflammatory process, they cause damage to the airways through release of toxic granular proteins, reactive oxygen species, and pro-inflammatory lipid mediators (92). They also contribute to its chronicity through secretion of Th1 and Th2 cytokines (156), and possibly antigen presentation to T cells (157), and play a role in remodelling (158). Their role in AHR is controversial. Eosinophil deficiency in $IL\text{-}5^{-/-}$ and PHIL mice reduced AHR, and reconstitution of eosinophils restored AHR (159,160). However, in humans, depletion of eosinophils in the airway by anti-IL-5 had no effect on AHR (95), although it must be noted that eosinophil depletion was not complete (161). Dissociation of inflammation and AHR is also seen in mice, where the dblGATA strain of eosinophil-deficient mice demonstrated that AHR may arise by eosinophil independent pathways (158).

1.4.6.4. Mast cells

Mast cells are found in bronchial biopsies in human asthma (162), and degranulate after crosslinking of IgE bound to the high affinity FcεRI on their surface. They rapidly release preformed and newly synthesised pro-inflammatory and bronchoconstrictive mediators such as histamine, proteoglycans, serine proteases, prostaglandin D2 (PGD2), leukotriene C4 (LTC4) and platelet activating factor (PAF). In addition to mediating this early phase hypersensitivity

response to allergen, they can promote Th2 differentiation and effector responses through Th2 cytokine and chemokine production (163,164) and TNFα production (165). However, some models have shown mast cells are not essential for allergic inflammation or AHR (166).

Mast cells are present in the submucosa of normal subjects, asthmatic patients, and patients with eosinophilic bronchitis – a disease with a very similar inflammatory infiltrate to asthma, but no AHR (167). However, only the asthmatic subjects had increased mast cell numbers in the airway smooth muscle, implying a close relationship between these two cell types is important in the development of AHR in asthma, rather than just eosinophilic inflammation.

1.4.6.5. Neutrophils

Neutrophilic inflammation, which is promoted by TNFα and IL-17, is associated with some exacerbations and also different phenotypes of asthma, including severe asthma and steroid resistant asthma (168). The possible role of Th17 cells and IL-17 in neutrophilic airway inflammation has been discussed above. In addition, elevated levels of TNFα are seen in BAL and biopsies from asthmatics (169), inhalation of TNFα results in AHR and airway neutrophil infiltration (170), and treatment with etanercept, a TNFα antagonist, reduced AHR and asthma symptoms in a clinical trial (171). Thus there may be an important role for neutrophils in certain situations, promoted by Th17 cells and/or TNFα.

1.4.7. Structural cells and asthma

1.4.7.1. Epithelial cells

In addition to their role as a barrier, epithelial cells can also play an active role in the inflammatory process. Recently it was shown thymic stromal lymphopoietin (TSLP) is released after activation of epithelial cells by TLRs and other innate signals (36,172), thereby providing a link between innate and adaptive immunity, and initiation of inflammation. Overexpression of transgenic TSLP in the epithelium results in airway inflammation and AHR (143), and TSLPR deficient mice have reduced responses to inhaled antigen (173). Human bronchial epithelial cells have also been shown to produce leukotrienes after LPS stimulation, and may therefore contribute directly to AHR and inflammation (174).

A relatively recent concept to develop is that in asthmatic patients the epithelium may be more susceptible to damage by viral infection (175,176), or toxins (177), and aberrant healing may contribute to the development of asthma (178). This may contribute to the early remodelling changes seen, and create an environment more susceptible to future infection/damage and inflammation. Whether these changes in epithelial function predate inflammation, or are caused by it, remains to be elucidated.

1.4.7.2. Smooth muscle and fibroblasts

Smooth muscle hypertrophy, under the influence of cytokines and inflammatory cells, contributes to airway thickening and remodelling (179). Structural elements have also been implicated in the pathogenesis of asthma by the identification of polymorphisms in the *ADAM33* gene (a disintegrin and metalloproteinase 33) that are strongly associated with asthma, AHR, and remodelling with a decline in lung function (180). Although the exact role of ADAM33 in asthma has yet to be elucidated, it is only expressed on smooth muscle and fibroblasts. Both smooth muscle cells and fibroblasts also release cytokines and chemokines contributing to ongoing inflammation.

1.4.8. Chemokines and asthma

Chemokines and their receptors are key mediators of the coordinated movement of cells into inflamed tissues in asthma (181,182), although adhesion molecules also play important roles. A full discussion of chemokine biology is beyond the scope of this introduction, but I will summarise some of the key concepts. They are broadly split into families based on the relative position of conserved cysteine residues:

- C-C family: CCL1 to CCL28 binding to CCR1 to CCR10
- C-X-C family: CXCL1 to CXCL16 binding to CXCR1 to CXCR6
- XCL1 and XCL2 binding to XCR1
- CX_3CL1 binding to CX_3CR1

In addition there are decoy receptors, such as D6, which bind to ligands and are internalised without signalling, thus removing them from the tissue. Kinetic changes in expression allow for movement of cells into specific sites at specific times. Different cell types tend to have specific patterns of chemokine receptors,

and respond to the corresponding ligands. However, ascertaining which the most important interactions are is difficult, as chemokines are promiscuous and can bind to multiple receptors, and chemokine receptors can bind multiple chemokines. Also, expression *in vitro* and *in vivo* may be different. There is a huge amount of data from experimental models, much of it examining very specific interactions in specific models, and is therefore often conflicting. Some of the key interactions in asthma are summarised in table 1.2

CELL	RECEPTOR	LIGANDS
Th2 Eosinophil Mast cell	CCR3	CCL11 (eotaxin) CCL24 (eotaxin-2) CCL26 (eotaxin-3)
Th2	CCR4	CCL17 (TARC)[1] CCL22 (MDC)[2]
Th2	CCR8	CCL1 (TCA-3)[3]
Th1	CCR5	CCL5 (RANTES)[4] CCL3 (MIP-1α)[5]
Th1	CXCR3	CXCL9 (MIG)[6] CXCL10 (IP-10)[7]

Table 1.2 Chemokines in asthma

[1] thymus and activation regulated chemokine; [2] macrophage-derived chemokine; [3] T cell activation protein 3; [4] regulated on activation normal T-cell expressed and secreted chemokine; [5] macrophage inhibitory protein 1α; [6] monokine induced by IFNγ; [10] IFNγ inducible protein 10.

Allergic asthma is associated with increased expression of CCR3 on eosinophils and CCR4 and CCR8 on T cells (183,184), and they may be expressed during different phases of inflammation (185). A role for these receptors is at least partly supported by experimental data. The eotaxins are all upregulated during allergen challenge (186), and whilst $CCR3^{-/-}$ mice have reduced eosinophils, the effect on AHR is variable depending on the involvement of mast cells (187,188). Whilst $CCR4^{-/-}$ had no effect on allergic airway inflammation or AHR (189), neutralisation of TARC or MDC did (190,191), suggesting that there may be an additional receptor for these chemokines. CCR8 and TCA-3 do not appear to play an important role in experimental asthma (192). RANTES is increased in the serum of patients with asthma, and correlates with severity, whereas IP-10 is reduced (193).

1.5. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a relatively common inflammatory condition affecting up to 1% of the population in the UK (194). It is a systemic autoimmune disease, although the clinical features are usually limited to the joints. Chronic inflammation of the synovium leads to erosion of cartilage and bone and destruction of the joint. This results in pain, swelling, stiffness and joint deformity, with eventual loss of joint function. Chronic inflammation may also affect other organs, such as the lung and kidneys, and result in systemic features, such as cachexia and depressed mood.

Pharmacological treatment has focused on the control of pain and inflammation, with the use of non-steroidal anti-inflammatory drugs (NSAIDs), and early use of disease modifying anti-rheumatoid drugs (DMARDs) (195). However, these drugs have significant toxicity and side effects, and although they may slow down the process of joint destruction, they are not completely effective. Thus there is significant morbidity and excess mortality in patients with RA, and much focus on the inflammatory pathways present to identify new therapeutic targets. Hence agents targeting TNFα and IL-1 are being used a number of inflammatory diseases, including RA (196).

1.5.1. Autoimmunity in RA

The pathophysiology of RA is complex and heterogeneous (see figure 1.3). There is undoubtedly a genetic predisposition, as shown by the association of various HLA-DR alleles with RA (197). Other contributor polymorphisms in the gene loci of many of the cytokines involved in RA have also been found, and also in the gene for peptidyl arginine deiminase IV (*PADI4*) (198), which may play a crucial role in the development of autoimmunity. Environmental factors have also been shown to be important, particularly smoking. Thus in susceptible individuals with the right environmental triggers, a pre-clinical phase of autoimmunity can arise. The precise mechanisms by which T and B cell tolerance are overcome is not clear. The best characterised autoantibodies are against cyclic citrullinated peptides (anti-CCP) and other citrullinated proteins, which are more sensitive and diagnostic of RA than rheumatoid factors (RFs). Citrullination of self-proteins is carried out by PADI4, rendering them antigenic, and is promoted by smoking in susceptible individuals (199). RFs are anti-IgG antibodies that recognise the Fc portion of IgG

autoantibodies to form immune complexes in the serum, which initiate and perpetuate inflammation through the activation of complement and binding to FcγRs on inflammatory cells. The mechanism by which this becomes localised to the joint is not known, but immune complexes are found prominently in synovial fluid, where they contribute to the pro-inflammatory milieu. It is also likely that the destruction of cartilage and bone results in the exposure of neoantigens which can also trigger autoimmunity.

1.5.2. Chronic synovitis in RA

The synovium is usually scarcely populated with cells, but in chronic synovitis in RA there is a marked increase in cellular infiltration and proliferation. There is a superficial lining layer comprised of macrophages and synovial fibroblasts, overlying an interstitial zone which consists of more of these cells, and also a cellular infiltrate of T cells, B cells, neutrophils, mast cells, NK cells and NKT cells. Cytokines, chemokines, immune complexes and complement fragments play a key role in activating and regulating this process. Many of these cells and mediators are also present in the synovial fluid. The joint is a relatively hypoxic environment, and so angiogenesis is also crucial, mediated by vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).

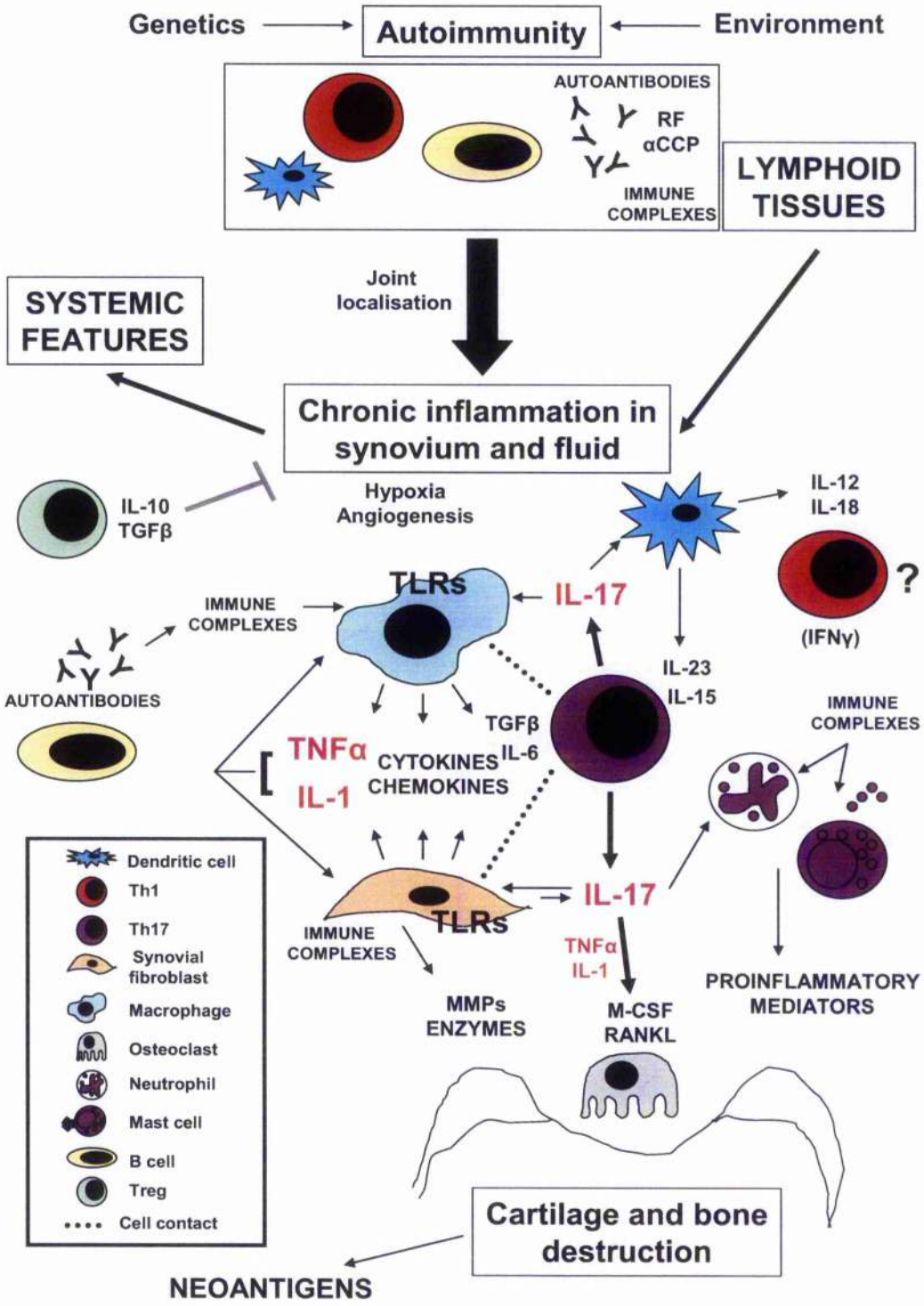

Figure 1.3 The pathogenesis of rheumatoid arthritis

A preclinical Th1 mediated autoimmune phase arises due to a combination of genetic predisposition and environmental factors, resulting in autoantibody production. Rheumatoid factors (RF) and anti-cyclic citrullinated protein (αCCP) antibodies are the best characterised of these. These autoantibodies form immune complexes which localise to the joint by an unknown mechanism. This

initiates a chronic inflammatory response in the synovium, which is augmented by the resulting tissue hypoxia and angiogenesis. Immune complexes are thought to initiate inflammation through activation of macrophages and synovial fibroblasts. Toll-like receptor (TLR) activation by endogenous tissue factors or pathogen-associated molecular patterns (PAMPs) may also play a role. Macrophages and synovial fibroblasts produce many proinflammatory cytokines. Interleukin (IL)-1 and tumour necrosis factor (TNF) α act as a positive feedback mechanism, promoting further cell activation and cytokine release. B and T cells from lymphoid tissues are also recruited into the synovium. Transforming growth factor (TGF) β and IL-6 promote Th17 cell differentiation and IL-17 release. IL-17 further activates macrophages and synovial fibroblasts, and stimulates dendritic cells in the joint to produce IL-23, which maintains the Th17 cell phenotype, and also IL-12 and IL-18 which promote Th1 responses. Although Th1 cells play a role in autoantibody production, their role in synovial inflammation is currently not clear. TNFα, IL-1 and IL-17 promote osteoclast maturation in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator for NFκB ligand (RANKL), which leads to bone resorption. In the presence of immune complexes they also stimulate synovial fibroblasts to produce matrix metalloproteinases (MMPs) and other enzymes, and neutrophils and mast cells to produce proinflammatory mediators, all of which contribute to cartilage destruction. Destruction of bone and cartilage reveals previously hidden self antigens (neoantigens) which can provoke further autoantibody production and help to perpetuate the inflammation. Inflammatory mediators released into the blood also cause systemic symptoms such as fatigue, pyrexia, anorexia and weight loss. Regulatory T cells (Tregs) have been shown to suppress joint inflammation through IL-10 and TGFβ production.

1.5.2.1. T cells in RA

There is a large body of evidence implicating T cells as one of the key regulators of RA. In humans they are present in large numbers in the synovium, and MHCII alleles are strongly associated with the development of RA. This is backed up by the requirement for T cells in many animal models of RA. It was previously thought that RA pathology was mediated by Th1 cells producing IFNγ, TNFα and IL-17. However, more recently it has been recognised Th17 cells are probably the more important effector cell in the joint. It is not clear where T cells become differentiated. It may begin in the lymphoid tissues, but the presence of DCs and the rich cytokine milieu in the joint would support the expansion of both Th1 and Th17 cells. However, Th1 and Th17 cells can mutually inhibit each other, which may further complicate the picture.

In human RA, Th1 cells expressing IFNγ are more prominent in the synovium in early disease, then reduce in frequency in established disease (200). IFNγ levels are also low in the synovium and serum (201). In murine CIA, blockade of IFNγ in early disease reduced anti-CII antibody levels and disease severity, but aggravated established disease (202). However, blocking IFNγ and deficiency of the IFNγR has also been show to result in earlier disease onset (203), mice deficient in IL-12p35 have exacerbated disease (52), and IFNγ inhibits osteoclastogenesis and bone resorption (204). Thus the role of Th1 cells and IFNγ is not yet clear, but may be more important in establishing autoimmunity than in joint pathology.

There is no direct evidence of Th17 cells in RA synovium, but IL-17 is present in the synovial fluid of RA patients and is produced by cells in the T cell area (205). In addition, mice deficient in IL-6 (206) and IL-23 (52) are resistant to CIA, and both of these are required for Th17 differentiation. IL-17 is a potent pro-inflammatory cytokine with effects on many cell types in the synovium. In conjunction with IL-1 and TNFα it can attract and activate synovial DCs through increased MIP3α expression in the synovium (207). It drives neutrophil accumulation and activation (208), and activates synovial macrophages and fibroblasts to produce pro-inflammatory cytokines and chemokines, and tissue destructive enzymes such as matrix metalloproteinases (MMPs), as well as pro-angiogenic factors. IL-17 is not dependent on TNFα or IL-1 for its arthritogenic properties (208,209). In murine models of arthritis, neutralisation of IL-17 (210) and IL-17R deficiency (211) both reduce experimental arthritis, whilst overexpression of IL-17 exacerbates it (212). Additional cytokines such as IL-22 and osteopontin from Th17 cells may also have roles in promoting pro-inflammatory cytokine production in the joint. In addition to cytokine production, cell-cell contact between Th17 cells and synovial macrophages and fibroblasts is important in promoting their activation, either through receptor-ligand interactions, or presentation of membrane-bound cytokines. Th17 cells are therefore important in the induction and persistence of inflammation. They are also involved in bone destruction (213).

Naturally occurring Tregs ($CD4^+CD25^+FoxP3^+CD62L^+$) have also been detected in patients with RA, although they have reduced suppressive activity (214).

Treatment with anti-TNFα antibodies overcome this by inducing a distinct subset of $CD62L^-$ Tregs that act through IL-10 and TGFβ production (215).

NKT cells are also present in RA, and much of the experimental data is conflicting (reviewed in (216)). However, on balance it would appear they are protective against autoimmunity, and so have a suppressive role in early disease. However, activation may exacerbate established disease, probably through the production of pro-inflammatory cytokines.

1.5.2.2. B cells in RA

An important role for B cells in RA was indicated by the clinical benefit of a B cell depleting monoclonal antibody (217). They produce autoantibodies in the joint that contribute to immune complex formation, which in turn bind to inflammatory cells through FcγR and activate them. They also trigger the formation of germinal centres, which results in increased affinity of auto-reactive antibodies, and are associated with a poorer outcome. B cell contact with synovial fibroblasts can promote pro-inflammatory cytokine and chemokine release, and MMP production (218).

1.5.2.3. Synovial macrophages and fibroblasts

As discussed above, synovial macrophages and fibroblasts may be activated by cell contact with Th17 cells, IL-17, and immune complexes. They may also be activated by innate signalling through TLRs (219). Thus exogenous bacterial and viral PAMPs, and endogenous ligands such as heat shock proteins, could play a role initiating and perpetuating inflammation. Once activated they are the key source of many of the proinflammatory cytokines and chemokines. They are also activated by these cytokines, especially IL-1 and TNFα, and so a positive feedback loop is established.

TNFα is one of the key pro-inflammatory cytokines in RA. It is present in most synovial biopsies, and up to 70% of patients respond to anti-TNFα therapy. In addition to its autocrine activation of macrophages and fibroblasts, it also activates neutrophils, endothelial cells and adipocytes to release pro-inflammatory mediators, and plays a role in joint destruction. IL-1 has a similar range of actions and is a very potent pro-inflammatory cytokine. This is counterbalanced by the expression of IL-1RA, and the balance between them may affect the clinical

outcome (220). IL-1RA is now also being used clinically in RA, although with less success. It is more successful as a treatment for autoimmune diseases caused by *NALP3* mutations and excess IL-1β production (221).

There are many more cytokines and chemokines that also contribute to the rich pro-inflammatory milieu established in the joint (222), but for clarity I will only mention a few here. IL-6, IL-23 and TGFβ contribute to Th17 cell differentiation and proliferation in the joint. TGFβ may also contribute to Treg development and repair mechanisms. IL-15 is important for T cell proliferation and survival, but also activates neutrophils and promotes B cell differentiation and isotype switching. IL-18 is present in the inflamed synovium (223), and IL-18$^{-/-}$ mice have reduced incidence and severity of CIA (224).

1.5.2.4. Other Innate cells in RA

Neutrophils are rapidly recruited into the arthritic joint, and are present in high numbers in both the synovium and synovial fluid. They are activated by TNFα, immune complexes, and complement fragments to produce cytokines (including TNFα, IL-1, IL-6 and IL15), chemokines, prostaglandins, and reactive oxygen and nitrogen species which contribute to inflammation in the synovium. Mast cells are present in the synovium, and are activated in similar ways to neutrophils. They also produce pro-inflammatory cytokines, and in addition protease enzymes, which break down matrix, but also activate macrophages. Mast cells produce IL-1 when stimulated through FCγRIII, and this may play a role in the initiation of synovial inflammation (225). Mast cell deficient mice are resistant to autoantibody induced arthritis (226). NK cells are also present in human RA, and release pro-inflammatory cytokines (227), but their role is not fully understood.

1.5.3. Cartilage and bone erosion in RA

Inflammation of the synovium triggers a cascade of events leading to cartilage and bone erosion. The current aim of treatment is to intervene in the inflammatory process as early as possible to avoid the inevitable joint destruction and irreversible deformity.

Activated synovial fibroblasts, neutrophils and mast cells produce matrix degrading enzymes, such as MMPs, which begin to break down cartilage and allows local influx of cells. Chondrocytes switch to a catabolic state and also produce matrix

degrading enzymes and pro-inflammatory cytokines, thus speeding up the process. IL-1 seems to be the critical cytokine for cartilage damage. Direct injection of IL-1 into joints leads to cartilage erosion (228), and cartilage erosion is prominent in IL-1RA deficient mice with spontaneous arthritis (228,229). IL-17 also promotes cartilage loss by TNFα and IL-1 dependent and independent pathways (230).

Under physiological conditions there is a balance between bone formation by osteoblasts, and bone resorption by osteoclasts. This balance is heavily tipped in favour of bone resorption in RA, through inhibition of osteoblast function and activation of osteoclast function. Osteoclast deficient mice develop inflammatory arthritis, but not bone erosion (231). Osteoclasts at the bone surface adjacent to the inflamed synovium are activated to resorb bone (232), which allows invasion of the bone by vascularised inflammatory pannus tissue. M-CSF is essential for early differentiation of osteoclast precursors into active osteoclasts, and is induced by TNFα from synovial fluid cells and T cells. RANKL is produced by synovial fibroblasts and T cells in response to a number of cytokines and pro-inflammatory mediators, especially IL-1, TNFα and IL-17. RANKL binds to RANK on the osteoclast to induce final differentiation and bone-resorbing activity. RANKL is antagonised by osteoprotegerin (OPG), a soluble decoy receptor, which completely prevents bone lesions in experimental arthritis (233). There is an imbalance between RANKL and OPG in arthritis.

1.6. The role of IL-33 and ST2 in inflammation

The *ST2* gene has long been known to encode an orphan receptor of the IL-1R family, with functions in innate and adaptive immunity, particularly Th2 responses. Recently IL-33 was described as the ligand for this receptor, and as expected was found to be a member of the IL-1 family of cytokines, and to have pro-Th2 functions. I will now discuss some of the features of the IL-1 families of cytokines and receptors, and the current evidence for the role of the IL-33/ST2 interaction in inflammation.

1.6.1. The IL-1 family of cytokines

The IL-1 cytokine family currently consists of 11 members (summarised in table 1.3; (1,234,235)), found close together on chromosome 2 in humans, except for IL-18 on chromosome 11. They share similar nucleotide sequences and protein structure, and exert their biological effects by binding to the IL-1R family of receptors.

LIGAND	INTERACTIONS	FUNCTION
IL-1α (IL-1F1)	IL-1R1 + IL-1RAcP	Cytokine
	IL-1RII	Decoy receptor
	None (intracellular)	Transcription factor
IL-1β (IL-1F2)	IL-1R1 + IL-1RAcP	Cytokine
	IL-1RII	Decoy receptor
IL-1ra[1] (IL-1F3)	IL-1R1	Cytokine antagonist
IL-18 (IL-1F4)	IL-18R + IL-18AP	Cytokine
	IL-18bp[2]	Cytokine antagonist
IL-1F5	Unknown	Unknown
IL-1F6	IL-1Rrp2 + IL-1RAcP	Unknown
IL-1F7	IL-18R	Unknown
IL-1F8	IL-1Rrp2 + IL-1RAcP	Unknown
IL-1F9	IL-1Rrp2 + IL-1RAcP	Unknown
IL-1F10	Soluble IL-1R	Unknown
IL-33 (IL-1F11)	ST2L	Cytokine
	sST2?[3]	Decoy receptor?
	None (intracellular)	Nuclear factor

Table 1.3 Members of the IL-1 family of cytokines

[1]IL-1 receptor antagonist binds to IL-1R1 with higher affinity than IL-1α or IL-1β but without recruiting IL-1RAcp thus does not activate signalling; [2]IL-18 binding protein binds to IL-18 to block binding to IL-18R; [3]putative interaction yet to be directly shown.

1.6.2. The IL-1R family of receptors

The IL-1 family of cytokines bind to members of the IL-1R family of receptors, which are part of the TLR/IL-1R superfamily (summarised in table 1.4; (6,236)). These are a rapidly expanding group of receptors with wide-ranging effects on innate and adaptive immune function. They all share an intracellular signalling region (the so-called Toll-IL-1R (TIR) domain) and similar signalling pathways. They are divided into three groups according to the extracellular ligand binding portion, or lack of it (see Figure 1.4). The IL-1R family have 3 immunoglobulin domains and bind cytokines of the IL-1 family, the TLR group have leucine-rich repeats and bind PAMPs and the adaptor group are intracellular and modify the signal transduction of these receptors.

FAMILY	RECEPTOR	INTERACTIONS	FUNCTIONS
Type 1	IL-1RI[1]	IL-1α, Il-1β, IL-1ra	Cytokine receptor
(IL-1R)	IL-1RII[2]	IL-1α, Il-1β	Decoy receptor
	IL-18R[3]	IL-18	Cytokine receptor
	IL-1RAcp[4]	IL-1RI	Accessory protein
	IL-18AP[5]	IL-18R	Accessory protein
	ST2L[6]	IL-33	Cytokine receptor
		MyD88	IL-1R/TLR regulation
	IL-1Rrp2[7]	IL-1F6,7, and 9	Unknown
	SIGIRR[8]	Unknown	IL-1R/TLR regulation
	TIGIRR[9]	Unknown	Unknown
	IL-1RAPL[9]	Unknown	Unknown
	B15R[10]	IL-1β	Antagonist
Type 2	TLR 1-11	PAMPs	Danger signals
(TLR)	Toll, 18 wheeler	Spätzl, Unknown	*Drosophila* proteins
Type 3	MyD88	TLRs and IL-1Rs	Adaptor
(Adaptors)	Mal / Tirap	TLRs and IL-1Rs	Adaptor
	TRAM	TLRs and IL-1Rs	Adaptor
	TRIF	TLRs and IL-1Rs	Adaptor

Table 1.4 TLR/IL-1R superfamily of receptors

[1]Type 1 IL-1 receptor; [2]Type 2 IL-1 receptor not signal as it only has a short cytoplasmic tail; [3]IL-18 receptor; [4]IL-1 receptor accessory protein required for high affinity binding and signalling; [5]IL-18 receptor accessory protein required for high affinity binding and signalling; [6]Membrane bound form of ST2, the putative second member of the receptor complex is unknown; [7]IL-1 receptor related protein 2 has no known function; [8]Single immunoglobulin IL-1R related protein (237) is a negative regulator of IL-1R and TLRs (238,239) expressed in the epithelium of the kidney, lung and gut (240); [9]Three immunoglobulin IL-1R related protein; IL-1 receptor accessory protein like protein (241); [10]B15R is a soluble mediator from the *Vaccinia* virus with homology to IL-1R which binds and neutralises IL-1β, thus subverting the immune response against it (242).

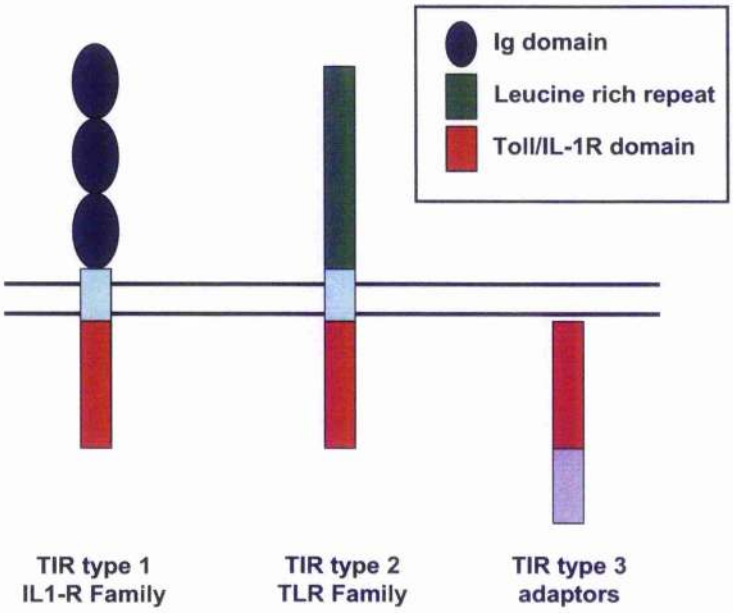

Figure 1.4 The TLR/IL-1R family of receptors

1.6.3. IL-1 family cytokines are pro-inflammatory

There is a huge body of work concerning the roles of IL-1α, IL-1β, and IL-18, and their receptors, in inflammation and other processes (reviewed in (243-246)). I will give just a brief overview of their key functions.

IL-1β is the main secreted form of IL-1, and despite being only 24% identical, IL-α and IL-1β bind to the same receptor, IL-1R1, and have identical biological effects (thus referred to collectively as IL-1). IL-1 and IL-18 are expressed to some degree by a large variety of cell types depending on the stimuli given. However, in both humans and mice monocytes/macrophages are a key source of both secreted IL-1β and IL-18. Murine macrophages express IL-1α as they become activated, and secrete it at much higher levels than is found in humans. IL-18 is also produced by dendritic cells (247). Epithelial cells of the lung, gut and skin (keratinocytes) produce IL-18 and IL-1. Keratinocytes in particular produce a large amount of IL-1α in both humans and mice. IL-1 and IL-18 are also found in the central nervous and endocrine systems, and so play an important role in linking these with the immune system.

IL-1 can affect almost every cell in the body as IL-1R is ubiquitously expressed. Whilst its major role is in immunity and inflammation, it also has roles in regulation

of cell proliferation and differentiation (embryogenesis, tumourigenesis and invasion (248)), regulating the hypothalamus-pituitary-adrenal axis (appetite (244), sleep (249), temperature (250)), energy metabolism (glycaemic control, lipid and protein catabolism), bone resorption (251) and atherosclerosis (252). IL-18 is also an important pro-inflammatory mediator, although IL-18R expression is more restricted, being predominantly on Th1 cells and NK cells, and therefore so are its effects. Additionally, it functionally antagonises IL-1 in bone metabolism by inhibiting osteoclast function (253), and has anti-tumour activities.

IL-1 is the prototypic proinflammatory cytokine due to its ability to regulate the expression of a wide range of mediators. This includes cytokines, chemokines, acute phase proteins, adhesion molecules, growth factors, clotting factors and enzymes. In fact, many of the actions of IL-1 are through increased inducible nitric oxide synthase (iNOS) and NO production, and increased cyclo-oxygenase 2 (COX-2) and prostaglandin E2 (PGE2) production. Thus IL-1 directly and indirectly enhances innate protective mechanisms, T cell function through IL-2 and IL-2R, antibody production, haematopoiesis and inflammatory cell infiltration and activation.

IL-18 is also a very important pro-inflammatory cytokine, but does not have the wide-ranging effects IL-1 has. Many of the biological roles of IL-18 studied thus far are dependent on its induction of IFNγ from NK cells, T cells and macrophages, and their activation to increase NO production, cytotoxic cell functions and intracellular microbe clearance. In this respect it shares many functions with IL-12, and the two are synergistic because they induce the expression of each others' receptors. IL-12 is essential for Th1 polarisation and memory cell production, and IL-18 from dendritic cells augments this (247). However, IL-12 and IL-18 together can induce more IFNγ production from naïve T cells in the absence of antigen than its presence (29). These cells do not become memory cells but may have a role in innate immunity. A similar phenomenon is seen in the absence of IL-12 but the presence of IL-2. In this scenario IL-18 augments IL-4 and IL-13 production in the presence of antigen, and therefore Th2 polarisation and atopic inflammation, and IL-13 in the absence of antigen, with a potential role in innate inflammation (246).

The functions of IL-33 are in keeping with being a member of the IL-1 family, but will be discussed in detail later (section 1.6.8.).

1.6.4. Regulation of IL-1 / IL1R family interactions

IL-1 and IL-18 have potent biological effects, and are therefore tightly regulated. Regulation occurs at the level of gene expression, and protein translation, processing and release from the cell. In addition there are a number of soluble and cell-membrane associated factors which can antagonise IL-1 and IL-18 function (see figure 1.5). IL-1 in particular is barely detectable *in vivo,* but evidence for its potency comes from the biological effects detected after injection of only ng/ml amounts, despite the constitutive presence of a huge excess of antagonistic sIL-RII in the serum. In addition, administration of IL-1ra is being used clinically to rapidly and effectively neutralise IL-1 and induce remission of a number of inflammatory diseases (221).

1.6.5. IL-33 is a member of the IL-1 family of cytokines

The *IL-33* gene was identified in 2005 in both humans and mice (1), and had previously been identified in humans and mice as *NF-HEV*, a nuclear factor (254,255), and in dogs as *DSV27*, a gene upregulated in cerebral artery vasospasm (256). Unlike most of the other members of the IL-1 family it is found on chromosome 9 in humans, and chromosome 19 in mice. The structure of the gene in humans and mice is very similar (254).

The nucleotide sequences of murine and human IL-33 share approximately 50% homology (254) (see figure 1.4), and of the other family members it is most similar to IL-18 (1). Human and murine IL-33 have a predicted 12 β strand trefoil structure, and so are very similar in size and shape to IL-1α, IL-1β, and IL-18. IL-1β and IL-18 have no signal peptide and require cleavage by caspase-1 in the IL-1β inflammasome before secretion of the active C terminus cytokine moiety (257). Similarly, IL-33 has no signal peptide and, at least *in vitro*, it is cleaved by caspase-1 at aa112. This yields a C terminus fragment which when used as a recombinant protein was found to be active as a cytokine (1). The *in vivo* role of caspase-1 cleavage and the mechanism of IL-33 secretion are unknown.

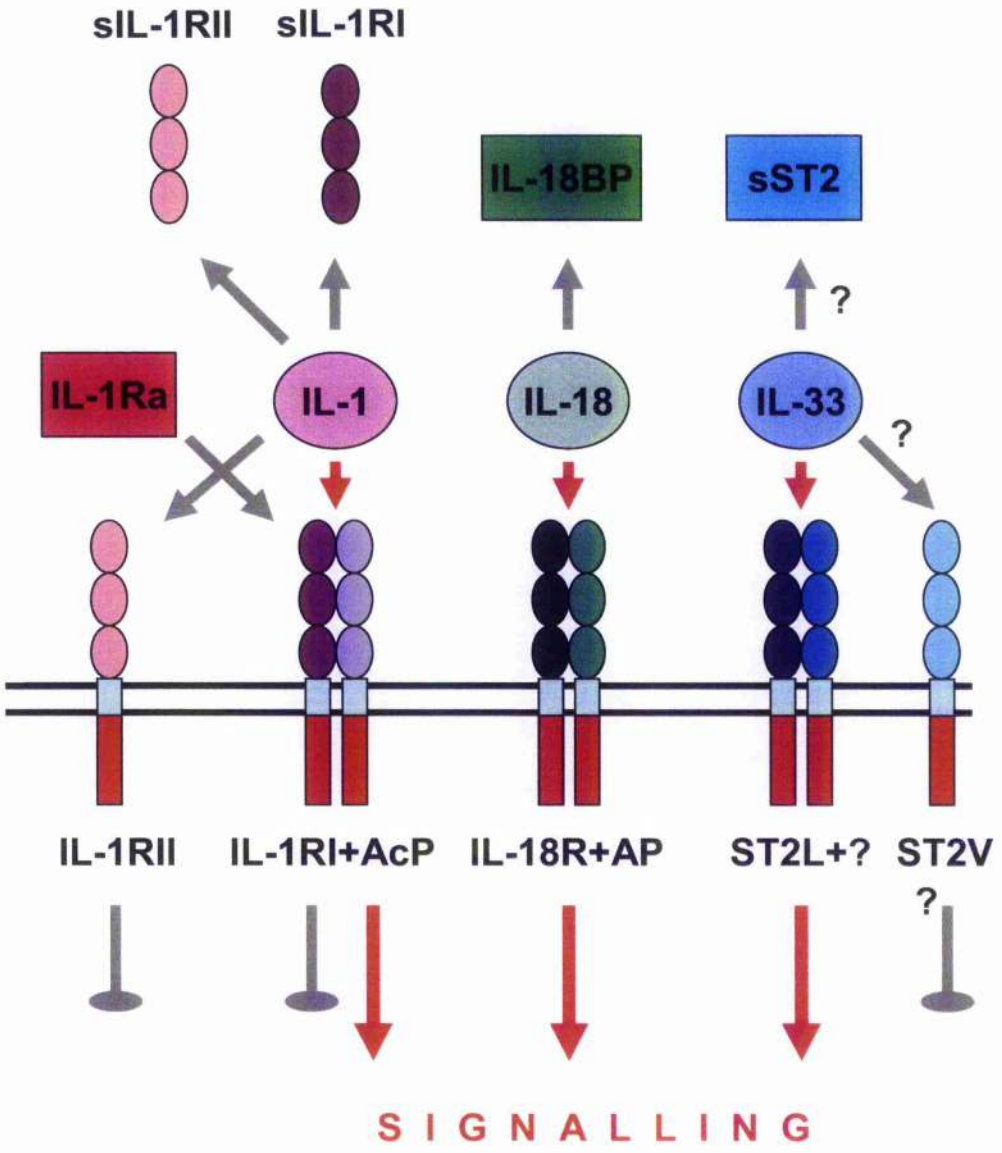

Figure 1.5 IL-1, IL-18 and IL-33 agonists and antagonists

The IL-1 family of cytokines and their receptors interact in similar ways to allow tight regulation of their function. IL-1 binds to IL-1RI and recruits IL-1RAcP resulting in active signalling. This is a very potent interaction requiring the presence of only a few receptors, and so is regulated in a number of ways: i) binding to the decoy receptor IL-1RII does not result in signalling; ii) IL-Ra is homologous to IL-1β and binds IL-1R1 with higher affinity than IL-1β or Il-1α but does not recruit IL-1RAcp and so does not initiate signalling; iii) soluble forms of IL-1RI and IL-1RII bind IL-1 to prevent its interaction with the functional IL-1RI receptor on the cell membrane. IL-18 binds to the IL-18R and recruits IL-18AP for signalling. IL-18BP binds to IL-18 and prevents its interaction with the receptor. IL-33 binds to ST2L and results in signalling. The presence of an accessory protein is predicted, but has not yet been found. sST2 and ST2V are splice variants of ST2L that are secreted or membrane associated respectively. Possible regulatory interactions are indicated by question marks (?).

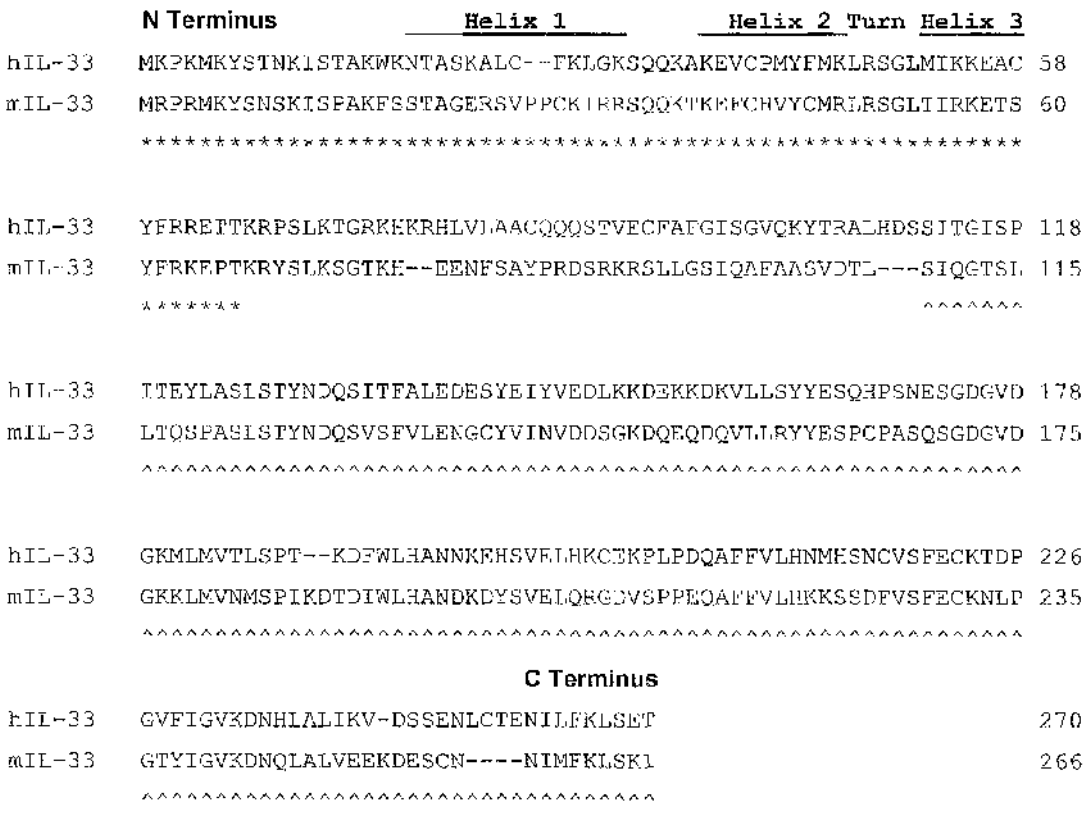

Figure 1.6 Amino acid sequences of human and mouse IL-33

The sequences of human and murine IL-33 (1), GenBank accession numbers AY905581 and AY905582 respectively, aligned to show homology (55%). The full-length human protein is predicted to be 30kDa, and the murine 29.9kDa. Caspase-1 cleavage takes place at aa112, yielding 2 fragments. The N terminus fragment contains a homeodomain-like helix-turn-helix (HTH) motif (***) required for localisation to the nucleus and its role as a nuclear factor. The C terminus is an 18kDa IL-1-like cytokine domain (^^^) that is also the sequence used for the recombinant protein.

IL-1α is quite distinct from IL-1β and IL-18 in that it is largely an intracellular factor rather than a cytokine. It is rarely found extracellularly, although it can be presented at the cell surface or released from dying cells, where it has identical activity to IL-1β. It is active without being cleaved and the N terminus region (aa1-115) was found to target it to the nucleus where it may have transcription factor function (258,259). The N terminus of IL-33 (aa1-65) was found to contain a homeodomain-like helix-turn-helix (HTH) DNA-binding motif and was also found to localise IL-33 to the nucleus (254,255), where it may also play a role in transcriptional regulation. Thus IL-33 has structural and functional elements in common with both IL-1β (C terminus) and IL-1α (N terminus).

The expression of IL-33 mRNA at the tissue level is shown in table 1.5 and at the cellular level in table 1.6 (summarised from (1,254,255), and is broadly similar to IL-1α, IL-1β and IL-18 combined. The level of expression runs in descending order from top to bottom in each table. In contrast to ST2, the expression of IL-33 has not been examined in embryogenesis.

Thus IL-33 mRNA is expressed to some extent in virtually every murine tissue, although it is highest in the central nervous system (CNS) and at epithelial surfaces interacting with the environment (skin, lungs and gut). The major source would appear to be activated macrophages and resting dendritic cells, although both of these were derived by *in vitro* methods. In humans the CNS was not examined, but expression was highest in skin and lung tissues. In contrast to mice it seemed to be expressed more in tissue cells such as fibroblasts and smooth muscle cells.

Expression is seen in murine lymphoid tissues, but not to any significant extent in lymphocytes. In keeping with this it is seen mostly in high endothelial venule endothelial cells (HEVECs) of human lymphoid tissue, as well as some scattered cells in the T and B cell areas, although the specific cell types were not identified. Chronically inflamed tissues take on some of the features of lymphoid tissue with HEVs surrounded by lymphoid aggregrates, and IL-33 was also found in HEVECs of Crohn's intestine and rheumatoid arthritis synovium. Expression in HEVECs was much higher than in other types of endothelial cell, implying a specific role here. It should be pointed out that IL-33 protein expression has only been shown in the nucleus of HEVECs thus far, so its cellular source as a cytokine is unknown.

	LOW	MEDIUM	HIGH
Mouse	Colon	Lymph node	Spinal cord
	Thymus	Spleen	Brain
		Pancreas	Lung
		Peyer's Patch	Skin (ears)
		Heart	Stomach
		Kidney	
Human[1]		T and B cell areas:	
		Tonsils	
		Peyer's Patch	
		Mesenteric lymph node	

Table 1.5 Tissue expression of IL-33 mRNA

[1]Human lymphoid tissue IL-33 protein expressed in the nucleus

		RESTING	ACTIVATED
Mouse	BM[1] derived macrophage	-	++++
RT-PCR[2]	BM derived dendritic cell	++++	-
	Epithelial cell	+	+
	Th2 cell	+	
	B cell	+	
	Peritoneal macrophage	-	
	Th1 cell	-	
	Naïve T cell	-	
	L fibroblast	-	
Human			
RT-PCR	Dermal fibroblast	+	+++++
	Bronchial smooth muscle	+++	+++++
	Pulmonary artery smooth muscle	+++++	
	Coronary artery smooth muscle	++++	
	Bronchial epithelial cell	+++	
	Keratinocyte	+	++
	Mesangial cell (kidney)	++	
	Small airway epithelial cell	+	+
	Lung fibroblast	-	+
	Monocyte derived dendritic cell	-	+
	Monocyte	-	+
	Mammary epithelial cell	+	
	Renal epithelial cell	+	
	Splenocyte	+	
	Prostate epithelial cell	-	
	Th0/Th1/Th2 cells	-	
	NK cell	-	
	B cell	-	
	Peripheral blood mononuclear cell	-	
ISH[3]	Tonsillar HEVEC[4,5]	+++	
	Peyer's patch HEVEC	+++	
	Mesenteric LN HEVEC	+++	
	Nasal polyp EC[6]	+	
	Human umbilical vein EC	+	
	Placenta	-	
	Crohn's intestine EC	+++	
	RA synovium EC	+++	
	HeLa epithelial cancer cell line	-	

Table 1.6 Cellular expression of IL-33 mRNA

[1]BM = bone marrow; [2]RT-PCR = real-time quantitative PCR; [3]ISH = *in situ* hybridisation; [4]HEVEC = high endothelial venule endothelial cell; [5]Tonsillar HEVEC IL-33 protein expressed in the nucleus; [6]EC = endothelial cell.

1.6.6. ST2 is a member of the IL-1R receptor family

The *ST2* gene was first identified in 1989 as a delayed early cell cycle gene in the BALB/c murine fibroblast cell line 3T3 stimulated by serum (260). It has also been identified as T1, induced by Ha-*ras* and v-*mos* oncogenes, and serum, in quiescent murine NIH 3T3 cells (261,262), and glucocorticoid in proliferating cells (262). Homologous *ST2* genes have been found in humans (263) (also identified

as *DER4* (264)), chicken (265) and salmon (266). The rat homolog is *Fit-1*, found as a Fos-responsive gene of rat fibroblasts (267). In humans it is on chromosome 2 (268), and in mice on chromosome 1 (269), both closely linked to other members of the IL-1R family.

The human gene structure (270) is shown in Figure 1.7, and the intron/extron structure is well preserved between species and other members of the IL-1R family (271). As with many members of the IL-1R family there are a number of mRNAs generated from the same gene by differential splicing, resulting in several protein products (see Figure 1.5). The product of a short 2.7kb mRNA (272) is secreted from cells and is therefore termed soluble ST2 (sST2). It comprises 3 immunoglobulin domains with a short tail which is homologous to the extracellular portions of IL-1R1 and IL-1R2. A longer 5kb mRNA gives rise to ST2L, the membrane anchored receptor (273). The 5' end is identical to sST2, but at the 3' end it has a transmembrane portion and a cytoplasmic tail containing the TIR domain, and has homology to IL-1R1 along its length.

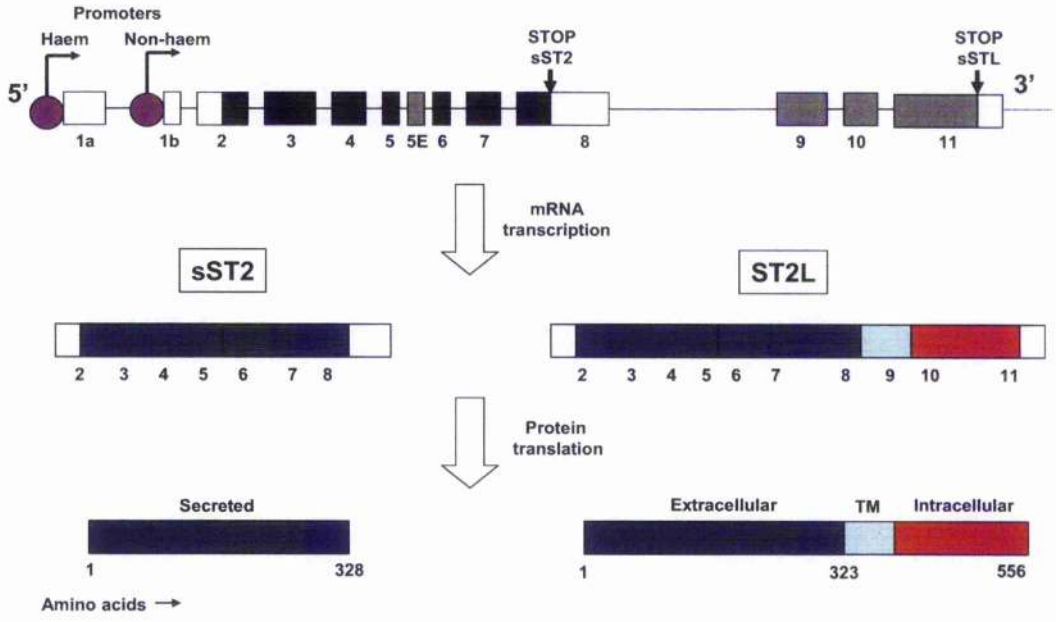

Figure 1.7 Structure of the human ST2 gene

The human *ST2* gene contains two promoters, specific to haematopoietic or non-haematopoietic cells. Exon usage for mRNA transcription is indicated. Exon 5E is only used in ST2V (not shown). sST2 is identical to the extracellular portion of ST2L, with the addition of 5 aa's.

There are 2 promoters which regulate production of the mRNA. In humans (274) and mice (275) the promoters are cell-specific, with the distal promoter dominant in haematopoietic cells, and the proximal promoter dominant in non-haematopoietic cells. Either promoter can result in production of sST2 or ST2L mRNA by differential 3' processing. The distal promoter is constitutive and serum independent (276) and has GATA consensus sites in human and mouse. One of these binds GATA-3 with enhanced activity (277), and enforced GATA-3 expression induced ST2L expression on $CD4^+$ cells (278). The proximal promoter is growth responsive and interacts with AP-1 and helix-loop-helix transcription factors (279). In rats (280) the situation is different, with the distal promoter specific to ST2L and the proximal specific to sST2, but both can be used in the same cell. The function of these 2 products will be discussed in more detail later.

More recently other variants have been found. ST2V is a truncated form of sST2 found so far in humans (281) and chickens (265). It uses the stop codon containing exon 5E (see Figure 1.5) and therefore has a hydrophobic tail instead of the third immunoglobulin domain, which allows it to be anchored in the cell membrane. In humans it is expressed mostly in stomach, small intestine and colon tissue (282). ST2LV is a form of ST2L found in chickens (265) which has the transmembrane portion deleted, and is released as a soluble protein from transfected cells. The functions of these are unknown at the moment.

The expression profile of ST2 mRNAs has been more extensively studied than IL-33. Tissue distribution is shown in table 1.7, and a summary of the cellular distribution in table 1.8.

		HUMAN[1]		MOUSE[2]	
		sST2	ST2L	sST2	ST2L
Adult	Spleen	y	y	y	y
	Bone marrow			y	y
	Thymus			y	y
	Lung	y	y	y[3]	y[3]
	Stomach	y	y	n	n
	Small intestine	y	y	n	n
	Colon	y	y	n	n
	Liver	y	y	n	n
	Kidney	y	y	n	n
	Placenta	y	y	n	n
	Testis	y	y	n	n
	Brain[4]	y/n	n	n	n
	Heart[4]	y/n	y/n	n	n
	Skeletal muscle[4]	n	n	n	n
	Pancreas[4]	n	n	n	n
	Skin (ears)[4]			y	y
Embryo	Liver			y	y
	Spleen			y	y
	BM			y	y
	Eye (retina)			y	n
	Bone (osteoblast)			y	n
	Skin (dermis)			y	n

Table 1.7 Expression of ST2 mRNAs in mouse and human tissues

[1]Human data from (270) unless otherwise stated. [2]Mouse data from (283) unless otherwise stated. [3]ST2L constitutively expressed in the lung, sST2 only after LPS stimulation (284). [4]Brain and heart expression found by Li (270), but not by Kumar (285). Expression of sST2 and ST2L in skin only after UV irradiation.

Soluble ST2 is widely expressed during embryogenesis, deposited in the extracellular matrix of non-haemopoietic tissues (283), and may be an early marker of osteogenesis (286). ST2L expression in the embryonic mouse is restricted to haemopoietic tissues (283). In the adult mouse both sST2 and ST2L are restricted to haematopoietic tissues (283) and to the lung (284). They are inducible in the skin by inflammation, but it is not known if this expression is from resident or infiltrating cells (285). In contrast, ST2 mRNAs are found almost ubiquitously in human tissues (270). It is likely this reflects the presence of inflammation within the human tissues at the time of collection.

		MOUSE		HUMAN	
		RESTING	ACTIVATED	RESTING	ACTIVATED
sST2					
Cell lines	Fibroblast	N	y	y	
	Alveolar macrophage	Y	increased		
	Th2			y	increased
	Th1			n	
	B cell			n	
	Non-lymphoid[1]	Y		y	
1° Cells	Fibroblast	N	y		
	Mammary epithelium	Y			
	Bronchial epithelium			y	increased
	PA endothelium			y	increased
	PA smooth muscle			y	increased
	Lung fibroblast			y	increased
	Alveolar macrophage			y	increased
	Th2			y	y
ST2L					
Cell lines	Fibroblast	Y	y	y	increased
	HUVEC			y	increased
	Alveolar macrophage	Y			
	Mast cell	Y		y	
	Th2	Y		y	
	Th1	N		n	
	B cell	N		n	
	Non-lymphoid[1]	Y		y	
1° cells	Fibroblast	N	y	n	n
	Bronchial epithelium			n	y
	PA endothelium			y	decreased
	PA smooth muscle			n	y
	Alveolar macrophage			y	increased
	Astrocyte	Y			
	Tissue mast cell	Y			
	BM mast cell	Y			
	Th2	Y	increased	y[2]	increased
	Th1	N		n	
	Natural Killer	N			
	DC	N			
	Monocyte			y	increased
	Macrophage	N			
	Keratinocytes			y	
	B cells	N			

Table 1.8 Expression of ST2 mRNAs in various cell types

[1]Many immortalised cell lines have been found to express sST2 or ST2L, largely non-lymphoid lines.

[2]Human Tc2, NK2 and NKT2 cells express ST2L also (287).

The predominant cells expressing ST2L constitutively in mice are mast cells, which express high levels of ST2L at all stages of development (288,289). It is also found on a murine alveolar macrophage cell line, and in human alveolar

macrophages (284) and circulating monocytes (290). Importantly, ST2L is a specific marker for antigen-experienced T helper type 2 (Th2) cells (291,292) and not Th1, which express the IL-18R (293). ST2L also identifies human Th2 cells (293), but these cells may need to be activated first (294). It is also on IL-10 producing Tregs in mice (295), but not humans (294).

Soluble ST2 is generally only expressed in cells activated by growth or inflammatory mediators. It is expressed in the developing mammary epithelium at puberty, but not at terminal differentiation during pregnancy and lactation (296). Fibroblasts (297,298), alveolar macrophages (284) and Th2 cells (294) have all been shown to release sST2 when stimulated by pro-inflammatory mediators. Low level sST2 expression is seen in a number of bronchial and pulmonary artery cell types, but this is markedly upregulated after activation (284).

Despite their similarity to the extracellular portion of IL-1R1 neither sST2 nor ST2L bind IL-1α or IL-1β (283). Several putative ligands for ST2L were reported (299,300), but did not have any functional relevance *in vivo*. sST2-Fc bound to bone-marrow derived macrophages (293,301), dendritic cells (141) and a glioblastoma cell line (300). Human sST2 binds a to B cell myeloma line (302) and a monocyte line (303), but the mechanism is unknown. Partly because the ligand was unknown there has been some debate over the signalling pathways initiated by ST2L. Initial studies indicated that, in common with other IL-1R family members, ST2L could activate NF-κB (304), but later studies suggested it did not (305). The recent discovery of IL-33 as the natural ligand for ST2L (1) showed definitively that ST2L signalling pathways are similar to the other IL-1R and TLR family members (see Figure 1.6). However, in some situations ST2L may be a negative regulator of TLR signalling ((306); see section 1.6.7.1.)

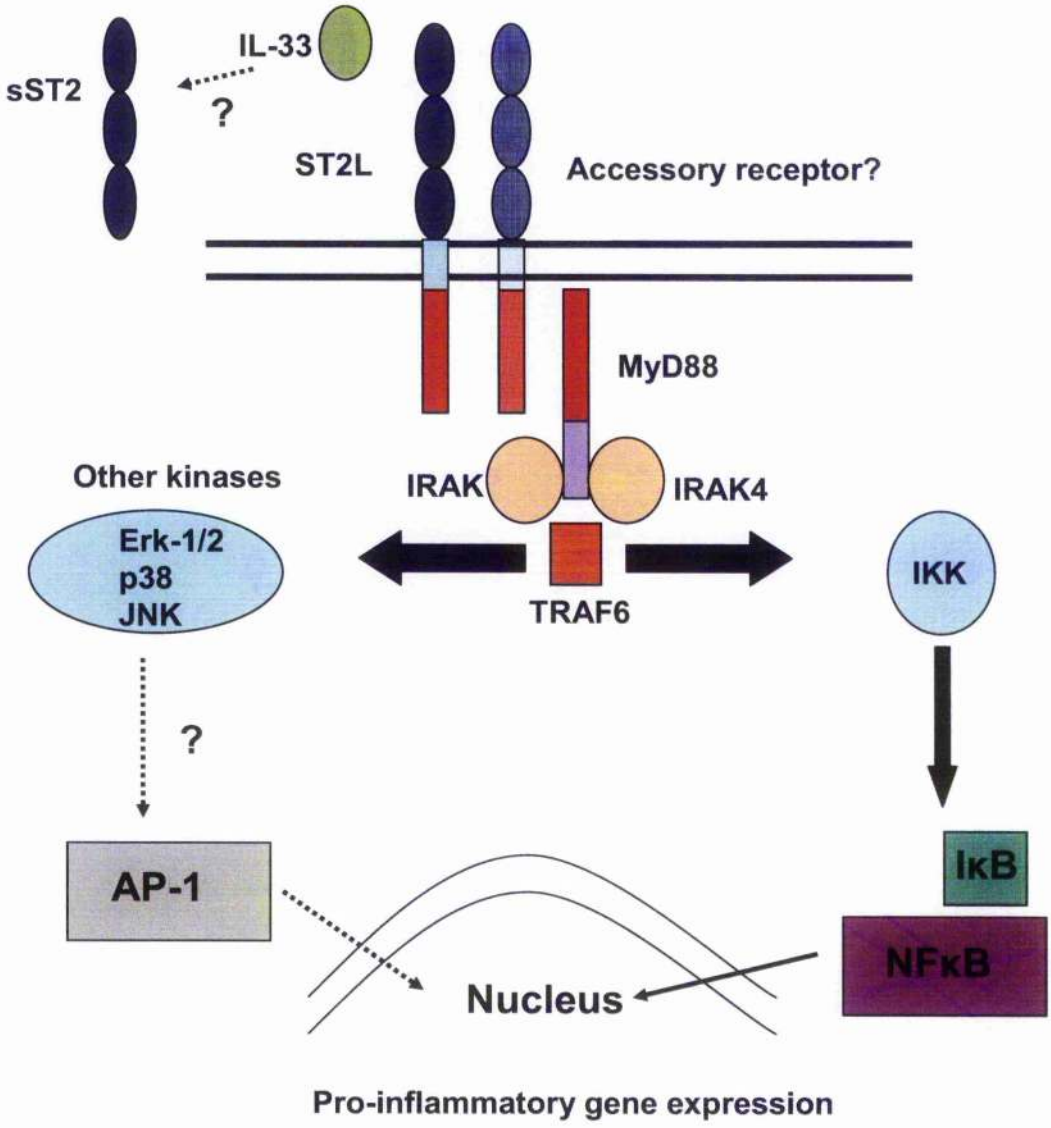

Figure 1.8 ST2L signalling pathways after IL-33 ligand binding

The signalling mechanism is very similar to IL-1R1 signalling (reviewed in (6,307)). IL-1R and IL-18R bind IL-1 and IL-18 respectively, but require an accessory protein for signalling. This has not been identified yet for ST2L. There are a number of soluble antagonists for IL-1 and IL-18. sST2 may perform this role for IL-33, but this has not yet been shown directly. On ligand binding ST2L initially interacts with MyD88 through the TIR domain. This is indispensable for signalling. IRAK, IRAK4 and TRAF6 then assemble and activate NF-κB through degradation of IκB. IL-1R and IL-18R also do this, and in addition activate AP-1. ST2L was previously shown to activate AP-1 when over-expressed or stimulated by an anti-ST2 antibody (305). The end result is up-regulation of pro-inflammatory genes in the nucleus.

1.6.7. Innate Immune functions of IL-33 and ST2

Most members of the Toll/IL-1R superfamily are involved in innate immunity through activation of NFκB. Expression of IL-33 and ST2 proteins can occur in many structural and immune cells involved in the innate response, as detailed above. In a model of innate immunity, treatment of mouse ears by UV irradiation causes a marked local inflammatory response in the skin. An increase in ST2 mRNAs was seen 48hrs later, but it is not known if this is from resident or infiltrating cells (285). However, the functional contribution of IL-33/ST2 to innate immunity has focused mainly on the role of sST2 and ST2L on macrophage function, and the ability of IL-33 to induce a type 2 innate response.

1.6.7.1. ST2 modulates macrophage function

In addition to fibroblasts, alveolar macrophages produce sST2 when stimulated by LPS or pro-inflammatory cytokines (284). sST2 has been shown to bind to monocytes/macrophages by an unknown receptor, resulting in downregulation of LPS-induced pro-inflammatory cytokine release (284,301,303,308). LPS can even increase the binding of sST2 to macrophages (301). Treatment with sST2 *in vivo* can reduce LPS-induced acute lung injury (284) and endotoxic shock (301), and ischaemic reperfusion injury in the intestine (308) and liver (309). Anti-ST2 antibodies caused an increase in endotoxic shock mortality (301). In the ALI model the peak level of sST2 in BALF occurred after peak inflammatory cytokine release, in keeping with the role of sST2 as a regulatory mechanism to limit innate inflammation. The exact mechanism of action of sST2 is not yet clear, but there does appear to be a direct effect through binding to a cell-surface receptor, as well as potentially sequestering the ligand for ST2L. Downstream events may include downregulation of TLR1 and TLR4 expression (301,309), reduced IκB degradation and NFκB nuclear translocation (303), and increased IL-10 expression (308).

Alveolar macrophages and monocytes expresses ST2L constitutively (284,290), and levels are upregulated by activation. In contrast BM-derived macrophages do not express ST2L at all (301). When co-transfected with IL-1R or TLR4, ST2L was able to downregulate signalling by sequestering MyD88 and Mal and preventing NF-κB activation (306). This is in stark contrast to ST2L activating NFκB when bound by IL-33, but may reflect the fact that this is an artificial system where ST2L may be acting without a natural ligand or accessory receptor, whereas IL-33 signalling was investigated in mast cells naturally expressing high levels of ST2L

(1). However, these results do indicate ST2L could also act as a negative regulator of innate immunity. In keeping with this, macrophages from $ST2^{-/-}$ mice produced more pro-inflammatory cytokines in response to TLR2, 4 and 9 stimulation than wildtype (306). Endotoxic shock can be prevented by pre-treatment with a sub-lethal dose of LPS (endotoxin tolerance), thought to act in several ways to reduce the severity of inflammation and mortality. $ST2^{-/-}$ resulted in increased LPS-induced pro-inflammatory cytokines *in vivo* and loss of endotoxin tolerance, although mortality from LPS shock was not affected (306). It is not clear if these results could be in part due to the loss of sST2 regulation also. Similar to endotoxin tolerance, pre-treatment with TLR2 agonists can protect against peritonitis-induced shock. ST2 mRNA was upregulated in the spleens on mice with tolerance to TLR2 ligands (310), though it is not clear if this is sST2 or ST2L mRNA, or both.

Overall there is no doubt that sST2 and ST2L may be powerful modulators of innate inflammation, but the respective roles of each, and in particular the possible antagonistic or direct actions of sST2 remain to be elucidated. ST2L may be involved in the transition from innate to adaptive immunity, as inhibiting TLRs tends to predispose to Th2 responses. The role of IL-33 in these models has not yet been examined.

1.6.7.2. IL-33 induces a systemic type 2 innate response

Structurally, IL-33 has much in common with the prototypic pro-inflammatory cytokines IL-1α, IL-1β and IL-18, although thus far there is very little functional information on IL-33. Schmitz et al (1) used recombinant IL-33 to induce a potent type 2 innate response in mice. This was characterised by an increase in type 2 cytokines and total IgE and IgA in the serum, eosinophils in the blood, and an increase in eosinophils and mucous producing Goblet cells at mucosal surfaces in the lung and gut. Although they also demonstrated IL-33 activates NFκB through binding to ST2L on mast cells *in vitro*, the cellular source of IL-33 and the responding cells *in vivo* are not known, and it has not yet been confirmed that IL-33 acts through ST2L *in vivo*.

1.6.8. Th2 immune functions of IL-33 and ST2

The main focus of research on the *ST2* gene has been its role as a marker for Th2 cells, and its potential role in the initiation of Th2 responses, and in Th2 effector mechanisms. This evidence is summarised below.

1.6.8.1. ST2 is expressed in CD4$^+$ Th2 cells

A number of studies have demonstrated *ST2* gene expression preferentially in CD4$^+$ Th2 cells. For instance, Th2 cell lines, and not Th1, express ST2L (292,302), and while naïve CD4$^+$ cells do not express ST2L (311,312), it has been established as a cell surface marker distinguishing Th2 cells from Th1 or Th17 in mice (291,292,313) and humans (293). However, the situation is not as simple as this.

In vitro studies have shown that ST2L is specifically a marker of well-differentiated antigen-experienced Th2 cells. The haemopoietic cell promoter for ST2 contains a number of GATA binding sites and ST2L expression is increased by GATA-3 binding (277), and over-expression of GATA-3 results in naïve CD4$^+$ cells taking on a CD44^{hi}CD45RB^{lo}CD62L^{lo} antigen-experienced phenotype (278). *Ex vivo* it is on cells with the CD4$^+$CD62L^{lo} phenotype, and which co-express IL-4 and IL-5, and not IFNγ (291,314). The percentage of cells expressing ST2L *in vitro* increases with every round of antigen stimulation under Th2 polarising conditions whether polyclonal (311), or antigen-specific (315). Initially IL-4 and IL-10 are produced, then ST2L and IL-5 expression increase in parallel.

ST2L expression is influenced by other factors. Cytokines such as TNFα, IL-1, IL-4, IL-5 and IL-6 all increase expression of ST2L *in vitro*, and IFNγ decreases it (311,312). IL-13 has no effect (311). However, ST2L expression is not dependent on these, as it is expressed at normal levels in IL-4$^{-/-}$ (291,312,316), IL-10$^{-/-}$ (291) and IL-4/13$^{-/-}$ (48) mice. Although it is expressed in IL-5$^{-/-}$ mice, it is only at one third of the level seen in wildtype mice (291), suggesting a link between IL-5 and ST2L regulation (see section 1.6.8.4.).

Antigen dose also influences ST2L expression. In neutral *in vitro* culture conditions a lose dose of antigen resulted in increased ST2L expression and IL-4 production, whereas at high doses, ST2L expression was reduced and IFNγ was produced (312). However, *in vivo* ST2L$^+$ cells are found in the highest number at the site of

inflammation, such as the footpad in *L major* (317), the lung in airways inflammation (141,318) and *S mansoni* (314) then with reducing frequency in draining, then distant, lymph nodes.

Thus although ST2L is a marker for Th2 cells, it is not a marker for all Th2 cells, and in humans is only expressed in activated Th2 cells, which can also produce sST2 (294).

1.6.8.2. ST2 is expressed in Th2 effector cells

As previously mentioned, mast cells are the major expressers of ST2L in mice, and an important Th2 effector cell, rapidly releasing pro-inflammatory mediators after degranulation by IgE binding. ST2L has also been found on human type 2 $CD8^+$ cytotoxic T cells, NK cells, and NKT cells (287,293). *ST2* expression in other Th2 effector cells such as eosinophils or basophils has not yet been demonstrated.

1.6.8.3. ST2 modulates Th2 inflammation

Apart from its presence on Th2 cells and effector cells, there is further evidence that *ST2* is expressed in and may play a role in Th2 responses. For instance, sST2 is elevated in the serum of mice with allergic airways disease very early in the response (3 hours), before even mRNA is detected (6 hours), and preceding the peak of Th2 cytokines (24 hours). There is also a second peak at 36 hours (319). ST2L expression on $CD4^+$ cells increases in acute airways inflammation (318) and in a model of chronic asthma (320). ST2 mRNAs are increased in rats 8 hrs after induction of contact hypersensitivity (321). The opposite is true for Th1 responses, where *ST2* expression is suppressed. Overexpression of IP-10, a Th1 chemokine, induces a Th1 response and reduces $ST2L^+$ cell numbers in airway inflammation by 60% (322).

However, although the expression of *ST2* has been shown in experimental Th2 diseases, assessing its function requires manipulating its expression or function directly. There are a number of approaches that have been taken to assess the role of sST2 and ST2L in modulating Th2 immune responses:

a) Polyclonal antibody serum inducing complement lysis

A polyclonal anti-ST2 antibody caused complement-induced lysis of $ST2L^+$ cells *in vitro*. When given to BALB/c mice susceptible to *L major* through a dominant Th2 response this induced healing through a shift towards a Th1 response (292). It is likely this is through loss of Th2 cells, as mast cells do not play a prominent role in the response to *L major*. It could also be due to lysis of cells binding sST2 at their surface (eg macrophages). Neither of these possibilities can be discounted, but these results do demonstrate that cells expressing ST2L (or binding sST2) have an important role in Th2 responses.

b) Non-lytic monoclonal antibodies

In vitro crosslinking of ST2L on Th2 cells with a non-lytic anti-ST2 monoclonal antibody promotes proliferation and cytokine production (311) via the JNK pathway (305), suggesting that ligation of ST2L *in vivo* may be important in Th2 cell activation.

In vivo monoclonal anti-ST2 has been shown to reduce allergic airways inflammation induced by adoptive transfer of either Th2 cells (291) or antigen-pulsed dendritic cells (141), and virally induced Th2 airway inflammation, but not Th1 (323). In each of these models anti-ST2 was used to putatively block interaction of ST2L with its ligand, but cell activating properties cannot be excluded.

c) ST2-Fc fusion protein

An sST2-Fc fusion protein has also been used to block ST2L function by sequestering the presumptive ligand. However, it must be remembered that sST2-Fc has been shown to bind to a number of cells, including macrophages, B cells and dendritic cells, and may have functions independent of ST2L. Nevertheless, administration of sST2-Fc has been used successfully to modulate Th2 responses. *In vitro* there was a reduction in cytokine release from polarised antigen-specific Th2 cells, no effect on Th1 cells, and Th0 cells had reduced IL-4 and IL-5, and increased IFNγ (315). *In vivo* there was a reduction in Th2 airway inflammation (141,291,315) but not Th1 (315). In addition, sST2-Fc was found to boost the Th2 response and pathology seen in carbon tetrachloride (CCl_4)-induced hepatic fibrosis, dependent on $CD4^+$ cells and IL-4Rα (IL-4 and IL-13 signalling) (324).

Thus sST2-Fc may have both pro- and anti-Th2 actions, the mechanisms of which may be different depending on the circumstances.

d) transgenic overexpression of sST2

Over-expression of sST2 by gene transfer resulted in reduced allergic airways disease (319) and although it did not prevent a Th2 response it did reduce the number of IL-5 secreting cells in *N brasiliensis* infected lungs (325).

e) ST2 gene disruption

The *ST2* gene has been knocked out in several different ways, all on the Th1 dominant C57Bl/6 background (325-327). In general, the phenotype seen is not a dramatic one. As with overexpression of sST2, $ST2^{-/-}$ did not reduce the Th2 response, but did reduce the number of IL-5 secreting cells in *N brasiliensis* infected lungs (325). There was reduced primary granuloma formation, but not secondary, in lungs challenged with *S mansoni* eggs (326).

1.6.8.4. ST2 and IL-5 regulation appear to be linked

A number of lines of evidence point to the fact that *ST2* and *IL-5* gene expression are linked, more so than with any other Th2 cytokine. Both *ST2* and *IL-5* genes contain GATA binding sites and are regulated by GATA-3, the archetypal Th2 transcription factor (41,277). In fact, *in vitro* all $ST2L^+$ cells produced IL-5, although not all IL-5 producers were $ST2L^+$ (314). In $IL\text{-}5^{-/-}$ mice the amount of ST2L expression was much reduced (291) whilst in $ST2^{-/-}$ mice the number of IL-5 producing cells was reduced (325). *In vivo* $ST2^{-/-}$ mice and ST2-Fc transgenic mice both have lower levels of IL-5 in *N brasiliensis* infection although all other responses are normal (325). Both $ST2L^+$ and $ST2L^-$ Th2 cells were found in *L major*, with $ST2L^+$ cells producing most of the IL-5 (328), and correlating with non-healing lesions (317).

1.6.8.5. ST2 may have a negative regulatory role in Th2 responses

Virtually all studies that have demonstrated a role for ST2 in Th2 inflammation have focused on a pro-inflammatory function. However, a very recent study has shown that ST2 may play a role as a negative regulator of Th2 function (329). This study showed that $ST2^{-/-}$ mice developed worse allergic airways inflammation than $ST2^{+/+}$ mice. It went on to demonstrate a key role for ST2 in regulating T cell function in this model. Th2 cells from $ST2^{-/-}$ mice produced more cytokines *in vitro*

than $ST2^{+/-}$, and transfer of $ST2^{-/-}$ Th2 cells resulted in worse allergic airways inflammation than transfer of $ST2^{+/+}$ Th2 cells, even in lymphocyte deficient $Rag^{-/-}$ recipient mice. This is supported by the finding that T cells from the $ST2^{-/-}$ mice produced significantly higher levels of cytokines, particularly Th2 cytokines, than cells from $ST2^{+/+}$ mice when stimulated by antigen, or polyclonally with anti-CD3 and anti-CD28 antibodies (data not shown). Thus *ST2* may act as a "brake" on cytokine production, although the mechanism is unknown.

1.6.8.6. ST2 is not essential for Th2 responses

Just as it has been shown that Th2 responses can develop in the absence of IL-4 (45-47), some studies have shown that blocking *ST2* function or $ST2^{-/-}$ does not affect Th2 responses. Interfering with ST2L signalling during *L major* infection using a non-lytic anti-ST2 mAb or sST2-Fc did not affect Th2 differentiation, the frequency of $ST2L^{+}$ or ST2L- cells, or the non-healing nature of the lesion (330). *In vitro* naïve T cell function, and Th1 and Th2 polarisation were unaffected by $ST2^{-/-}$, as were mast cell development and function (326,327). There was no difference in eosinophilic airway inflammation (327), outcome of *N brasiliensis* infection ((325,327)), or secondary granuloma formation in response to *S mansoni* eggs (326). Mice expressing high levels of an sST2-Fc transgene also cleared *N brasiliensis* normally (325).

Interestingly, even in these models where disease outcome was unaffected, there were detectable differences in Th1/Th2 cytokine production. It was noted that the percentage of IL-5 producing cells in the lungs of *N brasiliensis* infected mice was reduced in both $ST2^{-/-}$ and sST2-Fc transgenic mice, with a corresponding reduction in BAL eosinophilia (325), and that draining lymph nodes from the lungs of mice with 2° *S mansoni* granulomas produced less IL-4 and IL-5 (326). In addition, $CD4^{+}$ cells from *L major* infected mice with disrupted ST2 signalling were more responsive to IL-12 and produced more IFNγ (330). Whilst these provide further evidence of the link between ST2 and Th2 function, it was not sufficient to materially alter the course of the infection. It is likely that given the importance of Th2 responses to parasite clearance there will be a number of back-up mechanisms by which they can arise, particularly under strong stimulation.

1.6.8.7. IL-33 and Th2 responses

Whilst it stimulates the release of Th2 cytokines from *in vitro* polarised Th2 cells (1), IL-33 has not yet been shown to play a role in established Th2 responses, but given its potent type 2 innate inflammatory properties it is likely it will play a role in differentiation to Th2 responses, and/or in the Th2 effector response.

1.6.9. Th1 or Th17 autoimmune functions of IL-33 and ST2

Although there has been less work on the role of *ST2* and IL-33 in Th1 responses, there is evidence of a potentially important regulatory role. IL-33 is expressed in the nucleus of HEVs from Crohn's bowel and RA synovium, although its function there is unknown (255). Experimentally, ST2-Fc is highly effective in blocking the induction and disease progression of the Th1/Th17 mediated CIA model in DBA/1 mice (331). $ST2^{-/-}$ exacerbated a streptozocin-induced Th1 model of autoimmune diabetes (332).

1.6.10. IL-33 and ST2 in human disease

Soluble ST2 expression can be detected by ELISA (333) in a number of different human diseases, and often correlates with severity. However, a definite pathological role has yet to be identified.

1.6.10.1. Elevated sST2 in innate inflammation and trauma

Patients with sepsis have a markedly elevated serum sST2 compared to normal subjects (334). Trauma or abdominal surgery also elevated sST2 levels, but not by as much (334). Serum sST2 is elevated after cardiac surgery (335) and in cardiac muscle under strain (336), and is a predictor of mortality or the need for transplant in patients with heart failure (337). It is also elevated by myocardial ischaemia (336,338) and correlates with the rise in creatine kinase and the fall in left ventricular function (337), and can predict mortality after myocardial infarction (338). Subarachnoid haemorrhage (SAH) leads to an inflammatory reaction in the subarachnoid space, and can precipitate a systemic inflammatory syndrome. Cerebrospinal fluid has higher elevated levels of sST2 than the serum following SAH, implying it is being released locally (339). Thus sST2 is released after several very different triggers of innate inflammation.

1.6.10.2. Elevated sST2 in Th2 inflammation

Serum sST2 is elevated in a number of human diseases with significant Th2 pathology. It is elevated in stable atopic asthmatics, and a further rise during exacerbation correlated well with the severity (340). Serum and BAL sST2 were elevated in acute eosinophilic pneumonia, and returned to normal after successful treatment with corticosteroids (341). Serum sST2 was also elevated in patients with an exacerbation of interstitial pulmonary fibrosis (342). In addition, the *ST2* gene may have a role to play in the susceptibility to atopic inflammation. Functional SNP polymorphisms have been found in the promoter of the *ST2* gene, and are associated with atopic dermatitis. High risk polymorphisms resulted in increased promoter activity and were associated with an increase in serum sST2 and total IgE (343).

1.6.10.3. sST2 and Th1 inflammation

Elevated sST2 was found in the serum of some patients with autoimmune diseases including systemic lupus erythematosis, RA, Wegener's granulomatosis and Behcet's disease (344). However, the numbers were rather small, and not all patients had sST2 present, so larger studies are needed to see if this correlates with disease severity.

1.6.10.4. sST2 and malignancy

CD4+ cells isolated from malignant effusions expressed more *ST2* mRNA, and the effusion fluid contains more sST2, than patients with effusions secondary to TB or heart failure (345). ST2L expression could be used to distinguish mycosis fungoides lesions from skin lesions of adult T-cell leukaemias (346), and also to distinguish anaplastic large cell lymphoma from other lymphomas (347). Soluble ST2 is also an indicator of low risk of recurrence in lymph node negative breast cancer (348). Thus *ST2* expression can have significant implications for planning treatment and predicting survival. As well as being a marker for some kinds of tumour, *ST2* could also play a functional role. Levels of *ST2* expression were found to be low in a human glioblastoma cell line and tumour samples (349). Transfecting sST2 into the cell line reduced the ability of the cells to proliferate when not anchored, and suggests that sST2 can influence tumour growth and survival. *ST2* expression occurs in osteogenic osteosarcoma, and inhibiting it in these cells abrogated the formation of osteoid by these tumours, indicating a role for ST2 in osteoblast differentiation (350).

1.7 Hypothesis

On the basis of the current evidence I hypothesise that the IL-33/ST2 axis has important pro-inflammatory roles in different types of inflammation, and therefore is potentially an important target for modulation of immune responses.

1.8 Aims

In order to test this hypothesis I will demonstrate that:

1. IL-33 initiates an innate immune response in the lung in the absence of antigen
2. IL-33 is dependent on *ST2* gene expression for its pro-inflammatory effects
3. IL-33 may have role in asthma, a Th2 driven allergic disease, using murine allergic airways disease as a model.
4. IL-33 may have a role in rheumatoid arthritis, a Th1/Th17 driven autoimmune disease, using murine collagen-induced arthritis as a model.

These studies will identify IL-33 as a novel target for therapeutic intervention in many different types of inflammation and human diseases.

Chapter 2

Materials and Methods

2.1. Buffers and Reagents

Solutions/buffers and reagents used in all experiments are listed in appendix 1 (solutions/buffers) and appendix 2 (reagents).

2.2. Mouse Strains

All mice were housed in University of Glasgow Biological Services facilities according to United Kingdom Home Office regulations, with free access to food and water. Severe combined immunodeficient (SCID) mice were kept in filter top cages with sterilised food and water. All experiments were undertaken in the same facility under project licence 60/3119. I held personal licence number 60/9140. The strains of mice used and their sources are summarised in Table 2.1.

STRAIN	GENETIC MODIFICATION	SOURCE
BALB/c	None	Harlan Olac
BALB/c	ST2 gene knockout	Prof Andrew Mackenzie University of Cambridge
BALB/c	SCID	Harlan Olac
DBA/1	None	Harlan Olac
DBA/1	ST2 gene knockout	Bred at University of Glasgow

Table 2.1 Strains of mice used in experiments

2.2.1. Commercially available mice

Wildtype BALB/c (H-2^d), SCID mice (C.B-17 on the BALB/c background) and DBA/1 (H-2^q) mice were purchased from Harlan Olac (Bicester, Oxon, UK).

2.2.2. ST2 gene knockout mice

In some experiments I used mice which had the *ST2* gene disrupted to delete the majority of exons 4 and 5 (326). In homozygotes this results in ST2 knockout mice ($ST2^{-/-}$) with no expression of either soluble ST2 or ST2L. BALB/c $ST2^{-/-}$ mice had previously been obtained from Professor Andrew Mackenzie, BBRC, University of Cambridge.

2.2.3. Breeding DBA/1 $ST2^{-/-}$ mice

The BALB/c strain is resistant to arthritis so in order to carry out these experiments $ST2^{-/-}$ mice on the susceptible DBA/1 background were required. Female BALB/c $ST2^{-/-}$ mice were mated with male DBA/1 mice to produce $ST2^{+/-}$ heterozygous F_1 mice. Female F_1 heterozygotes were identified by polymerase chain reaction (PCR) and backcrossed for 7 further generations with wildtype male DBA/1 mice. Male and female F_8 heterozygotes were mated and $ST2^{-/-}$ homozygote offspring used to set up breeding pairs.

2.2.3.1. Screening for ST2 gene expression by PCR

Mice to be used in breeding were identified as wildtype ($ST2^{+/+}$), heterozygous ($ST2^{+/-}$) or homozygous ($ST2^{-/-}$) for the disrupted ST2 gene by polymerase chain reaction (PCR). The same method was used in both BALB/c and DBA/1 strains as the gene disruption was the same. All techniques were performed using gloves and guaranteed DNAase/RNAase free plasticware.

a) DNA extraction

All centrifugations were at 14000rpm at 4°C. Tail tips were digested overnight at 55°C in 0.5 ml tail lysis buffer. The tail digest was mixed with 0.5ml phenol/chloroform/isoamyl alcohol and centrifuged for 5 minutes. The top layer, containing the DNA, was decanted into a fresh eppendorf taking care not to disturb the protein layer (see Figure 2.1).

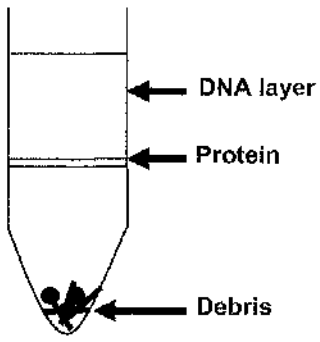

Figure 2.1 Extracted DNA is in the top buffer layer

This was mixed with 0.5ml chloroform and centrifuged for 5 minutes. The top DNA-containing layer was decanted again and 0.8ml of ice cold 100% ethanol was added, mixed well, and left at -20°C for 20 minutes for the DNA to precipitate. This was centrifuged for 15 mins, the supernatant was poured off, and 0.5ml of ice cold

70% ethanol added to the DNA pellet. This was centrifuged again for 15 minutes, and the supernatant poured off. The pellet was air-dried for 25 mins at 55°C in a vacuum-dryer, and resuspended in 50µl water for injection. The DNA was stored at 4°C until PCR was performed for genotyping.

b) Polymerase Chain Reaction for ST2 gene disruption

Reaction buffer (19µl) and sample DNA (1µl) were mixed in PCR tubes. DNA samples from mice known to be $ST^{+/+}$, $ST2^{+/-}$ or $ST2^{-/-}$ were used as positive controls. Pure water (1µl) was used as a negative control. PCR was performed using the following protocol:

1.	1 cycle	94°C	3 minutes
2.	30 cycles	94°C	45 seconds
		60°C	30 seconds
		72°C	90 seconds
3.	1 cycle	72°C	10 minutes
4.	Stored at 4°C until run on DNA detection gel		

c) DNA detection gel

The samples were mixed with 5µl loading buffer, run on a 2% agarose gel with 0.005% ethidium bromide for 30 mins at 100mV in TAE buffer, then viewed under ultraviolet (UV) light. A DNA sample ladder was used to estimate the size of the bands detected. Primers 1 and 3 amplified the 500 base pair (bp) wildtype band, and primers 2 and 3 amplified the 200bp disrupted *ST2* band (see appendix 2, table 1 for primer sequences). An example of the gels obtained is shown in Figure 2.2.

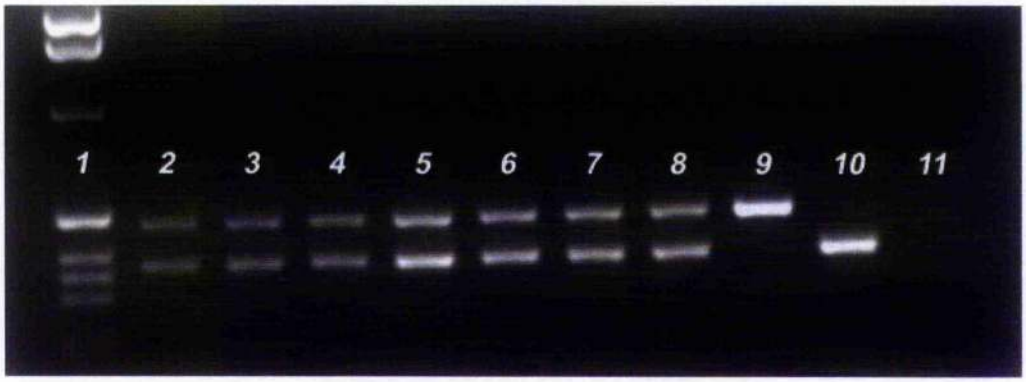

Figure 2.2 A DNA detection gel as viewed under UV light

1 = DNA ladder

2 – 7 = Samples. All heterozygous in this example

8 = $ST2^{+/-}$ heterozygous control

9 = $ST2^{+/+}$ wildtype control (500bp)

10 = $ST2^{-/-}$ homozygous control (200bp)

11 = Negative control (no sample DNA)

DNA from the tail-tips of mice to be used in breeding was amplified by PCR, run on a 2% agarose gel with 0.005% ethidium bromide, and then viewed under UV light to illuminate the resulting bands.

2.3. Cell culture

Cells were always handled under sterile conditions, and were washed in sterile wash medium by centrifuging at 1400rpm for 5 mins at 4˚C unless otherwise stated. Live cells were counted in a Neubauer haemocytometer (Weber Scientific International Ltd, UK) using an aliquot diluted with a 0.1% (w/v) solution of trypan blue to stain non-viable cells. The number of live cells in 25 central squares was counted and the number of cells per ml calculated using the following formula:

$$\text{Cells/ml} = \text{count} \times \text{dilution factor} \times 10^4$$

All cells were cultured at a final concentration of 1×10^6 per ml in complete RPMI medium, and at 37˚C in a 5% CO_2 atmosphere. Additional antigens for cell restimulation were added to cell culture where indicated

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2.3.1. Cytokine and chemokine production

To assess lymphocyte synthetic function cells were cultured in flat-bottomed 24 well culture plates (Nunc, Roskilde, Denmark) for 96 hrs. The supernatant was then removed and frozen at -20°C until further analysis.

2.3.2. Proliferation assay

To assess lymphocyte proliferation cells were cultured in triplicate for 72 hrs in round-bottomed 96 well tissue culture plates (Nunc, Roskilde, Denmark). Tritiated (^{3}H)-thymidine (15kBq in 10µl sterile wash medium) was added for the last 8 hrs of culture. Cells were harvested onto glass fibre filter paper (Wallac, Milton Keynes, UK) using a 295-0054 Betaplate 96 well harvester (Wallac). ^{3}H-thymidine incorporation was measured using a Matrix 96 Direct Beta Counter (Wallac). All procedures were performed according to local radiation safety guidelines.

2.4. IL-33 Purification

Recombinant human and murine interleukin (rh and rmIL)-33 were produced and purified within the department by Dr Damo Xu. Briefly, IL-33 cDNA was cloned from IL-1 stimulated human or murine fibroblasts by real time (RT)-PCR using specific primer pairs (see appendix 2, table 2 for sequences). The sequence-confirmed IL-33 cDNA was inserted into expression vector PQE (Quiagen) and fused with a His-tag before being transformed into *Escherichia coli* strain M15. The IL-33 protein was induced by IPTG and purified by Ni-NTA affinity chromatography (Quiagen). The IL-33 was then run through a polymyxin B column to remove endotoxin. The purity of IL-33 was more than 97% by SDS gel, and endotoxin levels were less than 0.01eu/ug of protein Limulus Amoebocyte Lysate QCL-1000 pyrogen testing (Biowhittaker).

2.4.1. Testing the bioactivity of IL-33

Peripheral lymph nodes were removed from naïve BALB/c ST2$^{+/+}$ and ST2$^{-/-}$ mice (Figure 2.3). A single cell suspension was created by crushing the lymph nodes gently through Nitex mesh (Cadisch, UK), washing them twice, resuspending in 5ml complete medium and filtering again through Nitex to remove debris. The cells were counted and kept on ice until plated out for cell culture. T lymphocytes were stimulated using plate-bound anti-CD3 with or without IL-33. Flat-bottomed 24 well

cell culture plates were coated by incubating with 200µl of 2µg/ml anti-CD3 in sterile PBS at 37°C for 3 hours. The wells were washed twice with complete medium before adding the cells at $1x10^6$ cells/ml. IL-33 was added at 10ng/ml to some wells. All conditions were cultured in triplicate for 3 days and supernatants collected for analysis.

The bioactivity of IL-33 was confirmed by measuring the increase in IL-5 concentration by enzyme-linked immunosorbant assay (ELISA). Batches of IL-33 with similar bioactivity were used in experiments. Specificity was confirmed by culture with $ST2^{-/-}$ cells, where no increase in IL-5 production was seen with the addition of IL-33 (Figure 2.4).

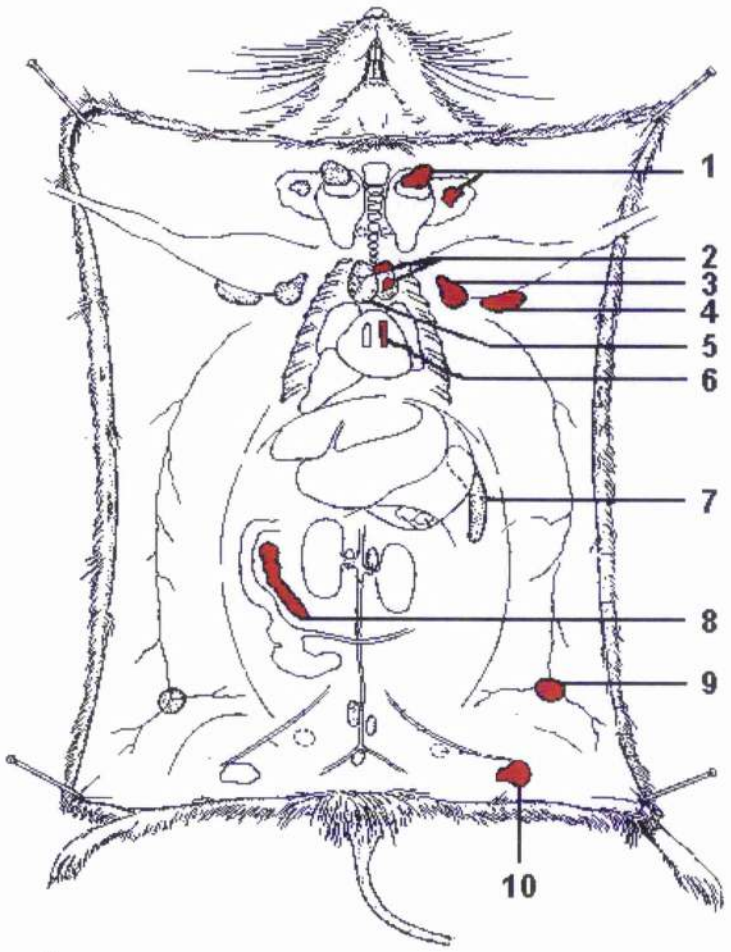


Figure 2.3 Murine lymphoid organ anatomy

1	Cervical	**6**	Sub-carinal (retrocardiac)
2	Mediastinal	**7**	Spleen
3	Axillary	**8**	Mesenteric
4	Brachial	**9**	Inguinal
5	Thymus	**10**	Popliteal

Adapted from the National Institute of Allergy and Infectious Diseases (part of the National Institute of Health) website: http://www.niaid.nih.gov/Dir/services/animalcare/MouseNecropsy/lymph.html

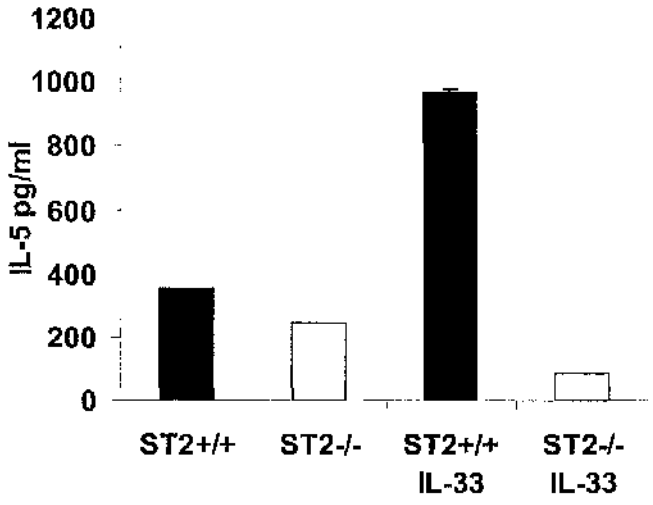


Figure 2.4 Testing the bioactivity of IL-33

Lymph node T cells were stimulated with anti-CD3 with or without IL-33 for 3 days. Culture supernatants were harvested and the concentration of IL-5 measured by ELISA. Results are the means ± SD of triplicate assay wells.

2.5. Murine models of human diseases

As discussed in the introduction, asthma and RA are complex multi-factorial diseases, with heterogeneity of genetics, pathophysiology, severity and response to treatment. The underlying mechanisms are not yet fully elucidated, and much research remains to be done. Although the study of human subjects is essential to guide research and translate experimental findings into clinically applicable data, it is limited by a number of factors. Firstly, it is usually only possible to study people who already have established disease, and so it is difficult to elucidate initiating factors. In addition, it can be almost impossible to separate the effects of just a single factor from many other confounding factors. Legal, ethical and safety considerations also restrict the amount and type of tissue that can be studied, often limiting the scope of investigation to snapshots of processes involved, rather than dynamic studies.

Therefore, much important work has been performed using *in vivo* animal models of disease. In particular the mouse has been used extensively for a number of reasons. On a practical level mice are not endangered, and a number of

genetically well characterised in-bred strains are readily available. There is also an extensive commercially available "toolbox" of immunological agents with which to carry out investigations. The mouse immune system is well studied and understood, and is similar to human in many regards. The mouse genome can be manipulated to selectively knock out or amplify factors of interest in order to assess their functional importance. Any tissue can be examined in detail at a number of time points, allowing exploration of dynamic processes, and experiments can be controlled to isolate the effect of a single factor at a time.

Disadvantages are that mice do not naturally develop asthma or arthritis, and so any disease model will be artificial. Also there remain important differences between human and murine immunity (reviewed in (351)) which means caution must be taken when applying murine findings to human disease. Despite these shortcomings, murine models have been invaluable in studying specific aspects of the inflammatory processes seen in asthma and arthritis, which can then be applied to humans for further study.

2.5.1. Murine allergic airways disease

The advantages and disadvantages of using mice to model asthma have been extensively debated and reviewed (352-356). Although none of the many models in use has all the hallmarks of human asthma, many features are present: a peribronchial and perivascular inflammatory infiltrate rich in eosinophils and lymphocytes, a Th2 cytokine bias, mucus hypersecretion, increased serum IgE, and changes in airway physiology. However, in addition to differences in immune function, mice have structurally and functionally different airways to humans, with fewer bronchial divisions, more bronchial-associated lymphoid tissue (BALT) and smooth muscle present only in the larger airways. Therefore, some key features of human asthma are not easily modelled or measured, in particular AHR and chronic inflammation (357).

2.5.1.1. Designing a murine model of asthma

A number of different approaches have been used to model asthma, including active sensitisation and challenge, transfer of antibody, transfer of allergen-specific lymphocytes and transient gene transfer (354). It should be remembered that differences in models can result in variable results even when studying the same phenomenon.

As I was most interested in studying the acute allergic response to an allergen I used a short sensitisation and challenge protocol in BALB/c mice using ovalbumin (OVA) as the allergen, adapted from Stock et al ((128); Figure 2.5). This type of model is widely used and gives reproducible results. The following factors were taken into account when considering what model to use:

a) Mouse strain

Strains of mice differ in their ability to exhibit various features of asthma. AHR is best demonstrated in A/J mice although it is also seen in BALB/c mice. BALB/c mice are pre-disposed to make strong Th2 type inflammatory responses, and have been shown to exhibit greater allergic airways inflammation than other strains.

b) Allergen and adjuvant

Various allergens have been used, including live organisms (fungi, parasites), complex proteins (from house dust mites and cockroaches) and simple proteins with or without adjuvants (OVA with alum). I used sterile chicken OVA as the model allergen as simple proteins give a reliable effective dose and reproducible response. Live organisms and complex proteins can vary widely in the response elicited even in congenic mice. Simple proteins alone do not stimulate strong responses so an adjuvant is given to boost the response. In this case I used alum as it also induces a Th2 type response (145).

c) Route and timing of administration

Active sensitisation and challenge mimics the events happening after allergen challenge in human asthma, albeit over a much shorter time-scale. Systemic sensitisation (intraperitoneal (IP)) followed by local challenge (intranasal (IN)) given from 7 days up to several weeks afterwards has been shown to give the optimal degree of allergic airways inflammation. In pilot experiments I found a single sensitisation and a single challenge 7 days later were sufficient to induce allergic airways disease, whilst 3 consecutive challenges gave maximal inflammation (data not shown). However, the dose of OVA and the number of challenges given could be varied to increase or reduce the level of inflammation.

2.5.1.2 Allergen sensitisation

All mice, including negative control groups, were sensitised on day 1 with 100µg of OVA in 100µl of sterile PBS mixed with 100µl of 2% alum injected intraperitoneally. The same sensitisation protocol was followed in all experiments.

2.5.1.3 Allergen challenge

The mice were anaesthetised using 250-350µl of avertin injected IP. Once they no longer withdrew from whisker stimulation they were challenged with either 2µg or 10µg of OVA in 30µl sterile PBS IN. PBS alone was used as a negative control. Where indicated rmIL-33 was added to the OVA or PBS and administered at the same time. The mice were placed in a warm box until they recovered consciousness, and supplementary oxygen given if required. Mice were challenged either 3 times (days 9, 10 and 11) or twice (days 9 and 11). The optimum dose and number of challenges were determined by pilot experiments (see section 4.2 for data).

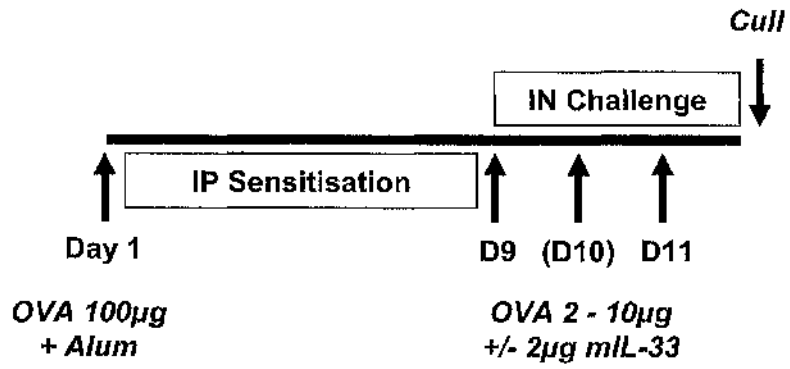


Figure 2.5 A murine model of allergic airways disease

Mice were sensitised to OVA by IP injection on day 1, with alum used as a Th2 adjuvant. The airways were challenged with IN OVA on days 9 – 11. Where indicated rmIL-33 was given IN at the same time as OVA. Mice were culled 24 hours after the final challenge and various tissues collected for analysis.

2.5.1.4 Tissue harvesting

The mice were culled 24 hours after the last challenge by anaesthetising with 500µl avertin IP, and then exsanguination to collect serum. Various tissues were then harvested and analysed.

a) Serum

The chest wall was opened and blood withdrawn from the right ventricle using a 1ml syringe and 23 gauge needle. It was then centrifuged at 14000rpm at 4°C for 30 mins, and the serum decanted and stored at -20°C until analysed.

b) Bronchoalveolar lavage

The trachea was exposed by dissection of the superficial neck structures and cannulated using a 1ml syringe with a 23 gauge needle. The needle was sheathed in plastic tubing (0.58mm ID, 0.78mm OD; VWR International) extending 1-2mm from the end to prevent the needle passing through the posterior wall of the trachea. The needle was held in place by blunt forceps to effect a seal. The lungs were inflated carefully with 800µl PBS and the fluid withdrawn after 10 seconds. This process was then repeated with a fresh 800µl aliquot of PBS and the two aliquots pooled and kept on ice until further processing. Total cell counts were performed as described (section 2.3). After removal of cells for differential cell counting (see below), the remaining BAL fluid was centrifuged at 1400rpm for 5 minutes at 4°C and the supernatant frozen at -20°C until analysed.

c) Differential cell count

In order to determine the relative frequency of different cell types $5x10^4$ cells were removed from the BAL and spun onto a slide at 300rpm for 6 mins using a Cytospin (Thermo Shandon, Cheshire, England). The resulting cytopreps were air-dried then fixed in methanol for 10 minutes. They were stained by the Romanovsky method using Rapi-Diff II and coverslips fixed in place with DPX. Macrophages, eosinophils, neutrophils and lymphocytes were identified using standard morphological criteria under x100 oil immersion microscopy, and the relative frequency of each was determined by counting 400 consecutive cells. The number of cells per ml in the original BAL was then calculated from the total cell count. Squamous epithelial cells were often seen, but were not included in the analysis. The slides were blinded and randomised before counting to eliminate any

bias. Figure 2.6 shows examples of these cell types as seen under x40 microscopy.

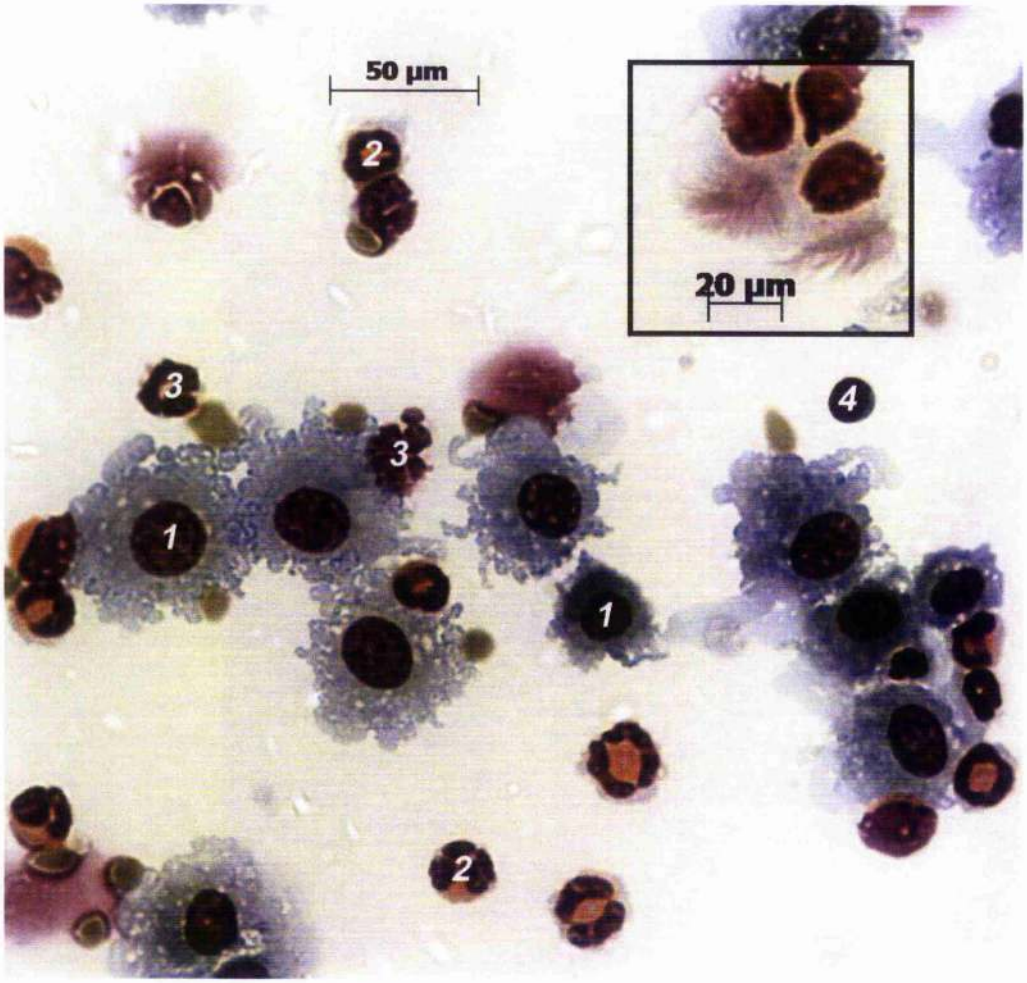


Figure 2.6 Leukocytes seen in mouse bronchoalveolar lavage fluid

This figure represents the standard morphology of various leukocytes seen in mouse bronchoalveolar lavage fluid at x40 magnification.

1 = macrophage – mononuclear with pale foamy cytoplasm

2 = eosinophil – multi-lobed or ring nucleus with eosinophilic cytoplasm

3 = neutrophil – multi-lobed nucleus with pale cytoplasm

4 = lymphocyte – mononuclear with very little cytoplasm

The insert shows ciliated squamous cells at the same magnification.

d) Draining lymph nodes

The heart and lungs were removed en bloc from the chest cavity and the mediastinal and subcarinal draining lymph nodes (DLNs) draining the lung were removed into ice cold wash medium and kept on ice. A single cell suspension was created by crushing the lymph nodes gently through Nitex, washing them twice, resuspending in 5ml complete medium and filtering again through Nitex to remove debris. The cells were counted and kept on ice until plated out for cell culture. Cells were cultured with or without 1mg/ml OVA for both proliferation and cytokine/chemokine cultures.

e) Lung histology

The heart/lung block was removed intact and the trachea cannulated. The lungs were inflated with 1ml 10% neutral buffered formalin (NBF), the trachea tied off using cotton thread, and then the whole block immersed in 10% NBF for at least 48 hrs. The lungs were mounted in paraffin blocks, and 6μm sections stained with haematoxylin and eosin (H&E; kindly performed by Mr. Roderick Ferrier, Department of Pathology, Western Infirmary, Glasgow). Sections were examined at x20-100 magnification and peribronchial and perivascular inflammation assessed.

Scoring system

There is no single validated scoring system for allergic airway inflammation seen in mice. I used a semi-quantitative system to assess the degree of eosinophilic infiltration seen (see Figure 2.7):

0	No eosinophils
1	Eosinophils make up less than 10% of total infiltrate or total infiltrate is <20 cells
2	Eosinophils make up 10%-50% total infiltrate
3	Eosinophils make up more than 50% total infiltrate

A total of 10 fields were scored for each mouse. Each field had to contain both a bronchus and a vessel in close proximity. The average field score for each mouse was calculated, and then the results expressed as the mean score for each group. All scoring was performed on slides which had been blinded. In some experiments

the scoring was validated by a second blinded observer to ensure inter-observer agreement. The results obtained were the same.

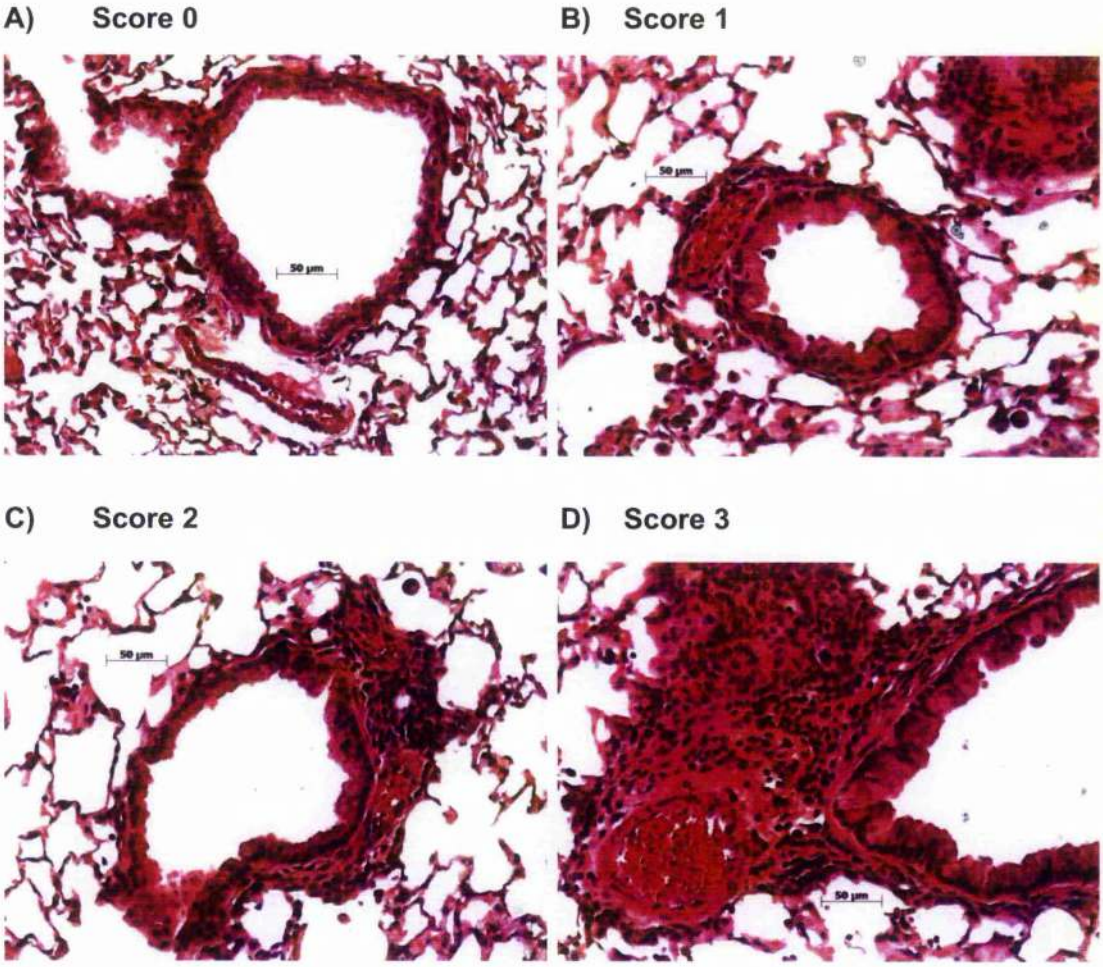


Figure 2.7 Lung histology from allergic airways inflammation

Shown here are examples of different grades of allergic airways inflammation in BALB/c mice using the experimental protocol described.

2.5.2. Murine collagen-induced arthritis

As with asthma, there is no single model which exactly mimics human RA, although various models have been developed which can exhibit some of the cardinal features of human disease, such as auto-antibody production, chronic synovitis and joint destruction, and even systemic features (vasculitis, interstitial pneumonitis, and subcutaneous nodules) (229,358-362).

2.5.2.1. Designing a model of rheumatoid arthritis

A model of antigen-specific collagen-induced arthritis (CIA) was used, which shares many of the immunological and pathological features of human RA (reviewed in (362)). The response to collagen is well categorised, and it has been widely used both in studying the pathogenesis of disease, and in testing novel therapeutics. It depends on both T and B cell immunity, resulting in a Th1/Th17 type response with production of arthritogenic antibodies.

The mice are immunised intradermally (ID) with heterologous collagen type-II (CII) and an adjuvant, and the response boosted by IP challenge with CII (see Figure 5.1). The following factors were taken into consideration when designing the model:

a) Mouse strain

Susceptibility to arthritis is linked to the expression of certain MHC-class II molecules. Most commonly used strains of mice, including BALB/c (H-2^d), are resistant to arthritis. However, DBA/1 mice (H-2^q) are highly susceptible to arthritis (362). Other genetic factors, such as variability in cytokine expression also play a role. As we only had $ST2^{-/-}$ mice on the BALB/c background, this necessitated breeding this trait onto the DBA/1 background (see section 2.2.3.).

b) Antigen and adjuvant

CII was used as the antigen as the anti-CII IgG2a antibodies produced cross-react with mouse CII in the joint, and activate complement there, thus localising the systemic response to cause joint pathology. DBA/1 mice will respond to chicken, bovine, porcine and human CII. I used bovine CII as it is widely used, results in a high incidence of disease and gives reproducible results.

Collagen alone will not induce arthritis. Co-administration of Complete Freund's Adjuvant (CFA) biases towards a Th1 type response, and is essential for the production of arthritogenic antibodies. The amount of antibody produced depends on the concentration of *Mycobacterium tuberculosis* present in the CFA (363). Recently, high doses were even shown to induce arthritis in strains previously thought to be resistant to CIA (364).

2.5.2.2. Antigen immunisation

I used a commercially available reagent kit (Chondrex) which contained both bovine CII (2mg/ml in 0.05M acetic acid) and CFA.

a) Emulsion preparation

An emulsion of CII and CFA was produced by mixing equal volumes of each in a small universal on ice to prevent denaturing. This was best achieved by rapidly drawing up and ejecting the mix using a 1ml syringe. The emulsion was considered stable when a drop remained as a solid clump and did not dissipate when placed on the surface of water.

b) Collagen Injection

On day 1 the mice were anaesthetised using halothane, and a single ID injection of 50µg CII in 50µl emulsion (1mg/ml) was made at the base of the tail using a 1ml syringe with a 27 gauge needle. The mice develop a local skin reaction with ulceration at the injection site.

c) Boosting the response

The response was boosted on day 21 by mixing equal volumes of CII and sterile PBS, and then injecting 50µg CII in 50µl PBS (1mg/ml) IP.

d) Treatment with rmIL-33

The effect of rmIL-33 on the severity of arthritis was assessed. Where indicated mice were treated by injecting 0.8µg rmIL-33 in 200µl sterile PBS IP for 5 consecutive days starting on day 21.

2.5.2.3. Footpad measurements

The thickness of the front and rear footpads was measured using callipers on day 21, as a baseline, and then every 1-2 days following this. The increase in footpad thickness was calculated from the baseline measurements. The clinical score for each footpad was also assessed using the following criteria:

	Score
No inflammation	0
Red toes or footpad	1
Red, swollen footpad	2
Loss of function (limping)	3

The incidence of disease and the average increase in footpad thickness and total clinical score per mouse were calculated for each group. Only those mice which developed disease were included in clinical score and footpad analysis.

2.5.2.4. Tissue harvest

At various time points after boosting, the immune response was assessed by culling the mice by neck dislocation, and harvesting the draining lymph nodes, spleens and joints.

a) Lymph node and spleen cultures

The popliteal DLNs and spleens were harvested (see Figure 2.3) and pooled for each group. They were kept on ice until single cell suspensions were prepared (as in section 2.5.1.5.d). In addition, the red blood cells from spleens were lysed after the first wash using 1ml of Red Cell Lysis buffer for 1 minute, then washing a further two times. Cells were cultured at $1x10^6$/ml in complete medium with 100µg/ml bovine CII for cytokine/chemokine production and proliferation.

For use in culture bovine CII was dissolved in sterile 0.05M acetic acid at 4°C overnight on a rotating mixer to give a concentration of 2mg/ml. This was then dialysed twice against 2 litres of PBS using dialysis cassettes. The resulting solution of CII in PBS was kept at -20°C until use. It was used in culture at 100µg/ml. For consistency the same batch of CII was used in all experiments. Chondrex CII was not used in culture as it tended to precipitate out and form large gel-like structures in culture medium, therefore CII from Sigma was used.

2.6. Administration of IL-33

In some experiments I used IL-33 alone to demonstrate its biological effects on the innate immune system. Recombinant IL-33 was produced as described previously (section 2.4) and administered as described below.

2.6.1. Intraperitoneal administration of IL-33

Recombinant IL-33 (human or murine) was injected IP for 7 days, at a dose of 0.8µg per mouse in 200µl sterile LPS-free saline (see Figure 3.1).

2.6.2.1. Tissue harvest

Mice were culled by neck dislocation 24 hours after the last injection, and various tissues harvested. Serum, BAL and lungs were collected and processed as described previously (section 2.5.1.5.), and in addition peritoneal wash and spleens were also collected.

a) Peritoneal Wash

The peritoneum was instilled with 6ml sterile LPS-free PBS using a 10ml syringe and 21 gauge needle. This was then withdrawn after 10 secs and kept in a universal on ice until processing. The total cell count was performed, and $5x10^4$ cells removed for cytospin and differential count as described for BAL fluid (section 2.5.1.5.c). The remaining sample was centrifuged at 1400rpm for 5 mins at 4°C to pellet the cells, and the supernatant kept at -20°C until further analysis.

b) Spleen harvest

The spleen was removed and kept in ice cold wash medium until processing. Spleens were weighed, then a single cell suspension prepared (see section 2.5.2.4.) Differential counts were performed on cytospins of $5x10^5$ cells as described for BAL fluid (see section 2.5.1.5.c).

2.6.2. Intranasal administration of IL-33

IN administration of rmIL-33 was undertaken in a similar fashion to OVA challenge in the airway inflammation model (see section 2.5.1.4.). Mice were anaesthetised using 250-350µl avertin IP, and then 2µg of rmIL-33 in 30µl sterile LPS-free PBS was given IN. Mice were allowed to recover in a warm box with supplemental oxygen if required. This was done for 7 consecutive days (see Figure 3.8).

2.6.2.1. Tissue harvest

Mice were anaesthetised 24 hours after the final dose with 500µl avertin, culled by exsanguination and tissues harvested. Serum, BAL, peritoneal wash, spleens and lungs were all processed as previously described (sections 2.5.1.5. and 2.6.2.1).

2.7. Enzyme-linked immunosorbant assay

Enzyme linked immunosorbant assays (ELISAs) were used to determine the concentration of cytokines, chemokines and antibodies in serum, BAL, peritoneal wash and cell culture supernatants. All ELISAs were carried out in flat-bottomed 96 well Immulon 4 assay plates (Thermo Labsystems, Franklin, USA) using 0.05% Tween-20 in PBS for washing. Analysis was performed on an MRX-II microplate reader (Dynex Technologies, UK) running Revelation software v1.5, and sample values calculated from the standard curves generated (see Figure 2.8).

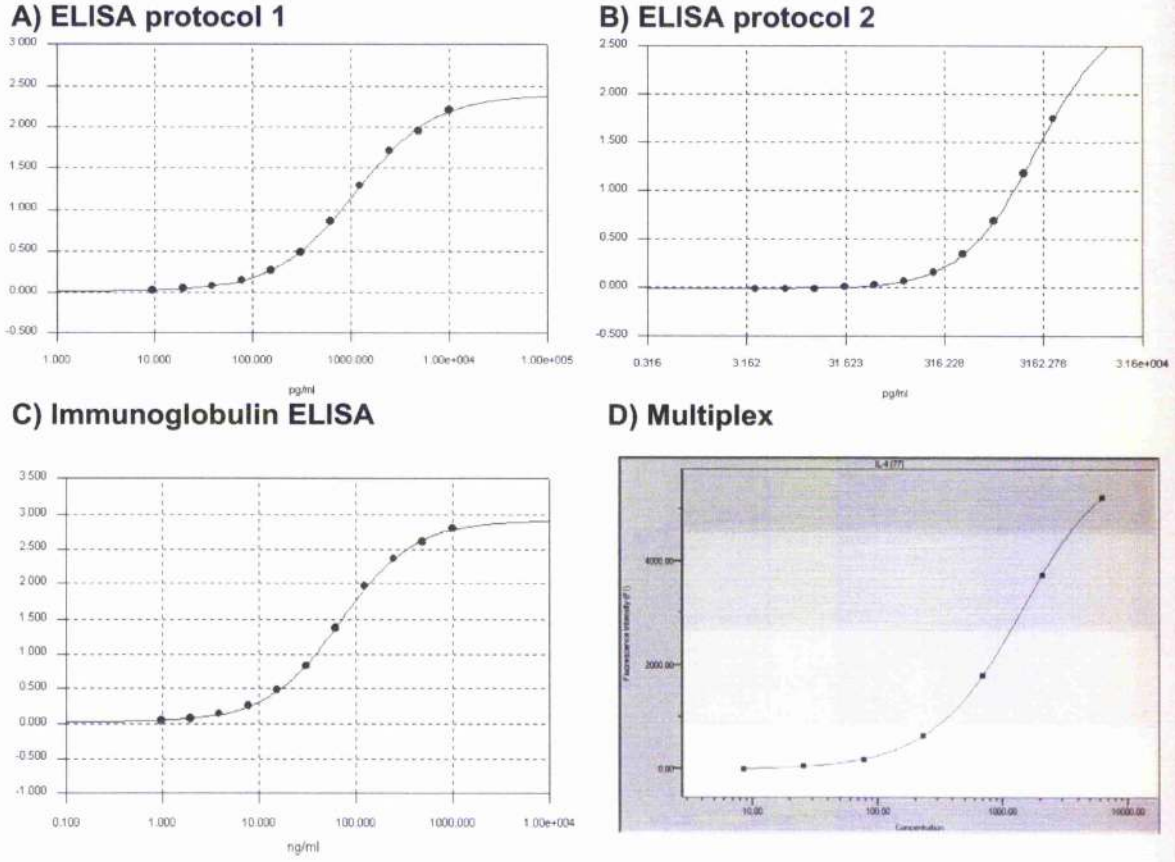


Figure 2.8 Standard curves for ELISA and multiplex

2.7.1. Cytokine and chemokines

Most cytokines and chemokines were measured using commercially available capture and detection antibody pairs, each recognising discrete epitopes. Recombinant murine cytokines and chemokines were used to create doubling dilution (1:2) standard curves on each plate. All samples were applied in triplicate where possible, and diluted 1:5 – 1:10 where concentrations were above the limit of the standard curve. The protocol followed depended on the source of the antibody pairs.

2.7.1.2. Protocol 1 – BD sourced antibodies and standards

Unless otherwise indicated, a volume of 50µl per well was used for all stages, antibodies, standards and samples were diluted in ELISA assay buffer A, and all incubations were at room temperature.

Cytokine	Capture Antibody (µg/ml)	Detection Antibody (µg/ml)	Top standard (ng/ml)
IL-4	2	2	10
IL-5	4	4	10
IFNγ	1	0.5	40

Table 2.2 BD sourced antibodies and standards

Plates were coated with the capture antibody overnight in ELISA coating buffer A at 4°C. They were then washed twice and non-specific binding blocked by incubating the plates at 37°C for 1 hr with 200µl ELISA assay buffer A. The plates were washed twice before applying the samples and standards, which were incubated for 2 hrs, and then the plates washed 4 times. The detection antibody, conjugated to biotin, was added and incubated for 1 hr, washed 4 times, then plates incubated with extravidin-peroxidase (1:1000) dilution for 30 mins. The plates were washed 6 times, and 100µl 3'3'5'5'-tetra-methylbenzidine (TMB) substrate added to each well. The plates were incubated for up to 30 mins in the dark and then colour development measured at 630nm.

2.7.1.3. Protocol 2 – R&D Systems sourced antibodies and standards

Unless otherwise stated a volume of 100µl per well was used, and antibodies, standards and samples were diluted in ELISA assay buffer B. All incubations were at room temperature.

Cytokine	Capture Antibody (µg/ml)	Detection Antibody (µg/ml)	Top standard (ng/ml)
IL-1β	4	0.3	2
IL-13	4	0.2	2.5
Eotaxin-1	0.8	0.4	0.5
Eotaxin-2	2	0.075	4
TCA-3	2	2	1
TARC	2	0.2	1

Table 2.3 R&D Systems sourced antibodies and standards

Plates were coated with the capture antibody overnight in ELISA coating buffer B. They were then washed twice and non-specific binding blocked by incubating the plates for 1 hr with 300µl ELISA blocking buffer. The plates were washed twice before applying the samples and standards, which were incubated for 2 hrs, then the plates washed 4 times. Eotaxin-2 detection antibodies were diluted in ELISA assay solution B with the addition of 2% heat-inactivated normal goat serum. The others were diluted as normal. The biotinylated detection antibody was incubated for 2 hrs, the plates washed 4 times, and 100µl streptavidin-peroxidase (1:1000) added and incubated for 20 mins. The plates were washed 6 times, and the TMB substrate added and incubated for 20 mins in the dark. The reaction was stopped using 100µl stop solution and colour development measured at 450nm with a 570nm correction filter.

2.7.2. Immunoglobulins

Total IgE and IgA, and antigen-specific IgE, IgG1 and IgG2a were measured using commercially available antibodies, kits and standards (R&D Systems). Standards were available for total IgE and IgA only, and were used at doubling dilutions in duplicate. Antigen-specific Igs were measured by comparing OD values directly. All samples were applied in triplicate where possible. Unless otherwise indicated a volume of 100µl per well was used, incubations were at room temperature, and antibodies, standards and samples were diluted in ELISA assay buffer A.

2.7.2.1. Total IgE

Total IgE was measured using a commercially available mouse anti-IgE kit. The concentrations of antibodies and avidin-horseradish peroxidase (HRP) varied according to batch and were used as recommended by manufacturer (typically 1:250). Plates were coated with the capture antibody overnight in ELISA coating buffer B at 4°C. They were then washed twice and non-specific binding blocked by incubating the plates for 1 hr with 200µl ELISA assay buffer A. The plates were washed twice before applying the standard (starting at 100ng/ml) and samples at 1:10 – 1:50 dilution. Samples and standards were incubated for 2 hrs, then the plates washed 4 times. The biotinylated detection antibody and avidin-HRP were added at the same time and the plate incubated for 1 hour. The plates were washed 6 times, and 100µl TMB substrate added. The plates were incubated for 30 mins in the dark, and then the reaction stopped using 100µl stop solution. The colour development was measured at 450nm with a 570nm correction filter.

2.7.2.2. Total IgA

Total IgA was measured using a commercially available antibody pair and purified monoclonal mouse IgA as a standards. Plates were coated with the capture antibody (2µg/ml) overnight in ELISA coating buffer B at 4°C. They were then washed twice and non-specific binding blocked by incubating the plates for 1 hr with 200µl ELISA assay buffer A. The plates were washed twice before applying the standard (starting at 1000ng/ml) and samples (BAL 1:10 – 1:50; peritoneal wash 1:20 – 1:100; serum 1:1000 – 1:5000). Samples and standards were incubated for 2 hrs, then the plates washed 4 times. The biotinylated detection antibody (2µg/ml) was added and the plate incubated for 1 hr. The plates were washed 4 times, and extravidin-peroxidase (1:1000) added and incubated for 30 minutes. The plates were washed 6 times and 100µl TMB substrate added for 30 mins in the dark. The reaction was stopped using 100µl stop solution and colour development was measured at 450nm with a 570nm correction filter.

2.7.2.3. Antigen-specific IgE

Antigen-specific IgE was measured using components of the total IgE kit. Plates were coated overnight with 10µg/ml OVA in ELISA coating buffer B at 4°C. They were then washed twice and non-specific binding blocked by incubating the plates at 37°C for 1 hr with 200µl ELISA assay buffer A. The plates were washed twice before applying the samples. No standards were available. Samples were diluted

to 1:50, applied in duplicate in doubling dilutions, and incubated at room temperature for 2 hrs. The remainder of the protocol was identical to the total IgE kit.

2.7.2.4. Antigen-specific IgG1 and IgG2a

Antigen-specific IgG1 and IgG2a were measured using commercially available anti-mouse IgG1 and IgG2a antibodies conjugated to biotin (R&D Systems). Plates were coated with overnight with 10µg/ml OVA in ELISA coating buffer B at 4˚C. They were then washed twice and non-specific binding blocked by incubating the plates at 37˚C for 1 hr with 200µl ELISA assay buffer A. The plates were washed twice before applying the samples. No standards were available. Samples were diluted to 1:250 – 1:500 for IgG1 and 1:25 – 1:50 for IgG2a, and applied in duplicate. Tripling (1:3) dilutions were used for IgG1 and doubling (1:2) dilutions for IgG2a. The samples were incubated for 2 hrs, then washed 4 times. Biotinylated detection antibody was added at 1:1000 for IgG1 and IgG2a and incubated for 1 hour. The plates were washed 4 times, and extravidin-peroxidase (1:1000) added for 30-60 mins. The plates were washed 6 times, and then 100µl of TMB substrate added for 30 mins. The reaction was stopped at this point using 100µl stop solution, and the plates analysed at 450nm with 570nm correction.

2.8. Multiplex Bead Analysis: Cytokines and Chemokines

Where indicated cytokines and chemokines in serum, BAL or culture supernatants were analysed using BioSource Multiplex Bead Immunoassays analysed on a Bio-Rad Bioplex analyser. Extensive instructions were provided with the kit used and followed closely (BioSource Mouse Cytokine Twenty-Plex – measuring FGF, VEGF, GM-CSF, IFNγ, TNFα, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL-13, IL-17, IP-10, KC, MCP-1, MIG, MIP-1α). The number of samples measured with one kit was doubled by diluting out the reagents to half the concentration using the recommended BioSource Mouse Extracellular Protein Buffer Reagent kit. This did not affect the sensitivity of any of the assays. A 50µl sample was sufficient, but where possible all assays were performed in duplicate.

The principle is similar to that of ELISAs, being a solid phase sandwich assay. Briefly, capture antibody coated beads were applied to the wells, and then the

plates incubated with standards and samples for 2 hrs. During this time analytes bound to the capture antibodies on beads with a defined spectral property specific to each analyte. After washing, biotinylated detection antibodies were added for 1 hr, and the plates washed again. Streptavidin conjugated to the fluorescent protein R-Phycoerythrin (RPE) was then added for 30 mins and the plates washed a final time. The Bio-Rad Bioplex then analysed the amount of fluorescence on each type of bead to determine the concentration of each analyte from the standard curves generated (see Figure 2.8).

The advantages of this method are that the sensitivity and range of values over which reliable concentrations are measured is greater than with ELISAs, and that multiple cytokines and chemokines can be measured from the same sample.

2.9. Statistics

All experiments were performed at least twice to ensure reproducibility of results. To compare different groups, data from individual mice are presented as means with standard error of the mean (SEM). For normally distributed data Student's 2 sample *t*-test was used to calculate p values, and for skewed data the Mann-Whitney U test was used (Minitab Statistical Software, Minitab Inc., State College, PA, USA). A p value of 0.05 or less indicates statistical significance.

2.9.1. Power Calculations

Power calculations were worked out using Minitab, with a significance level of 0.05 and a power value of 0.95.

2.9.1.1. Allergic airways inflammation

From previous experience a difference in eosinophilia of 10% was thought to represent a physiologically significant change, with a standard deviation of 5%. Using Minitab this gave a minimum group size of 8 mice (Figure 2.10A).

2.9.1.2. Collagen-induced arthritis

From previous experience a difference in clinical score per mouse of 3 would be regarded as significant, with a standard deviation of 2. Using Minitab this gave a minimum group size of 13 (Figure 2.10B).

A) Allergic Airways Inflammation

Power and Sample Size

2-Sample t Test

Testing mean 1 = mean 2 (versus not =)

Calculating power for mean 1 = mean 2 + difference

Alpha = 0.05 Assumed standard deviation = 5

Difference	Sample Size	Target Power	Actual Power
10	8	0.9500	0.9602

B) Collagen-Induced Arthritis

Power and Sample Size

2-Sample t Test

Testing mean 1 = mean 2 (versus not =)

Calculating power for mean 1 = mean 2 + difference

Alpha = 0.05 Assumed standard deviation = 2

Difference	Sample Size	Target Power	Actual Power
3	13	0.95	0.956112

Figure 2.9 Power calculations from Minitab

Chapter 3

The role of IL-33 in innate inflammation

3.1. Introduction

IL-33 is a novel cytokine which binds to and signals through ST2L. Current knowledge of its *in vivo* function is limited to C57Bl/6 mice, where systemic (IP) administration of human IL-33 over 7 days induced many of the features of a systemic Th2 effector response in naïve mice (1). In keeping with the known functions of ST2L, there was an increase in Th2 cytokines, total IgA and IgE, and systemic eosinophilia, with pathological changes seen in the lung and gut. Thus the IL-33/ST2L interaction can result in innate Th2 type eosinophilic inflammation.

Administration of IL-4 or IL-13 directly to the airways results in many of the features of asthma, including eosinophil accumulation, mucus hypersecretion and AHR, independent of antigen (89,103). IL-33 mRNA is expressed constitutively in murine lung tissue, and in human bronchial and pulmonary artery smooth muscle and resting bronchial epithelial cells (1). There are also cells constitutively expressing ST2L in the lungs, mainly mast cells (288). Pro-inflammatory signals can induce *ST2* expression in macrophages (284), and sST2 release from fibroblasts (285).

I hypothesised that administration of IL-33 to the airways of mice would result in innate Th2 type inflammation in the absence of antigen. Our lab had produced recombinant murine IL-33, and I would be using BALB/c mice to examine the role of IL-33 in inducing an innate inflammatory response by analysing the local and systemic effects of both intraperitoneal and intranasal routes of administration. By using $ST2^{-/-}$ mice I confirmed that the response seen was dependent on expression of the *ST2* gene, and by using SCID mice, which lack lymphocytes, I began to elucidate the cell types that may be involved.

3.2. The effects of rhIL-33 are not strain specific

As there is very limited published data on the use of recombinant IL-33 as a reagent, the first step was to demonstrate that the systemic effect of human IL-33 is not specific to C57Bl/6 mice. The *in vivo* effects in BALB/c mice were confirmed by following a similar protocol to Schmitz et al (1) and injecting 0.8µg of rhIL-33 IP for seven consecutive days (Figure 3.1). Tissues were collected for analysis the day after the final injection. A dose of 0.8µg was chosen as Schmitz had shown

that 0.4µg was sufficient and we wanted to ensure an adequate dose to see a response.

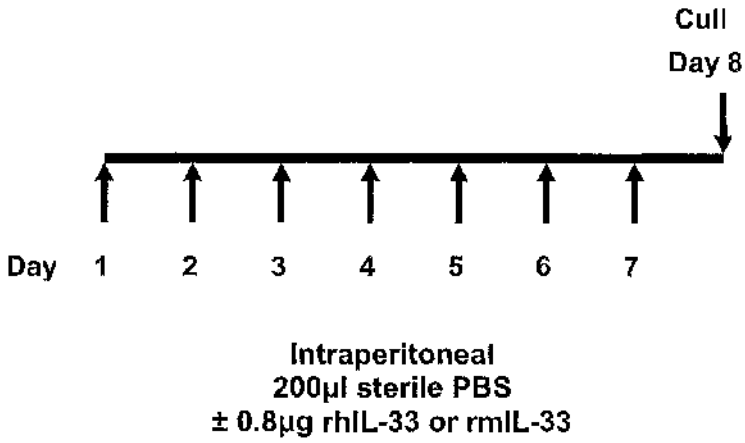


Figure 3.1 Intraperitoneal administration of IL-33

Recombinant human (rh) and murine (rm) IL-33 were produced as described and 0.8µg injected intraperitoneally in 200µl sterile PBS for 7 consecutive days. The day after the last injection the mice were culled and spleens, peritoneal wash, serum, BAL and lungs harvested for analysis.

3.2.1. IP rhIL-33 induces a systemic Th2 effector response in BALB/c mice

Similar to C57Bl/6 mice (1), treatment with IP rhIL-33 increased spleen weight (Figure 3.2A), reflecting an increase in total splenocytes, and increased the percentage of eosinophils in the spleen (Figure 3.2B). In keeping with the rise in eosinophil numbers, serum IL-5 was markedly elevated (Figure 3.2C). In contrast to C57Bl/6 mice, serum IL-13 was undetectable, as was IL-4. Other serum cytokines were either undetectable (IL-1β, IFNγ) or unchanged (IL-6, IL-10, IL-12).

3.2.2. IP rhIL-33 induces Th2 pathology in the lung

In C57Bl/6 mice IP rhIL-33 induced pathological changes at distant mucosal sites, with perivascular eosinophilic infiltrates, goblet cell hyperplasia and mucus hypersecretion in the lung (1). Although perivascular eosinophilia was also a feature in BALB/c mice, there was no goblet cell hypertrophy or mucus hypersecretion (no data shown). This is in keeping with the lack of IL-13 (89).

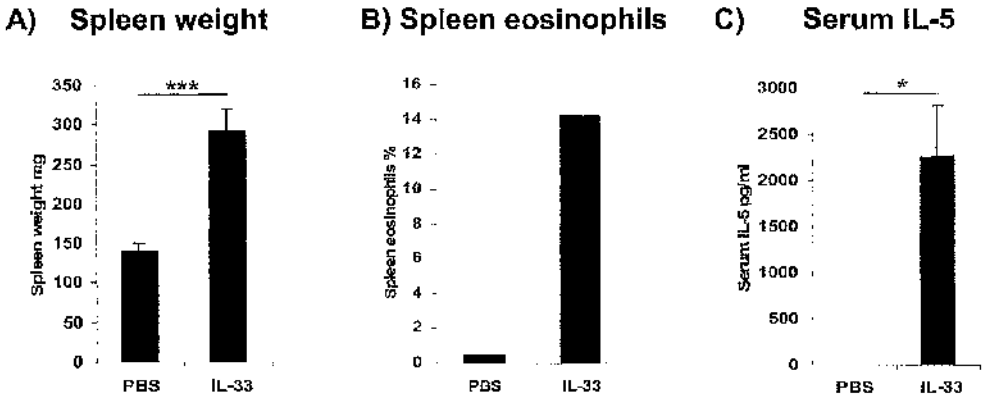


Figure 3.2 IP rhIL-33 is biologically active in BALB/c mice

BALB/c mice were given 0.8µg rhIL-33 IP for 7 days and tissues harvested. **(A)** Individual spleens were weighed. **(B)** Spleens were pooled, a single cell suspension prepared, and the percentage of eosinophils determined by differential cell counts of cytopreps. **(C)** The concentration of serum IL-5 was determined by sandwich ELISA. The results shown are means ± SEM (n=5;*p<0.05, ***p<0.001).

3.3. Murine IL-33 has the same effects as human IL-33

Having established that human IL-33 activity is not restricted to C57Bl/6 mice, I also needed to demonstrate that recombinant murine IL-33 has the same effect in mice as human IL-33, and is therefore just as useful, and more appropriate, as a reagent. In addition, I wanted to confirm that the activity of IL-33 is dependent on expression of the *ST2* gene. To achieve both these aims rmIL-33 was administered systemically to BALB/c $ST2^{+/+}$ and $ST2^{-/-}$ mice using the same protocol as before (figure 3.1). As the figures will indicate IL-33 had no effect in $ST2^{-/-}$ mice, so for simplicity the results are not discussed here.

3.3.1. IP rmIL-33 induces a systemic Th2 effector response

rmIL-33 increased the weight of the spleens (Figure 3.3A) and the percentage of eosinophils (Figure 3.3B) by the same amount as rhIL-33. In addition a rise in the percentage of neutrophils (Figure 3.3C) was also seen that had not occurred with rhIL-33 in either C57Bl/6 or BALB/c mice.

The serum cytokine profile was very similar to that seen after rhIL-33. In keeping with the rise in eosinophil numbers, IL-5 was markedly increased (Figure 3.4A),

but again no IL-13 or IL-4 were detected. Other cytokines were either undetectable (IL-1β, IFNγ, IL-6, IL-10) or unchanged (IL-12).

As eosinophils, and other inflammatory cells, infiltrated tissues distant from the injection site the levels of chemokines were also examined in serum. Both eotaxin-2 and TARC (Figures 3.4B and 3.4C) were elevated, although the rise in eotaxin-2 was not quite statistically significant (p=0.056). Eotaxin-1 levels were not increased by rmIL-33.

Serum total IgA was unaffected by rmIL-33, but total IgE was increased two-fold (data not shown).

3.3.2. IP rmIL-33 induces Th2 pathology in the lung

In keeping with the effect of rhIL-33 in C57Bl/6 mice and BALB/c mice, rmIL-33 induced a Th2 response in the lungs when given IP. A few eosinophils were seen perivascularly, at a similar level to that seen with human IL-33 (data not shown). There was no change in the BAL cell numbers (Figure 3.5A), and virtually all the cells were macrophages, with no eosinophils seen at all. There was, however, an increase in BAL IL-5 (Figure 3.5B) and some of the chemokines (eotaxin-1 and TARC; Figures 3.5C), albeit not statistically significant.

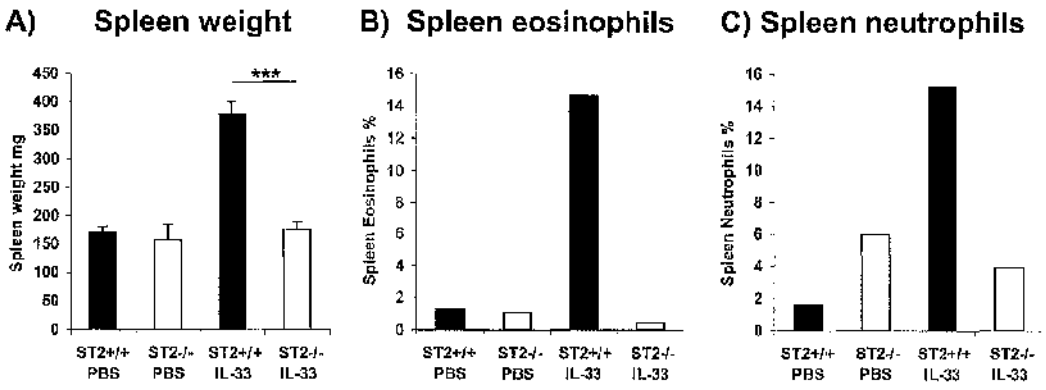


Figure 3.3 IP rmIL-33 increases spleen size and cellularity

BALB/c mice were given 0.8μg rmIL-33 IP for 7 days and tissues harvested. **(A)** Individual spleens were weighed. Spleens were pooled, a single cell suspension prepared, and the percentage of **(B)** eosinophils and **(C)** neutrophils determined by differential cell counts of cytopreps. The results shown are means ± SEM (n=2-4; ***p<0.001).

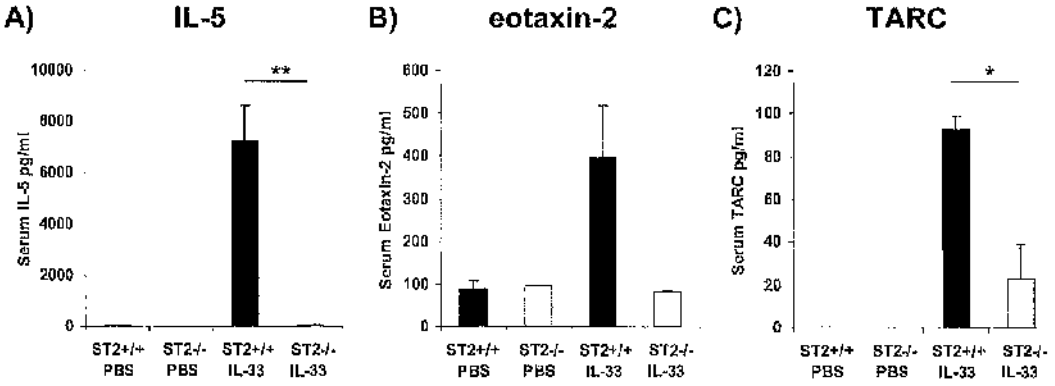


Figure 3.4 IP rmIL-33 increases serum IL-5 and chemokines

BALB/c mice were given 0.8µg rmIL-33 IP for 7 days and tissues harvested. Serum cytokines and chemokines were measured by sandwich ELISA; **(A)** IL-5 **(B)** eotaxin-2 **(C)** TARC. The results shown are means ± SEM (n=2-4; *p<0.05, **p<0.01).

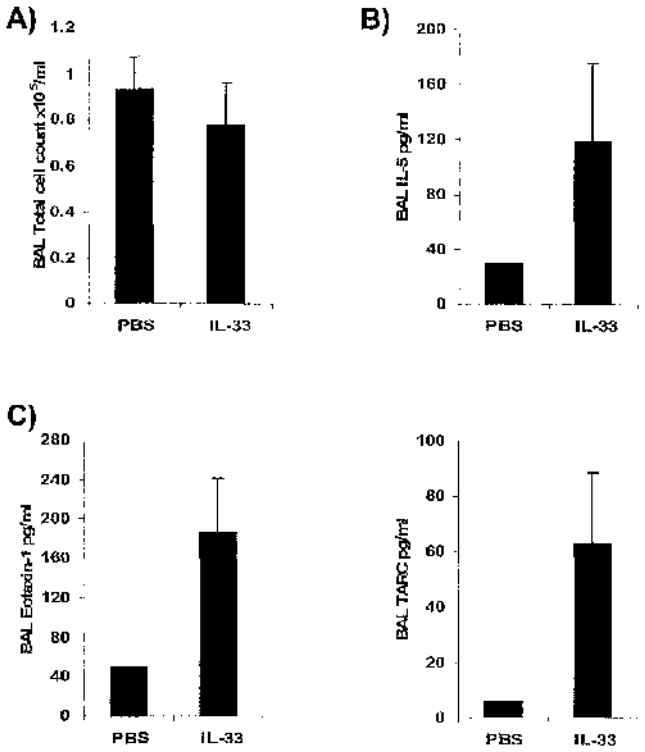


Figure 3.5 IP rmIL-33 induces Th2 pathology in the lung

BALB/c mice were given 0.8µg rmIL-33 IP for 7 days and tissues harvested. **(A)** H&E stained lung sections were examined for eosinophilic inflammation. **(A)** Cell counts, **(B)** IL-5 and **(C)** chemokines were measured in BAL fluid. The results shown are means ± SEM (n=2-4).

3.3.3. IP rmIL-33 induces a local Th2 effector response in the peritoneum

Having shown that IP injection of IL-33 causes a systemic inflammatory response it seemed logical to examine the peritoneal fluid to determine the extent of the local inflammatory response.

Cells in the peritoneal wash of PBS treated mice consisted almost exclusively of macrophages. After rmIL-33 administration there was an influx of inflammatory cells into the peritoneal cavity (Figure 3.6A). This consisted of eosinophils, neutrophils and macrophages (Figures 3.6B, 3.6C and 3.6D). There were proportionally more eosinophils, with an 80-fold increase (0.48 ± 0.48% to 39 ± 4.2%, $p<0.001$). Neutrophils increased 4-fold (0.85 ± 0.12% to 3.62 ± 1.84%, NS), and the percentage of macrophages decreased (98.2 ± 0.12% to 56.6 ± 3.6%, $p<0.001$). Very few lymphocytes were seen in PBS and rmIL-33 groups.

Very high levels of IL-5 were seen in the peritoneal wash after rmIL-33 treatment (Figure 3.7A). An increase in IL-13 was also seen (Figure 3.7B), which had not been seen in serum. Other cytokines were unchanged (IL-10) or undetectable (IL-4, IL-1β, IL-6, IL-12, TNFα and IFNγ).

As there had been a large influx of eosinophils and other inflammatory cells, chemokine levels were also examined. Eotaxin-1, eotaxin-2 and TARC (Figures 3.7C, 3.7D and 3.7E) were increased, although the increase in TARC was not quite statistically significant ($p=0.055$).

IgA levels in the peritoneum were increased almost ten-fold in the rmIL-33 treated group (Figure 3.7F), although serum levels were not affected (data not shown).

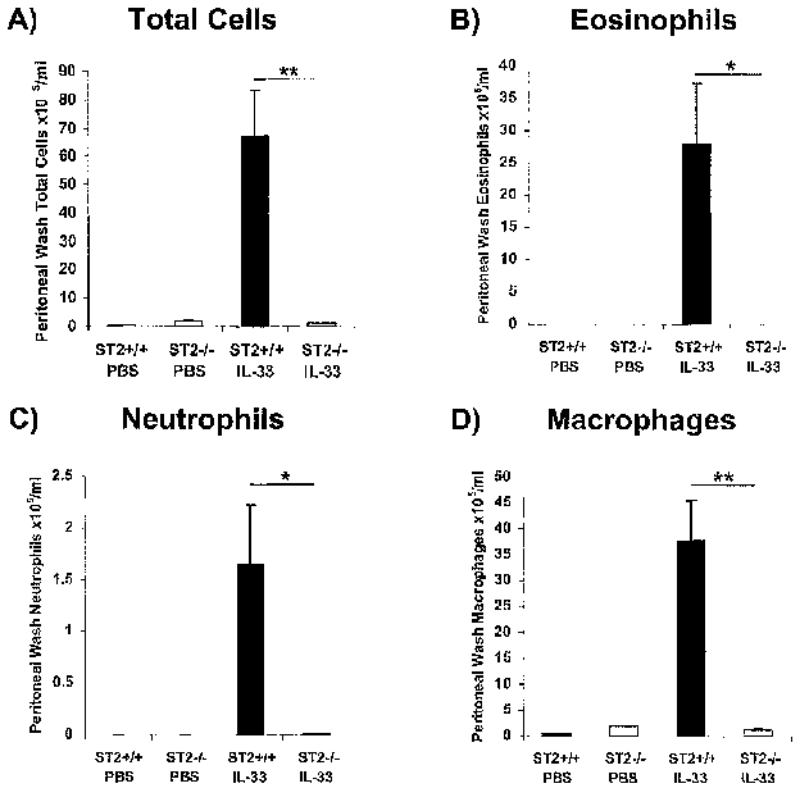


Figure 3.6 IP rmIL-33 increases the peritoneal inflammatory cell infiltrate

BALB/c mice were given 0.8µg rmIL-33 IP for 7 days and tissues harvested. **(A)** Total cell numbers in the peritoneal wash were counted. Differential cell counts were performed on cytopreps to determine the percentage of each cell type present, and the absolute number of **(B)** eosinophils, **(C)** neutrophils and **(D)** macrophages calculated. The results shown are means ± SEM (n=2-4; *p<0.05, **p<0.01).

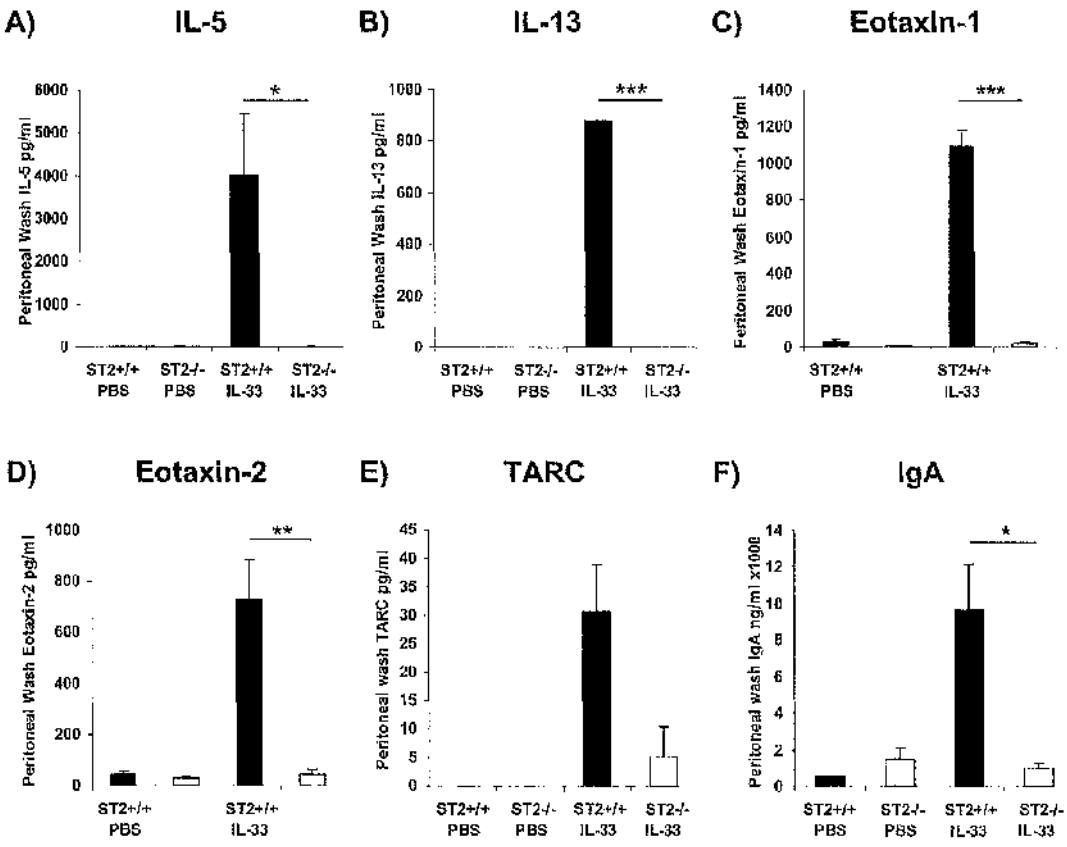


Figure 3.7 IP rmIL-33 increases peritoneal cytokines, chemokines and IgA

BALB/c mice were given 0.8µg rmIL-33 IP for 7 days and tissues harvested. Levels of cytokines, chemokines and IgA were determined by sandwich ELISA; **(A)** IL-5 **(B)** IL-13 **(C)** eotaxin-1 **(D)** eotaxin-2 **(E)** TARC and **(F)** IgA. The results shown are means ± SEM (n=2-4; *p<0.05, **p<0.01, ***p<0.001).

3.4. IL-33 initiates innate Th2 type eosinophilic inflammation in the lungs of wildtype and SCID mice

In the previous section I showed that rmIL-33 directly induced a strong local Th2 type response when injected intraperitoneally. Since both IL-33 and ST2 are expressed in the lung, I hypothesised that rmIL-33 given directly to the lungs would also induce a local Th2 type response.

The possible dosing schedule for IN IL-33 was investigated in initial pilot experiments. A dose response was seen over the range of 0.1 – 2µg per mouse, but doses above 2µg were not examined (Figure 3.8A). A single dose of 2µg rmIL-33 was sufficient to induce a measurable cytokine (IL-5 at 24 hours), chemokine (eotaxin-2 and TARC at 24 hours) and cellular (macrophages at 96 hours, eosinophils at 120 hours) response in the BAL (Figure 3.8B), but had no effect in $ST2^{-/-}$ mice (Figure 3.8C).The strength of the response was increased by repeated dosing for up to 7 days (results presented in this chapter), but dosing beyond this was not examined. Had more rmIL-33 been available it would have been ideal to extend these studies to higher doses and over longer periods at both low and high doses to more thoroughly investigate the *in vivo* biological function of IL-33, but the limiting factor was the rate it could be produced, purified and tested in the lab.

Therefore BALB/c mice were given 2µg intranasal rmIL-33 for seven consecutive days and tissues harvested to analyse the response (Figure 3.8C). In order to make preliminary studies of which cell types might be important in the response SCID mice, which lack lymphocytes, were also used.

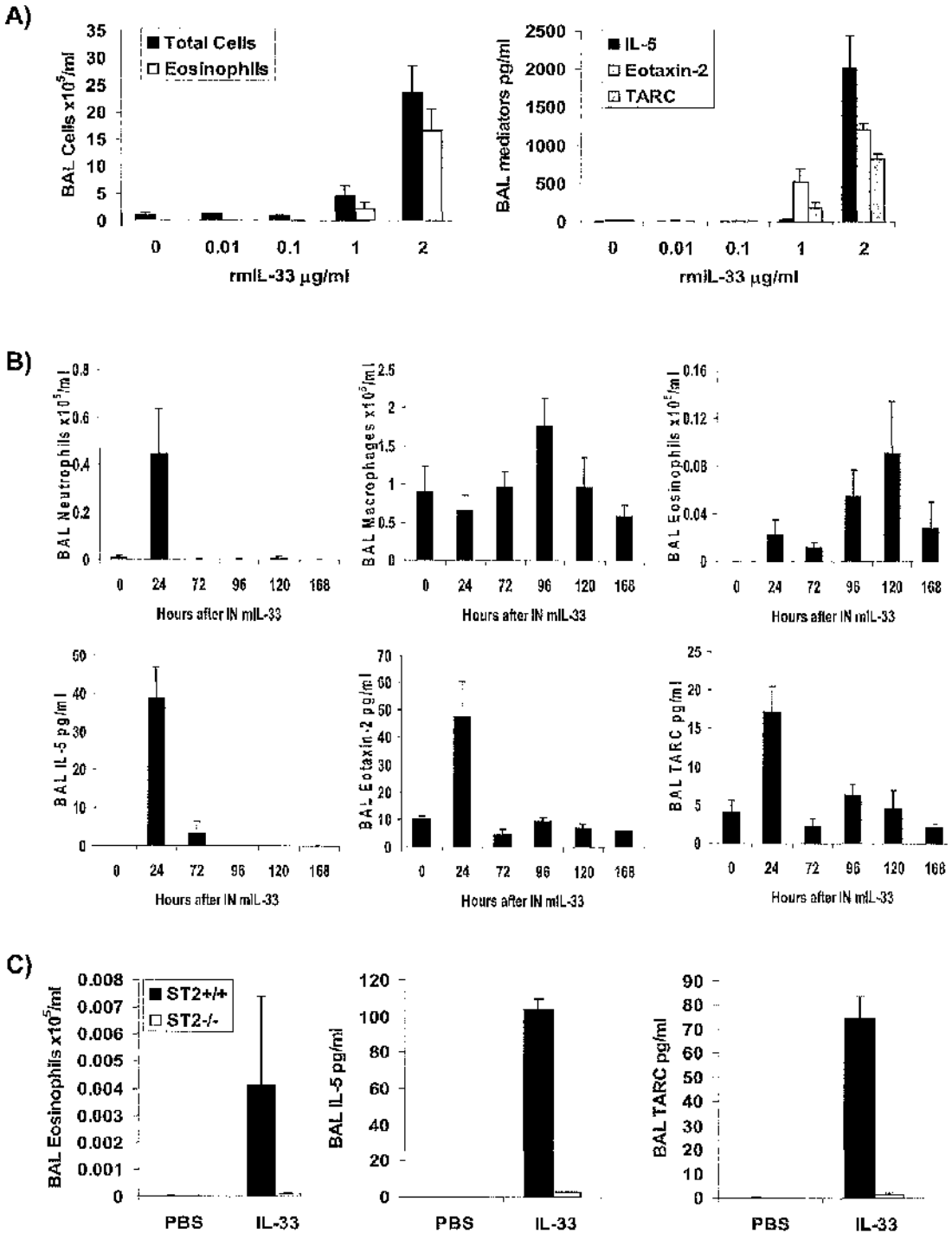


Figure 3.8 A-C. Figure 3.8D and legend over page

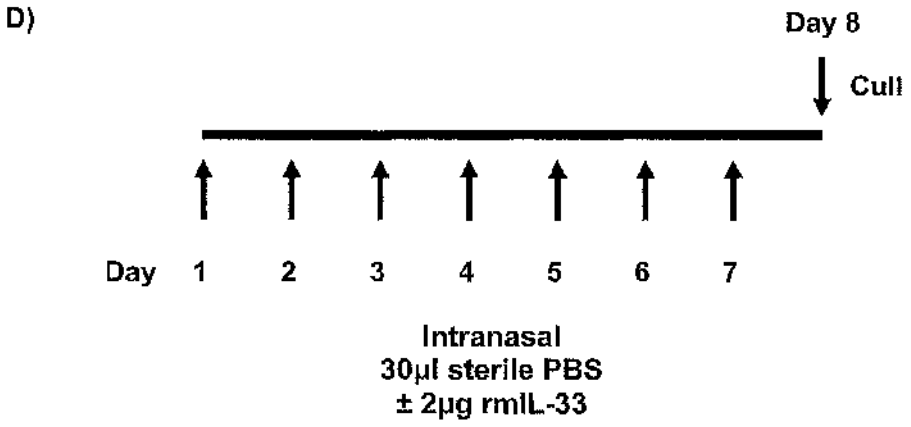


Figure 3.8 Intranasal administration of IL-33

Recombinant murine IL-33 was produced as described and given intranasally (IN) in 30µl sterile PBS. **(A)** Doses of 0 to 2µg/ml were given over 7 consecutive days and the mice culled on day 8. **(B)** A single dose of 2µg/ml was given and the mice culled at various time points thereafter. **(C)** A single dose of 2µg/ml was given to $ST2^{+/+}$ and $ST2^{-/-}$ mice and the mice culled at 24 hours (for cytokine analysis) or 120 hours (for eosinophil counts). In all experiments BAL fluid was collected and cell counts, cytokines and chemokines measured as described (n=3). **(D)** To investigate the effects of IL-33 in the airway more fully 2µg/ml was given for 7 consecutive days and the mice culled on day 8and serum, BAL and lungs collected for analysis.

3.4.1. IN rmIL33 induces a Th2 type response in the airway

IN administration of rmIL-33 increased BAL total cell counts (Figure 3.9A) in both wildtype and SCID mice. Analysis of the cell types involved revealed a similar pattern in wildtype and SCID mice. However, the magnitude of the cellular infiltrate was 10-fold less in SCID mice. The infiltrate consisted mostly of eosinophils (Figure 3.9B), although macrophages (Figure 3.9C) and neutrophils (Figure 3.9D) were also increased. Lymphocytes were seen at only very low levels and were not increased by mIL-33. The dominant cell in the BAL changed from virtually 100% alveolar macrophages in PBS challenged mice, to being over 70% eosinophils in wildtype and 40% in SCID mice.

The pattern of cytokines and chemokines in the BAL was also similar in wildtype and SCID mice, with the main difference again being the magnitude of the response. IL-5 and IL-13 were increased ten fold more in wildtype than SCID mice (Figure 3.10A and 3.10B). IL-10 was just detectable in both groups receiving rmIL-33, but there was no difference between wildtype and SCID mice. Other cytokines

were either undetectable (GM-CSF, IFNγ, IL-1α/β, IL-2, IL-4, IL-6, IL-12, and IL-17), or didn't change (TNFα), with rmIL-33.

Eotaxin-1, eotaxin-2, TARC and MIP-1α (figure 3.10C-F) were increased in wildtype mice, and less so in SCID mice. IP-10, KC, MCP-1 and MIG were not detectable in either.

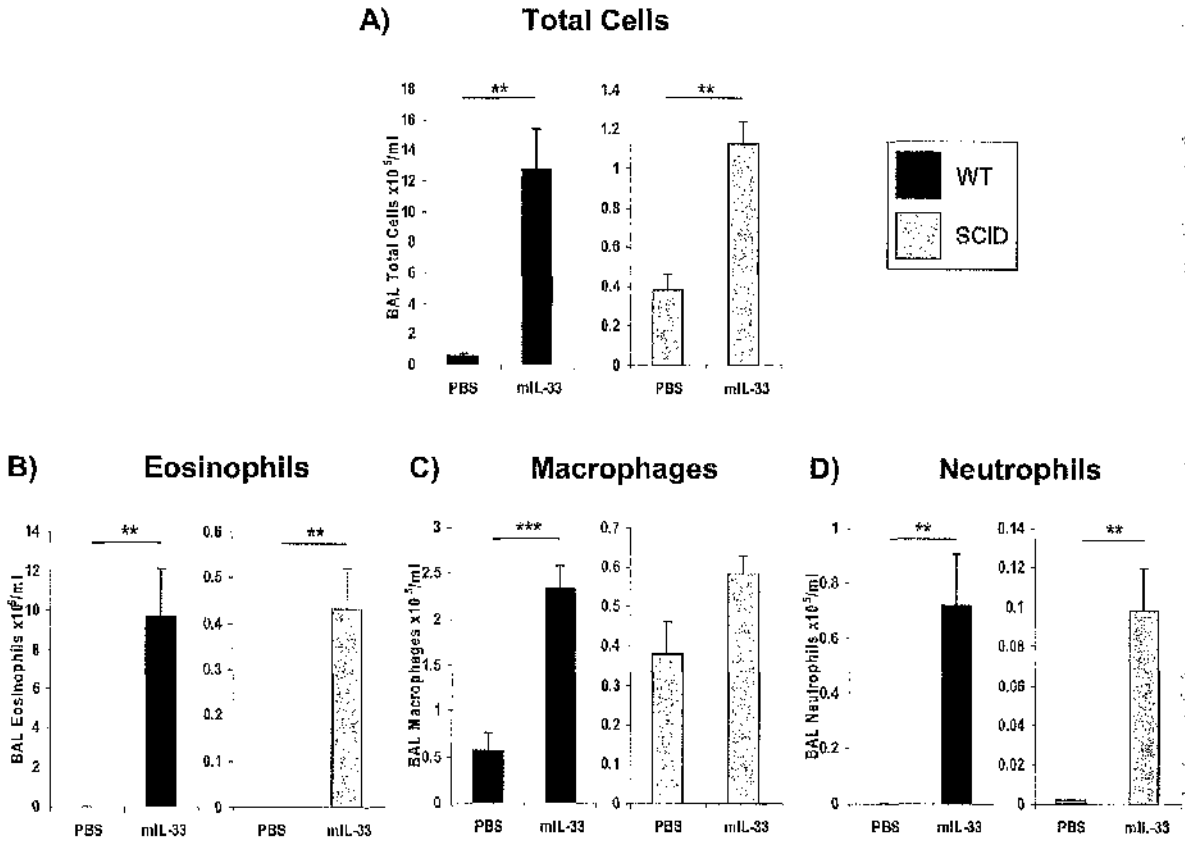


Figure 3.9 IN rmIL-33 increases BAL cell counts

BALB/c wildtype and SCID mice were given 2µg rmIL-33 IN for 7 days and tissues harvested. **(A)** BAL total cells were counted. Differential counts were performed on cytopreps and the number of **(B)** eosinophils, **(C)** macrophages and **(D)** neutrophils calculated. The results shown are means ± SEM (n=5; **p<0.01, ***p<0.001).

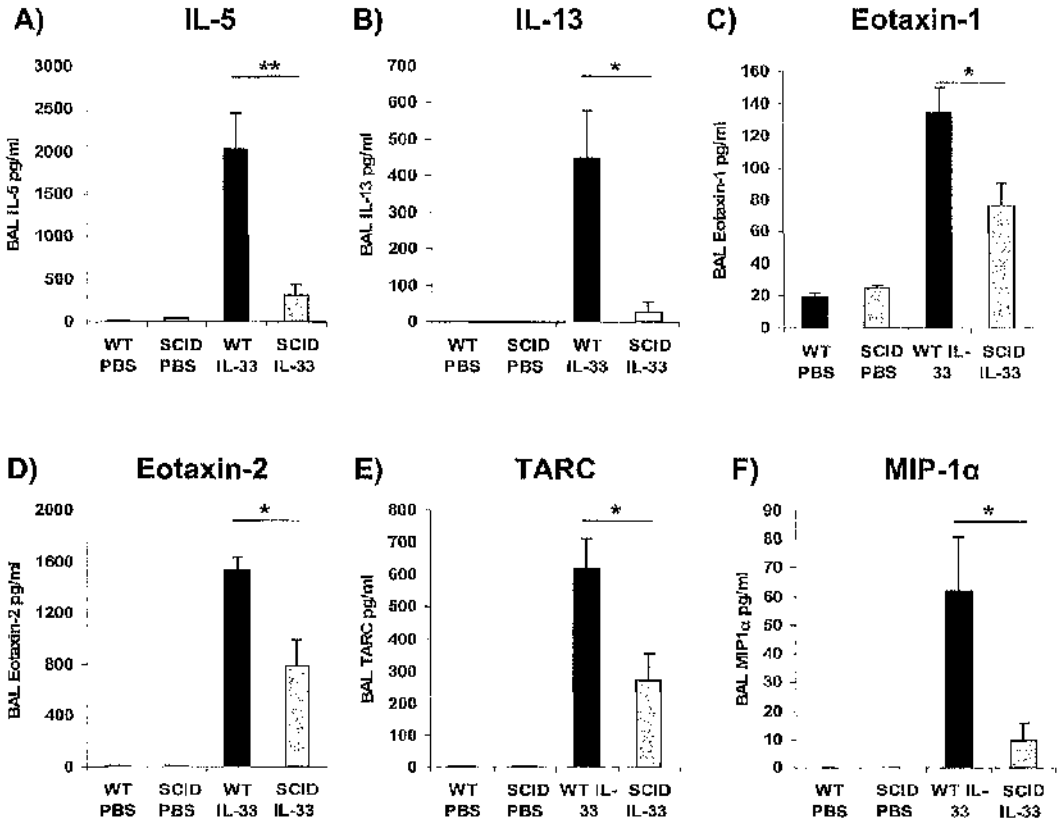


Figure 3.10 IN rmIL-33 increases BAL cytokines and chemokines

BALB/c wildtype and SCID mice were given 2µg rmIL-33 IN for 7 days and tissues harvested. BAL cytokines and chemokines were measured by sandwich ELISA or Multiplex; **(A)** IL-5 **(B)** IL-13 **(C)** eotaxin-1 **(D)** eotaxin-2 **(E)** TARC **(F)** MIP-1α. The results shown are means ± SEM (n=5; *p<0.05, **p<0.01).

3.4.2. IN rmIL-33 induces eosinophilic inflammation in the lung

Both wildtype and SCID mice treated with rmIL-33 exhibited marked eosinophilic inflammation in histological lung sections (Figure 3.11A-D), with larger airways affected more than smaller airways. The inflammatory response in wildtype mice appeared worse, with greater total number of cells, more eosinophils, and more areas of inflammation per section. The eosinophils tended to be more perivascular than peribronchial in both. The lung eosinophil score (Figure 3.11E) was increased markedly in both WT and SCID mice. Unfortunately the score was not sensitive enough to pick up a statistically significant difference as the WT group scored at the top of the scale on every section, and so the score is probably an underestimate. There appeared to be an increase in goblet cells in both, but much

more prominent in wildtype in keeping with the increased levels of IL-13. However, to assess goblet cells more accurately these sections would need to be stained specifically for mucus, for example using periodic acid Schiff (PAS) stain.

3.4.3. IN rmIL-33 increases BAL but not serum IgA

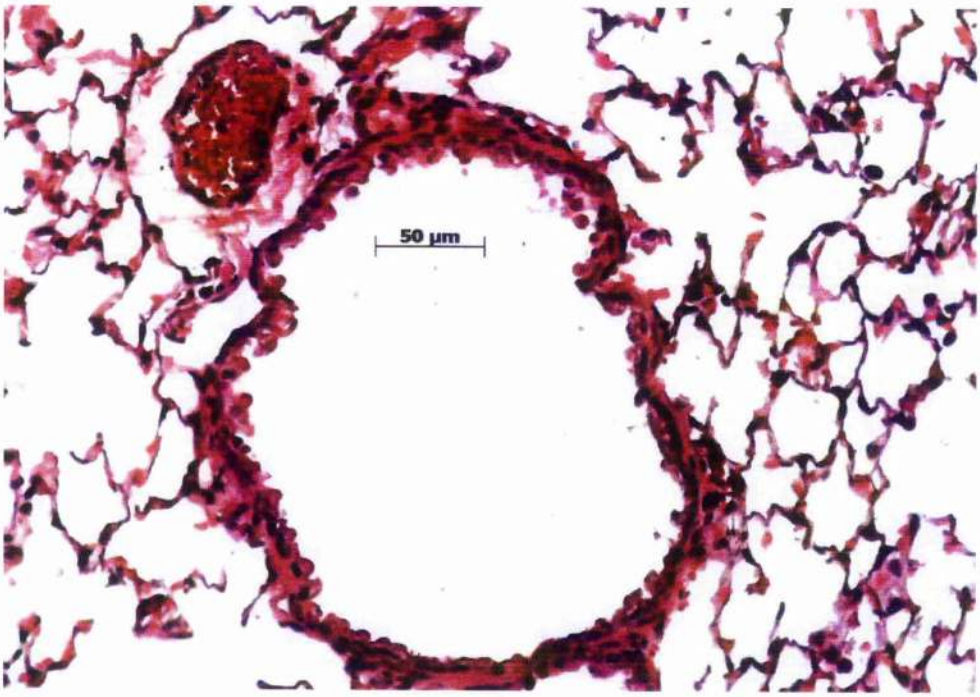
Treatment with IN rmIL-33 did not increase serum IgA levels (Figure 3.12A). However, as with IP rmIL-33 there was a marked increase in local IgA, this time in the BAL fluid (Figure 3.12B). As expected no IgA was detected in SCID mice as they do not possess B cells. IgE levels were not examined in BAL.

3.4.4. IN rmIL33 induces a systemic Th2 type response

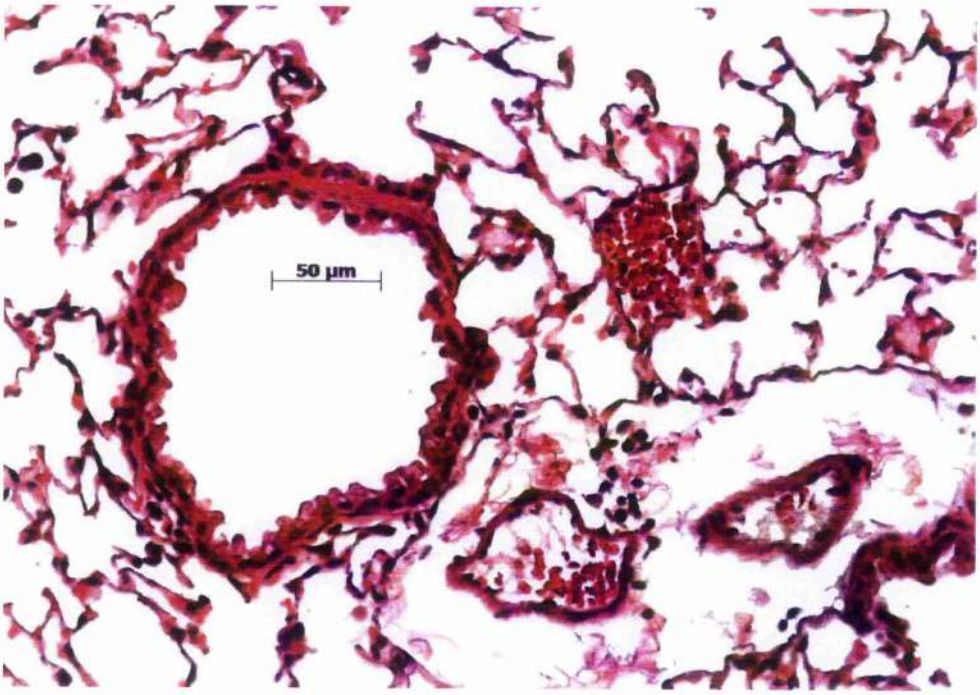
Both IP and IN routes of administration resulted in evidence of a systemic response. IN rmIL-33 induced a doubling in mean serum levels of total IgE (Figure 3.13A), although this did not quite reach significance ($p=0.06$). Serum IL-5 (Figure 3.13B) was increased, and this time serum IL-13 (Figure 3.13C) was also increased. A similar pattern was observed in SCID mice, but the magnitude was much less. The difference between wildtype and SCID was did not quite reach significance ($p=0.06$ for both IL-5 and IL-13). Other cytokines were either undetectable (GM-CSF, IFNγ, IL-1α/β, and IL-2) or not affected by rmIL-33 (IL-6, IL-10, IL-12, IL-17, and TNFα).

IN rmIL-33 resulted in a slight, but not significant, increase in many serum chemokines (eotaxin-2, TARC, IP-10, KC, MCP-1, MIG, and MIP-1α) but there was no difference between wildtype and SCID mice. Interestingly, rmIL-33 induced an increase in serum eotaxin-1 only in SCID mice (Figure 3.13D).

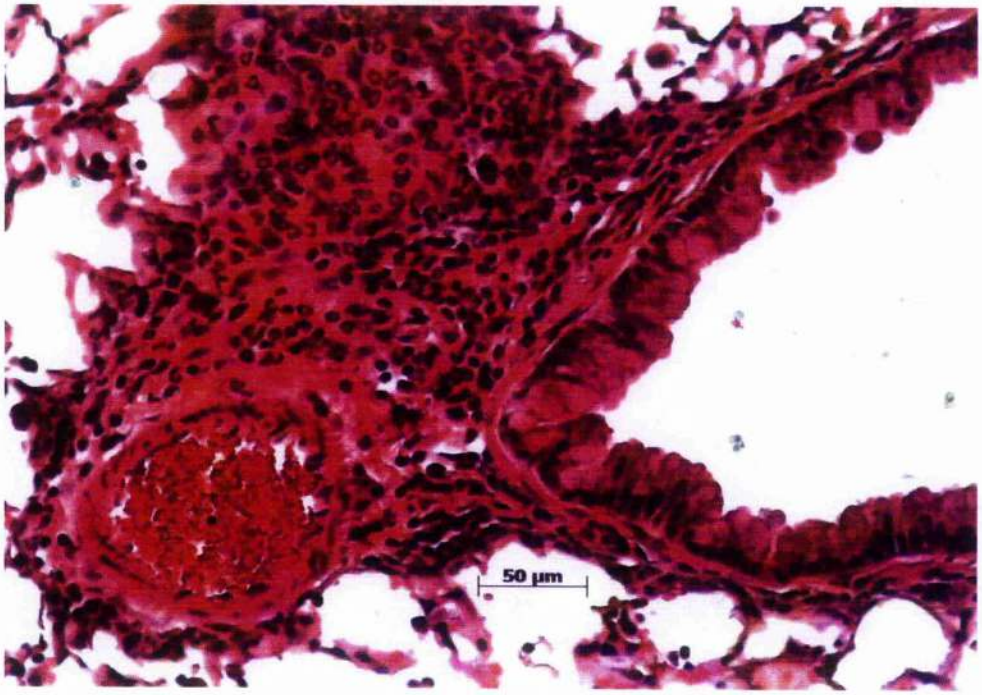
A) WT PBS



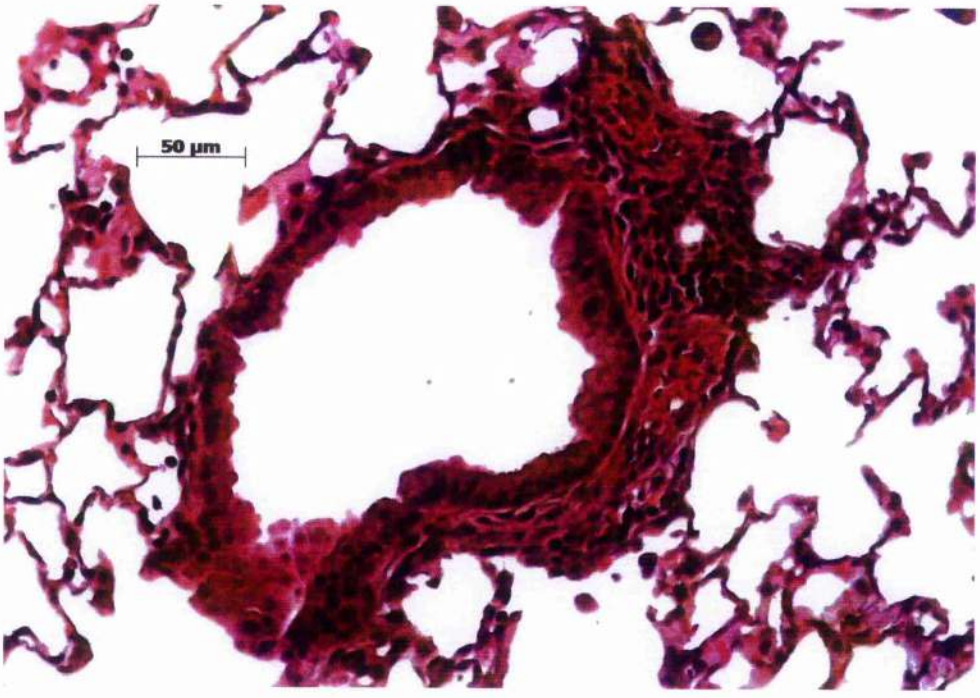
B) SCID PBS



C) WT IL-33



D) SCID IL-33



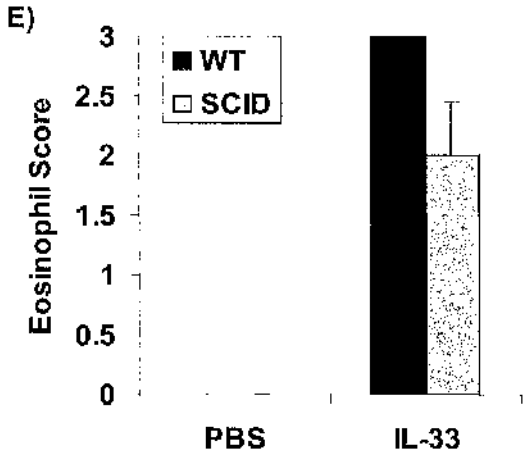


Figure 3.11 IN rmIL-33 Induces eosinophilic airway inflammation

BALB/c wildtype and SCID mice were given 2µg rmIL-33 IN for 7 days and tissues harvested. H&E stained lung sections were examined for eosinophilic inflammation; **(A)** WT PBS **(B)** SCID PBS **(C)** WT rmIL-33 **(D)** SCID rmIL-33. **(E)** The lung eosinophil score was assessed as described (n=5).

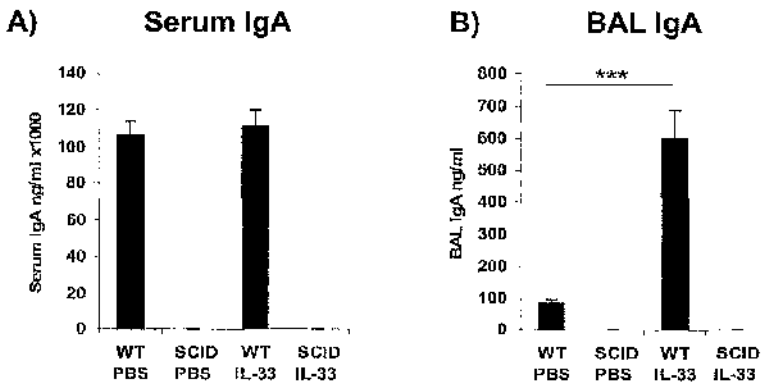


Figure 3.12 IN rmIL-33 increases BAL IgA

BALB/c wildtype and SCID mice were given 2µg rmIL-33 IN for 7 days and tissues harvested. The concentration of IgA in **(A)** serum and **(B)** BAL was measured by sandwich ELISA. The results shown are means ± SEM (n=5; ***p<0.001).

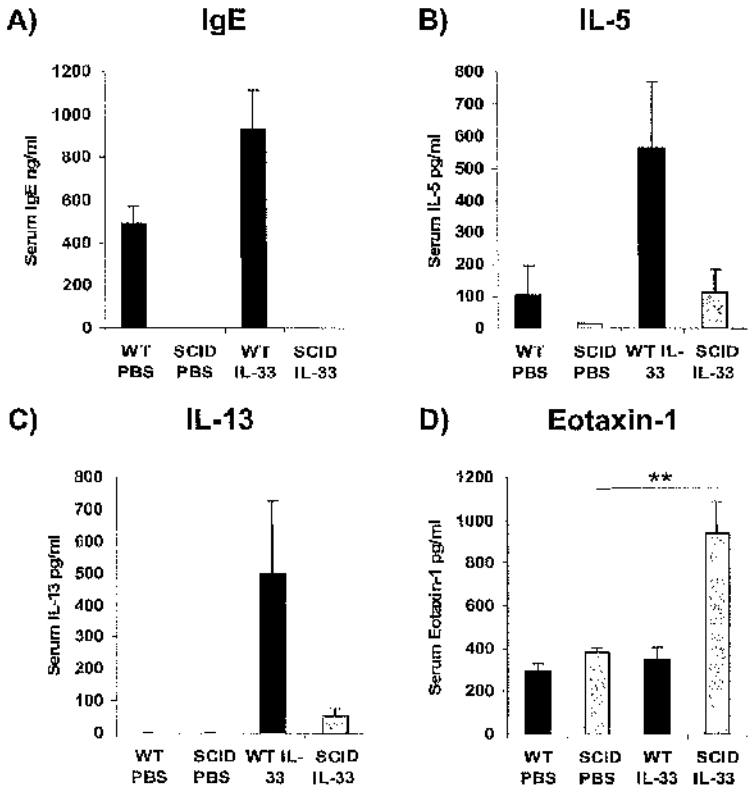


Figure 3.13 IN rmIL-33 increases serum IL-5, IL-13, eotaxin-1 and IgE

BALB/c wildtype and SCID mice were given 2µg rmIL-33 IN for 7 days and tissues harvested. The concentration of **(A)** IgE, **(B)** IL-5, **(C)** IL-13 and **(D)** eotaxin-1 were measured by sandwich ELISA. The results shown are means ± SEM (n=5; **p<0.01).

3.5. Discussion

In this chapter I have demonstrated that:

1. IL-33 has the same functions in BALB/c and C57Bl/6 mice
2. Murine and human IL-33 have the same systemic effects
3. IL-33 induces local innate inflammation
4. IL-33 activity is dependent on *ST2* gene expression
5. IL-33 can initiate innate Th2 inflammation in the lung
6. SCID mice have reduced lung inflammation compared to wildtype in response to IL-33

I will discuss each of these findings in more detail.

3.5.1. IL-33 has the same functions in BALB/c and C57Bl/6 mice

The systemic effects of human IL-33 in C57Bl/6 and BALB/c mice are very similar. In both there is an increase in spleen weight and eosinophil counts consistent with the rise in serum IL-5, which is vital for eosinophil maturation (reviewed in (365)) and mobilisation from the bone marrow (366). C57Bl/6 mice were shown to have perivascular eosinophil infiltration and goblet cell hyperplasia in the lung, whereas BALB/c mice had perivascular eosinophil infiltration, but airway goblet cell hyperplasia was absent. This is consistent with the lack of IL-13 in the serum and the essential role of IL-13 in mucus production (89). A local rise in IL-13 was seen with IP and IN administration, and there was a rise in serum IL-13 after IN IL-33, so the reason for the lack of serum IL-13 after IP IL-33 in BALB/c mice is unknown.

Laboratory mouse strains vary in their response to biological stimuli and this is particularly true when studying factors involved in Th1/Th2 regulation. For instance, C57Bl/6 mice have a Th1 biased immune response to the parasite *Leishmania major*, and quickly clear infection, whereas BALB/c mice have a predominantly Th2 response, and quickly succumb (367). The fact that both mice have such similar responses to IL-33 is important for 2 reasons. Firstly, I will use BALB/c mice to study the role of IL-33 in antigen-specific allergic airways inflammation (see Chapter 4), so to allow direct comparison it was necessary to use BALB/c mice to study innate inflammation. Secondly, this is evidence of the wider applicability and relevance of these effects, as they are not restricted to one strain.

3.5.2. Murine and human IL-33 have the same systemic effects

I went on to show that murine IL-33 has a very similar systemic effect to human IL-33 when given IP to BALB/c mice. The response is dominated by a rise in serum IL-5 associated with an increase in spleen size and eosinophil numbers. In addition a rise in spleen neutrophils was seen. LPS can induce a neutrophilic response, but no neutrophil rise was seen in $ST2^{-/-}$ mice implying this is a genuine IL-33 dependent phenomenon, and not due to LPS contamination. Serum IgE was also seen to rise. This was attributed to a rise in IL-4 in C57Bl/6 mice (1), although as with BALB/c, no IL-4 was detected in serum. C57Bl/6 mice had shown an increase in mRNA expression of IL-4, -5 and -13 in various tissues, but this was not examined in BALB/c mice. Perivascular eosinophilic inflammation was seen

with both human and murine IL-33, but no serum IL-13 or goblet cell hyperplasia. It is not clear if this is a generalised effect in all tissues, or if tissues with mucosal surfaces (the lung and the gut) are specifically affected. Further work examining other tissues is required. Thus the effect of recombinant murine IL-33 in BALB/c mice is consistent with the biological activity of human IL-33 in BALB/c and C57Bl/6 mice. These results indicate that not only is the amino acid sequence and structure conserved between species, but also the likely biological function.

In order to investigate the mechanisms by which eosinophils infiltrate the pulmonary tissue I also measured the level of chemokines in the serum and found eotaxin-2 and TARC to be elevated. Eotaxin-2 is a potent factor attracting eosinophils across the endothelium (368) by binding to CCR3. This transmigration is increased in the presence of IL-5, through eosinophil activation, and also in the presence of IL-1β, through endothelial cell activation. In this model it may be a combination of a direct effect of the IL-1β-like IL-33 absorbed into the circulation acting on endothelial cells, and an indirect effect of IL-5 on circulating eosinophils. TARC acts through CCR4 (369), which is not expressed on eosinophils, but is present on airway $CD3^+$ T cells (370) and on human $CD4^+$ iNKT cells (371), and is thought to be a major factor in the maintenance of Th2 dominance in asthma. There is likely to be a lot of overlap between these two subsets. It is not known whether any of these cells are found in the infiltrate. The cellular targets and source of cytokines and chemokines are unknown.

3.5.3. IL-33 induces local innate inflammation in the peritoneum

Having shown that IL-33 results in a systemic Th2 type response after IP administration, it was logical to examine the extent of local inflammation in the peritoneum. Treatment with IL-33 resulted in a massive influx of inflammatory cells, mostly eosinophils, but also neutrophils and macrophages. In keeping with this a large amount of IL-5 was found, and there was a marked increase in eotaxin-1 and -2, both of which are powerful eosinophil chemoattractants through binding to CCR3.

Neutrophils also express CCR3 and migrate in response to eotaxin-1 (372), which may explain the presence of some neutrophils in the peritoneum. However, it is not clear why human IL-33 did not attract neutrophils into the peritoneum, whereas murine IL-33 did. One possibility is contamination of the murine IL-33 with LPS or

another pro-inflammatory bacterial product, but the fact that $ST2^{-/-}$ mice did not have neutrophils in the peritoneum would argue against this, and suggests it is a genuine IL-33 mediated phenomenon. A further possibility is that the native murine IL-33 likely has different binding affinity for murine ST2, and therefore the downstream response generated may differ slightly. Further work examining the structure and kinetics of the IL-33 / ST2 interaction, and differential downstream signalling of human and murine IL-33, would be required to investigate this.

Macrophages were also elevated in the peritoneum. They express CCR4, which is thought to be important for their innate immune functions (373), so the elevated peritoneal levels of TARC, which acts via CCR4, would explain why IL-33 attracted macrophages into the peritoneum.

Mast cells are thought to constitute up to 5% of cells recovered from the peritoneum, but can be difficult to identify and purify. IL-33 has recently been shown to have a role in mast cell maturation and activation (374-376), but mast cells were not identified in the peritoneum after IL-33 treatment. However, I did not use any mast cell specific stains, nor any indirect measures of mast cell accumulation and function, such as tissue myeloperoxidase levels, so it may still be that mast cells play an important role in the peritoneal inflammation induced by IL-33. There was also a large increase in peritoneal IL-13, and it is possible that this reflects the presence of mast cells, as they have been shown to produce IL-13 after IL-33 stimulation even in the absence of IgE (377). Mast cells mature in the tissues, and the immature forms found in the blood are not active, which may explain why IL-13 is found at much higher levels in the peritoneum than the serum.

IgA levels were also markedly elevated in peritoneal fluid. It is not known if this is due to non-specific capillary leak of plasma proteins, including IgA, into the peritoneum, or IL-5 induced up-regulation of production by peritoneal B1 lymphocytes (378).

3.5.4. IL-33 activity is dependent on ST2 gene expression

All of the measures of inflammation, both systemic and local, were also examined in $ST2^{-/-}$ BALB/c mice, but were all negative, implying that these effects of IL-33 are dependent on *ST2* gene expression. This is important for a number of reasons.

As discussed in the introduction (section 1.6), the *IL-33* and *ST2* genes are expressed in various cells and tissues either constitutively or induced by pro-inflammatory mediators. IL-33 has been shown to bind to and signal through ST2L on the cell surface, and result in Th2 type inflammation in mice. $ST2^{-/-}$ mice and exogenous sST2 administration have been shown to modulate immune responses (306,315,331). Therefore IL-33 seems to play a role in initiating and regulating immune responses by acting as a cytokine. More recently IL-33 has been found intracellularly in the endothelium of chronically inflamed tissues, where it may function as a nuclear factor rather than a cytokine (255), and independent of *ST2* expression. However, its role in the nucleus is unknown. As the present results are *ST2* dependent they are in keeping with the role of IL-33 as a cytokine.

Human and murine IL-33 have only 55% homology at the amino acid level (1), therefore it is possible that the effects seen with human IL-33 are due to cross-reaction with a different receptor *in vivo*. It is also possible that both human and murine IL-33 could bind to an additional receptor other than ST2L. Also, rh and rmIL-33 were both produced from transfection into *E coli* which may result in the presence of pro-inflammatory contaminants. For instance, low doses of LPS have been shown to predispose to a Th2 type antigen-specific response in the airway (119). It is therefore possible that recombinant IL-33 could be processed as an antigen and a low dose of contaminating LPS bias towards a Th2 type antigen-specific response. These confounding possibilities are excluded by the negative results seen on $ST2^{-/-}$ mice.

Taken together these results further validate the use of rh and rmIL-33 for use as biological reagents, by demonstrating they have almost identical roles in different strains of mice, dependent on *ST2* gene expression. Initial studies of *ST2* gene function were hampered by the lack of a known ligand until it was found to be IL-33, and so some of the data presented were inconsistent or conflicting. This likely reflects not just differences in the model systems used, but also drawbacks in the

approaches taken. In some experiments $ST2^{-/-}$, lacking both ST2L and sST2, were used to define the role of ST2L. No conclusions can be reached about the relative contributions of each when both are lacking, and the effects seen after the loss of a receptor such as ST2L do not necessarily mirror the effects seen when that receptor is stimulated by ligand binding. In other experiments anti-ST2 antibodies or recombinant sST2 were used to block ST2 function, but a stimulating action either through ST2L or via another receptor could not be excluded. In addition, recombinant sST2 was conjugated to the Fc portion of IgG1 for purification purposes. Not only is this is a physically large molecule that could have steric hindering effects, it could be effective through binding to FcγR receptors, which are known to have potent immunomodulatory properties (reviewed in (379)). The identification of IL-33 as the ligand for ST2L opens up the potential to use recombinant IL-33, IL-33 gene knockout mice, and anti-IL-33 antibodies as reagents to confirm and extend previous results.

3.5.5. IL-33 can initiate innate Th2 inflammation in the lung

As mentioned in the introduction to this chapter, administration of IL-4 or IL-13 directly to the airways results in many of the features of asthma. Since *IL-33* and *ST2* are expressed in the lung, I hypothesised that IL-33 would induce an innate Th2 type response.

Administering IL-33 to the airways of mice did induce a strong Th2 type response with accumulation of inflammatory cells in the BAL dominated by eosinophils, but also including neutrophils and macrophages. In keeping with this, eotaxin-1 and -2 were elevated, as were TARC and MIP-1α. MIP-1α binds to CCR1, and is important in transendothelial migration of most inflammatory cells, but particularly monocytes/macrophages (380). IL-5 and IL-13 were also elevated in the BAL, as they both had been in the peritoneal wash, but IL-4 was not seen. No change in serum IgA was seen, but IgA was elevated in BAL. This could be due to capillary leak, but most IgA in bronchial secretions is the result of active transport into the lumen (381). Eosinophilic inflammation was also seen peribronchially and perivascularly, with goblet cell hyperplasia of the epithelium.

In addition a systemic Th2 type response was also seen. IL-5 and IL-13 were elevated in serum after IN IL-33, which was in contrast to the IP route of administration, where only IL-5 was elevated. In C57/Bl6 mice both IL-5 and IL-13

were elevated in serum after IP IL-33 (1). It is not clear why this difference occurs between IN and IP IL-33. The levels of IL-13 in BAL and in the peritoneal wash were similar. The level of IL-13 in serum after IN IL-33 was actually slightly higher than in BAL, suggesting that the IL-13 may be produced in perivascular cells, or even at distant sites, such as lymphoid tissues or the liver.

Total serum IgE was also found to be elevated. Interestingly the severity of asthma correlates better with an increase in total IgE rather than antigen-specific IgE (382).

Therefore, like IL-4 and IL-13, IL-33 can also induce many features of asthma independent of antigen, although which of these effects are direct, and which are through induction of cytokines such as IL-5 and IL-13 is not known. IL-25 has also been shown capable of inducing profound eosinophilic airway inflammation in naïve mice, also through increased IL-5, IL-13, eotaxin-1 and eotaxin-2, but also with the addition of increased IL-4 (109,110). In both these cases (IL-33 and IL-25) the pathological changes seen are very similar to those seen after direct instillation of IL-13 (89), which suggests these cytokines are acting upstream to induce IL-13 production. Further work is needed to elucidate the hierarchy of cytokine release and function in the allergic airway, and so determine the relative contribution of each.

3.5.6. SCID mice have reduced lung inflammation compared to wildtype

An almost identical response was seen in SCID mice as with wildtype mice, but ten-fold less in magnitude. SCID mice (383) have a lack of B and T lymphocytes, due to a defect in VDJ recombination of antigen receptors (384). However, other immune cell lineages are unaffected and function normally. In fact, they have increased numbers of some cells such as NK cells (385). Therefore the response to IL-33 must be largely, but not entirely, dependent on a lymphocyte subset. It seems unlikely that the effect of IL-33 was due to an effect on mainstream lymphocytes as they require antigen priming first, although this data does not exclude this possibility as there may be a population of pre-existing primed T cells in the naïve wildtype mice. However, SCID mice also lack iNKT cells (38). The possible role of iNKT cells in innate and adaptive inflammation has been discussed in more detail in Chapter 1. Of relevance here is that although they are constitutively present in the lungs in only small numbers (386), they can express

large amounts of cytokines immediately after activation, which can occur in the absence of antigen (387,388). In addition, repeated stimulation results in predominantly Th2 cytokine release (152). NKT2 cells have been shown to express ST2L (287) Therefore it is possible the innate effects of IL-33 in the lung are mediated by this subset of cells, which are increasingly shown to play major roles in autoimmunity, allergy, tumour immunity and response to infections (reviewed in (389)). The residual inflammation that occurs in SCID mice must be due to other non-lymphoid cells expressing ST2L in the lung, the most likely candidate being mast cells, which can produce Th2 type cytokines when stimulated. It may be that these are in fact the first cells to respond in wildtype mice, in which case iNKT or other lymphocytes may simply amplify the response. Further work is needed to define precisely which cells respond directly to IL-33, and which are activated indirectly.

The one aspect of the response where SCID mice did not have a reduction in the outcome measured was the serum level of eotaxin-1, which was increased quite markedly. The reason for this is not clear. It may be that SCID mice show different kinetics in the response to IL-33, and had we looked at other time points the pattern of release in both WT and SCID mice would be apparent. However, given that all other measures were reduced this seems unlikely. It is possible that other mediators we have not examined may also be increased in SCID mice and account for the increase in eotaxin-1. For instance, IL-25 can be produced by activated mast cells (390)), and when instilled into the airways can induce eotaxin-1 expression (110). Further studies are required to elucidate the downstream mechanism of actions of IL-33 in both WT and SCID mice.

3.6. Conclusions

In this chapter I have demonstrated that IL-33 has the same functions in BALB/c and C57Bl/6 mice and that murine and human IL-33 have the same systemic effects. I have shown that regardless of the route of administration IL-33 initiates a local and systemic type 2 innate inflammatory response that is dependent on *ST2* expression. The local response in the lung shares many features of asthma, and experiments with SCID mice suggest that both lymphoid and non-lymphoid cells may have an important role in the innate response triggered by IL-33.

Having shown that IL-33 may have a role in innate immunity, in the following chapters I will demonstrate the potential role of IL-33 in modulating established Th2 (Chapter 4) and Th1/Th17 (Chapter 5) antigen-specific immune responses.

Chapter 4

IL-33 and ST2 in allergic airways disease

4.1. Introduction

In Chapter 3 I demonstrated that rmIL-33 administered directly to the airways in the absence of antigen could elicit an innate Th2 type effector response. This effect was dependent on the presence of the *ST2* gene products, in particular ST2L, the receptor for IL-33. Th2 type responses in the airway play a vital role in the initiation and persistence of asthma. Previous investigations into the role of the *ST2* gene demonstrated that serum sST2 is elevated in exacerbations of asthma (340). In murine models of OVA-induced Th2 airway inflammation, ST2L and sST2 are expressed in acute and chronic disease (318,320) and inhibition of ST2L function, by blocking antibodies or sST2-Fc decoy receptors, reduced airway inflammation (141,291,315). This suggests a pro-inflammatory role for ST2L. In contrast to these findings, studies of OVA-induced Th2 airway inflammation using two strains of $ST2^{-/-}$ mice have demonstrated no effect with C57Bl/6 mice (327) or enhanced inflammation with BALB/c mice (329). Thus the roles of *ST2* gene products and IL-33 still require considerable clarification in allergic airways disease.

To investigate this we prepared rmIL-33 as the physiological ligand for ST2L, and used $ST2^{-/-}$ mice on the Th2-dominant BALB/c background. The experiments described in this chapter relate to the induction of allergic airways inflammation in BALB/c mice, and the effect of IL-33 in the effector phase by administering rmIL-33 into the airways during allergen challenge. I used $ST2^{-/-}$ mice to confirm the specificity of action of IL-33 in this model.

4.2. Intranasal administration of IL-33 exacerbates allergic airways inflammation

Allergic airways disease was induced in BALB/c mice as described in the materials and methods (section 2.5.1). In pilot experiments I determined the optimal dose of OVA and number of challenges in $ST2^{+/+}$ and $ST2^{-/-}$ mice. The response in $ST2^{-/-}$ mice was similar to $ST2^{+/+}$, but at a lower level. With 3 IN doses of OVA the airway cellular response followed a dose response up to 50µg per mouse, but the percentage of eosinophils in the BAL did not increase above 10µg (Figure 4.1A). In $ST2^{+/+}$ and $ST2^{-/-}$ mice a single dose of 10µg OVA was sufficient to induce a small but measurable airway response, and the kinetics were similar (Figure 4.1B). In ST2+/+ mice 2 doses were sufficient to induce a strong airway response, whereas in $ST2^{-/-}$ mice 3 doses were required (Figure 4.1C).

The results shown here are representative of two experiments performed using the optimised protocol for $ST2^{+/+}$ mice (Figure 4.1D). This protocol was designed to elicit a moderate amount of airway inflammation, thus allowing any exacerbating effects of rmIL-33 to be apparent. The dose of rmIL-33 used was the same as for inducing innate eosinophilic airway inflammation (section 3.4). These results demonstrate that rmIL-33 administered to the airways at the same time as OVA during allergen challenge exacerbates allergic airways inflammation.

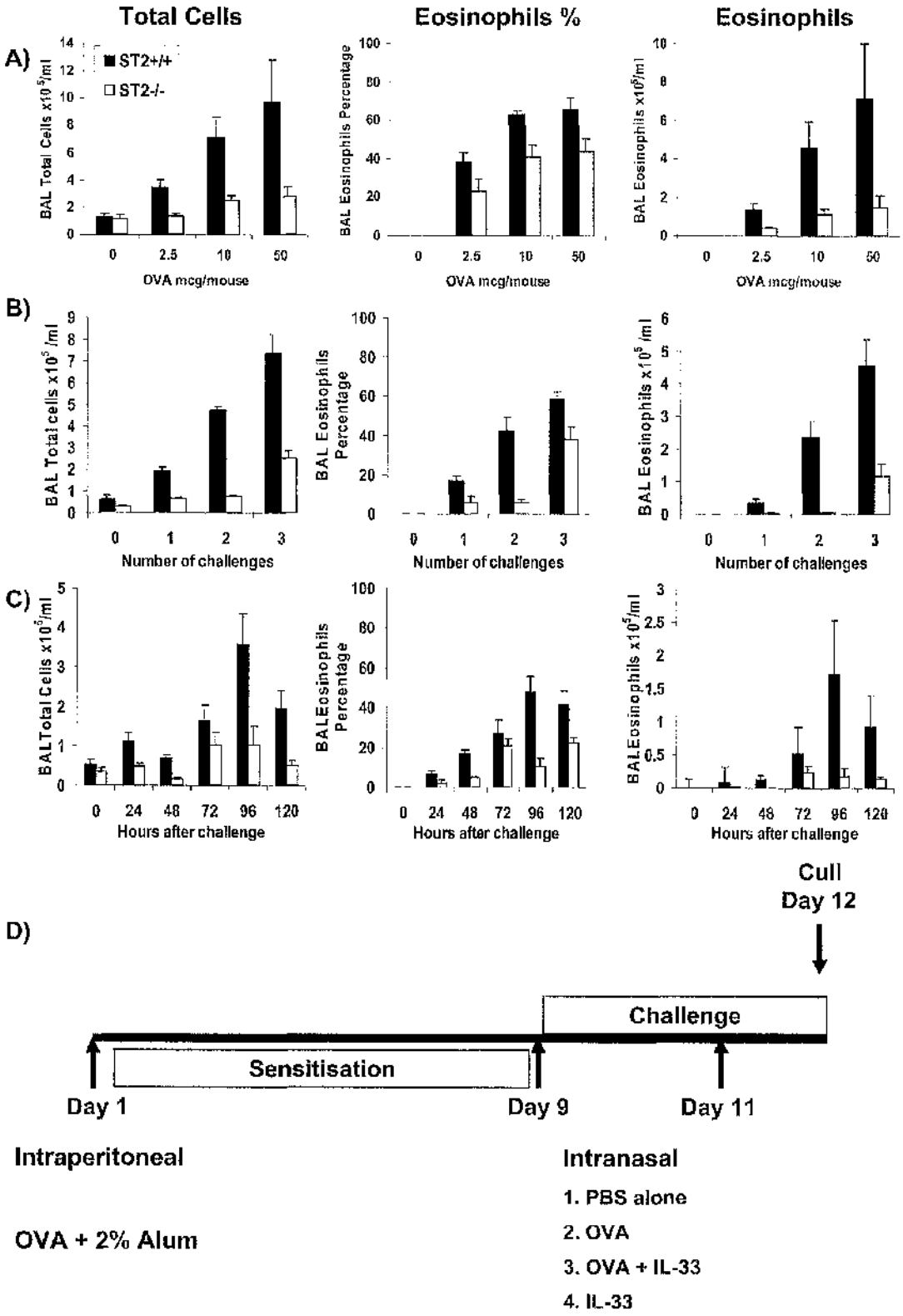


Figure 4.1 Administration of IL-33 in allergic airways inflammation

In all allergic airway disease experiments $ST2^{+/+}$ and $ST2^{-/-}$ BALB/c were sensitised on day 1 by IP injection of 100µg OVA in 100µl sterile PBS and 100µl of 2% alum. (A) Allergic airway inflammation was induced by IN instillation of 30µl sterile PBS containing 2.5 to 50µg OVA on days 9, 10 and 11 to

determine the optimum dose to induce eosinophilic inflammation. (B) An IN dose of 10µg per mouse was administered on 1 (day 9), 2 (days 9 and 11) or 3 (days 9, 10, and 11) occasions to determine the optimum number of allergen challenges. (C) A single IN dose of 10µg was administered and the airway response monitored to determine the kinetics of the response. BAL total and differential cell counts were performed as described in the materials and methods. (D) An optimised model of allergic airway inflammation was obtained with 2µg OVA ± 2µg rmIL-33 on days 9 and 11. Mice challenged with PBS or rmIL-33 alone were used as negative controls. Mice were culled 24 hours after the second challenge, and serum, BAL, lungs, and draining lymph nodes were obtained for analysis.

4.2.1. IL-33 exacerbates the inflammatory infiltrate in the lung

Following OVA sensitisation, airway challenge with OVA elicited typical peribronchial and perivascular eosinophilic inflammation, epithelial shedding and goblet cell hyperplasia. Challenge with rmIL-33 in the absence of antigen was able to induce a similar level of inflammation, and the combination of OVA and IL-33 was synergistic, producing a profound inflammatory response (Figure 4.2). A similar pattern was observed for the immunological parameters measured below.

4.2.2. IL-33 exacerbates inflammation in the airway lumen

Following OVA sensitisation, subsequent airway challenge with OVA or IL-33 significantly increased the BAL eosinophil, macrophage and neutrophil cell counts, and the concentration of cytokines and chemokines (Figure 4.3). These increases were markedly enhanced following challenge with a combination OVA and IL-33.

IL-5 and IL-13 were each increased above control by a similar amount in mice given OVA or IL-33, and the combination produced a marked increase (Figure 4.4A-B). The concentration of IL-4 was increased by OVA challenge, but not by IL-33, and the combination of OVA and IL-33 only increased IL-4 slightly (Figure 4.4C). Other cytokines were either undetectable (GM-CSF, IFNγ, IL-2, IL-12, TNFα, IL-1α, IL-1β and IL-17) or unchanged (IL-10).

Eotaxin-1, eotaxin-2 and TARC were increased by OVA or IL-33 challenge alone, and more so by the combination of both (Figure 4.4D-F). Monocyte chemotactic protein 1 (MCP-1) and MIP-1α were also increased, but not significantly, by the combination of OVA and IL-33. Other chemokines were either undetectable (TCA-3, IP-10, MIG) or unchanged (KC).

4.2.3. IL-33 increases the antigen-specific recall response

The effect of IL-33 on the antigen-specific cell-mediated immune response was examined by restimulating freshly isolated DLN cells with OVA *in vitro*. Lymph node cells from sensitised but not challenged mice (PBS group) proliferated in response to OVA, and this was increased in the groups of mice challenged with either OVA or IL-33. The group challenged with a combination of OVA and IL-33 had greater proliferation (Figure 4.5A), and also exhibited a high level of spontaneous proliferation *ex vivo* (Figure 4.5B).

The pattern of IL-5 production by lymph node cells was the same as that seen in BAL, with a small increase from mice treated with OVA or IL-33, and a marked increase with the combination (Figure 4.6A). This effect was even more marked for IL-13 production, where mice treated with OVA or IL-33 did not produce any increase above control, but the combination did (Figure 4.6B). IL-4 production was increased in mice given OVA, but not those given IL-33. IL-10 was not increased in either the OVA or IL-33 groups. However, IL-4 and IL-10 production were both increased markedly in mice given OVA and IL-33 (figure 4.6C-D). IFNγ and IL-12 were at the bottom of the assay detectable limits, and were unaffected by IL-33.

The production of chemokines eotaxin-1, eotaxin-2 and TCA-3 by lymph node cells was unaffected by IL-33, however TARC was increased by OVA or IL-33, and enhance by the combination of both (Figure 4.6E).

4.2.4. IL-33 Increases Th2 cytokines and chemokines in serum

IL-5 is a potent stimulator of eosinophil maturation and mobilisation from bone marrow. Although just detectable in mice given OVA or IL-33, serum IL-5 was increased by the combination (Figure 4.7A). The other key cytokines in this experiment (IL-4, 13, and IFNγ) were not detectable in serum

OVA or IL-33 increased the levels of eoataxin-1, eotaxin-2 and TARC (Figure 4.8B-D) in the serum, and IL-33 was able to enhance the effect of OVA. TCA-3 was not detected.

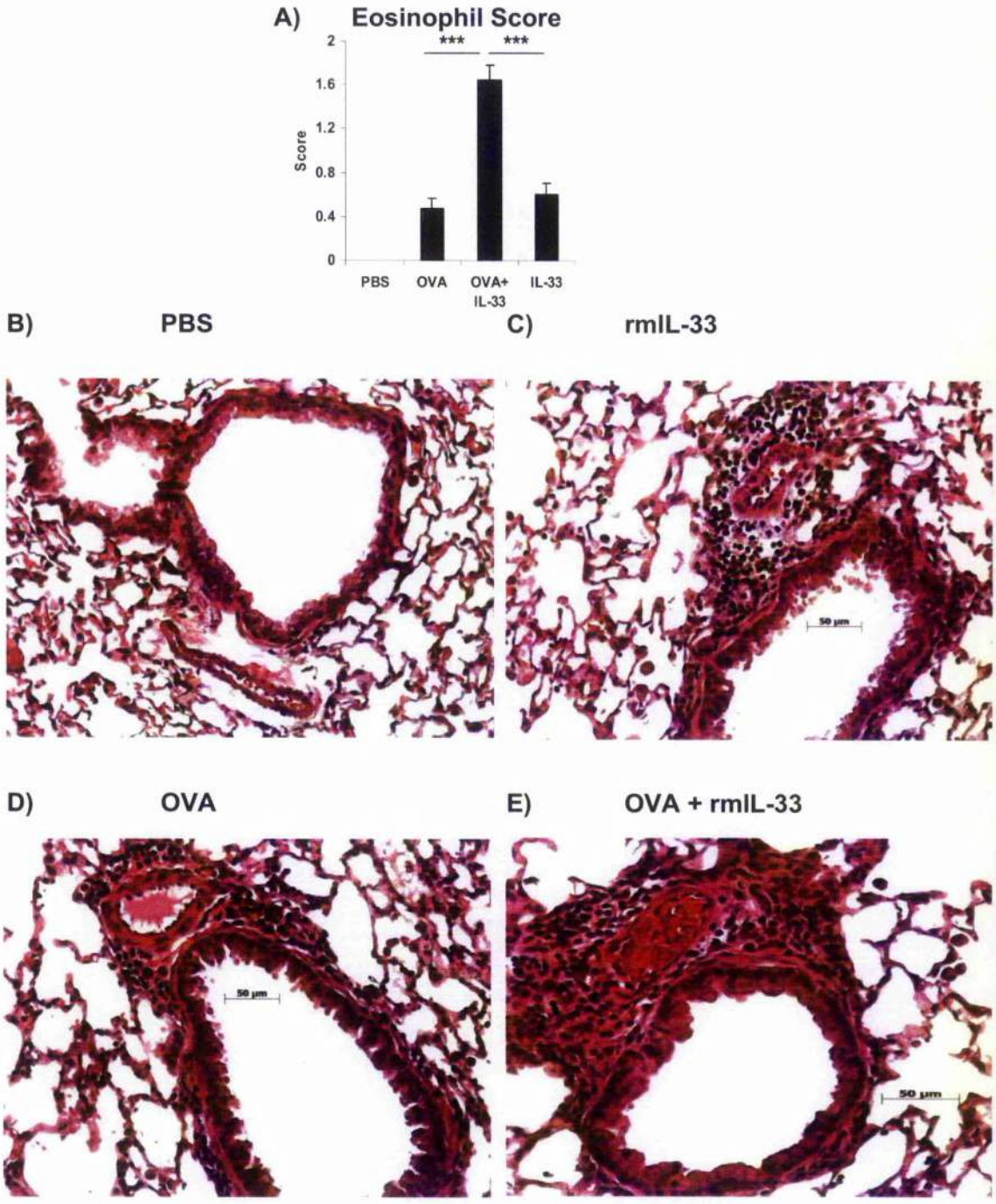


Figure 4.2 IL-33 increases the inflammatory cell infiltrate in the lungs

H&E stained lung sections were examined by light microscopy and eosinophils identified by morphological criteria. (A) The extent of peribronchial and perivascular inflammation was scored as described. Data are means ± SEM (***p<0.001, n=7). Photos show representative sections from mice challenged with (B) PBS, (C) rmIL-33, (D) OVA and (E) OVA + rmIL-33.

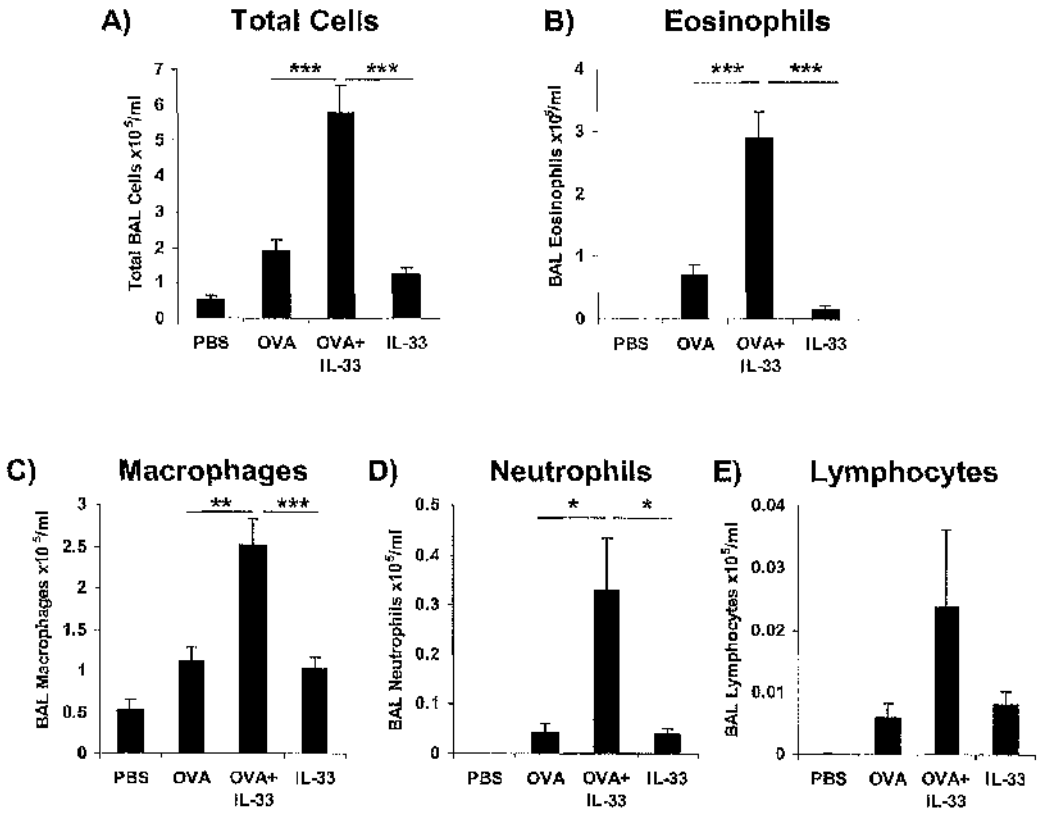


Figure 4.3 IL-33 increases BAL cell counts

BAL total and differential cell counts were performed as described in the materials and methods. Airway challenge with OVA and rmIL-33 resulted in increased (A) total cells, (B) eosinophils, (C) macrophages, (D) neutrophils and (E) lymphocytes, compared to OVA or IL-33 alone. Data are mean ± SEM (n=8; *p<0.05, **p<0.01, ***p<0.001).

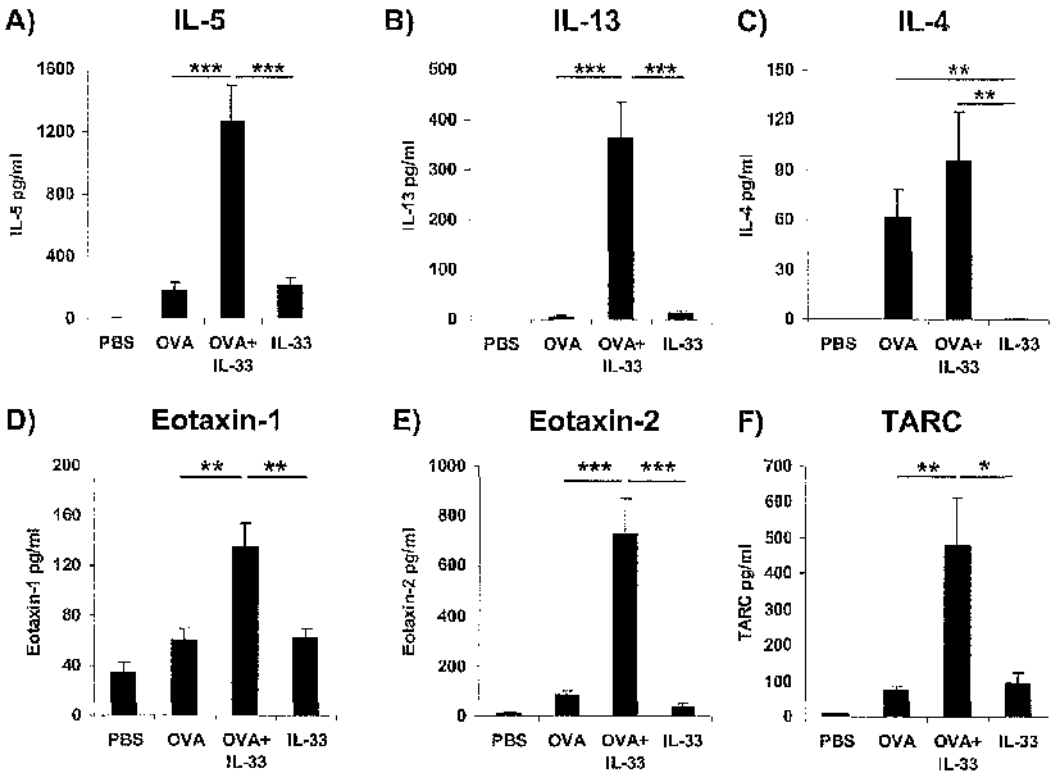


Figure 4.4 IL-33 increases BAL cytokines and chemokines

BAL supernatants were harvested and stored at -20˚C until cytokines and chemokines were measured by sandwich ELISA or multiplex. Airway challenge with OVA and rmIL-33 resulted in increased (A) IL-5, (B) IL-13, (C) IL-4 (D) eotaxin-1, (E) eotaxin-2 and (F) TARC compares to OVA or IL-33 alone. Data are means ± SEM (n=8; *p<0.05, **p<0.01, ***p<0.001).

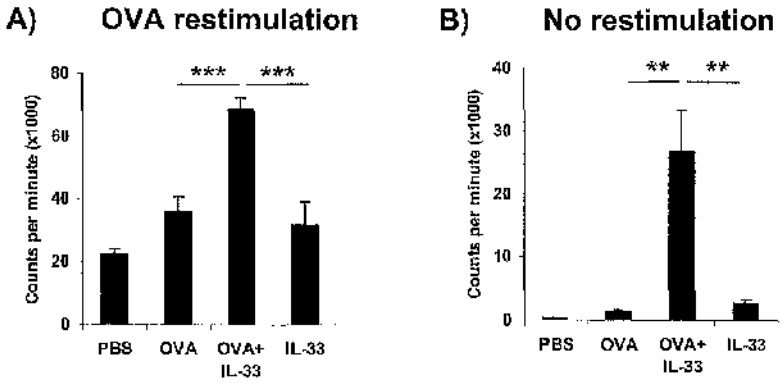


Figure 4.5 IL-33 increases proliferation of DLN cells

Thoracic lymph nodes draining the airways (DLN) were harvested and single cell suspensions obtained. Cells were cultured in 96 well plates in (A) medium alone or (B) with 1mg/ml OVA for the recall response. For the last 8 hours of culture ^{3}H-thymidine was added and incorporation into cells measured at 96 hours by betascintography. Lymph node cells from mice challenged with OVA and rmIL-33 had increased (A) antigen-specific and (B) spontaneous proliferation compared to OVA or IL-33 challenge alone. Data are means ± SEM (n=8; **p<0.01, ***p<0.001).

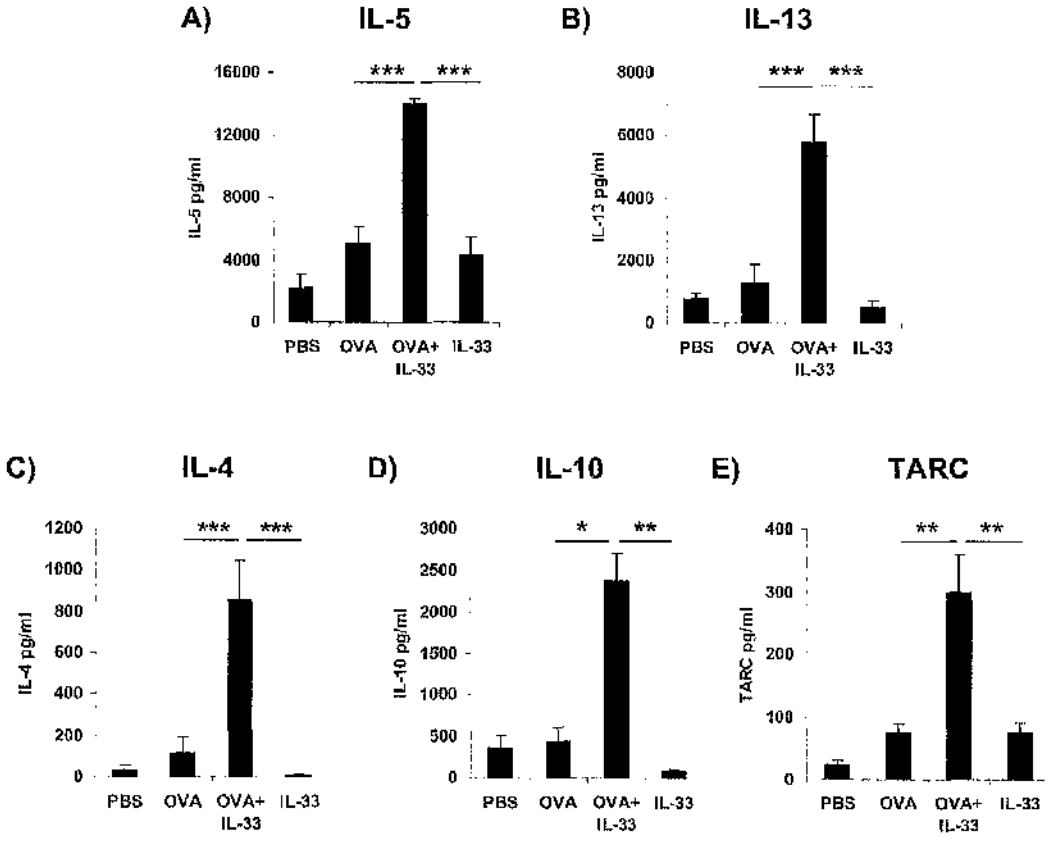


Figure 4.8 IL-33 increases DLN cytokine and chemokine production

Thoracic lymph nodes draining the airways (DLN) were harvested and single cell suspensions obtained. Cells were cultured in 24 well plates with 1mg/ml OVA for the recall response. Supernatants were harvested at 72 hours and frozen at -20˚C until cytokines and chemokines were measured by sandwich ELISA or multiplex. Lymph node cells from mice challenged with OVA and rmIL-33 had increased (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-10 and (E) TARC compared to OVA or IL-33 challenge alone. Data are means ± SEM (n=8; *p<0.05, **p<0.01, ***p<0.001).

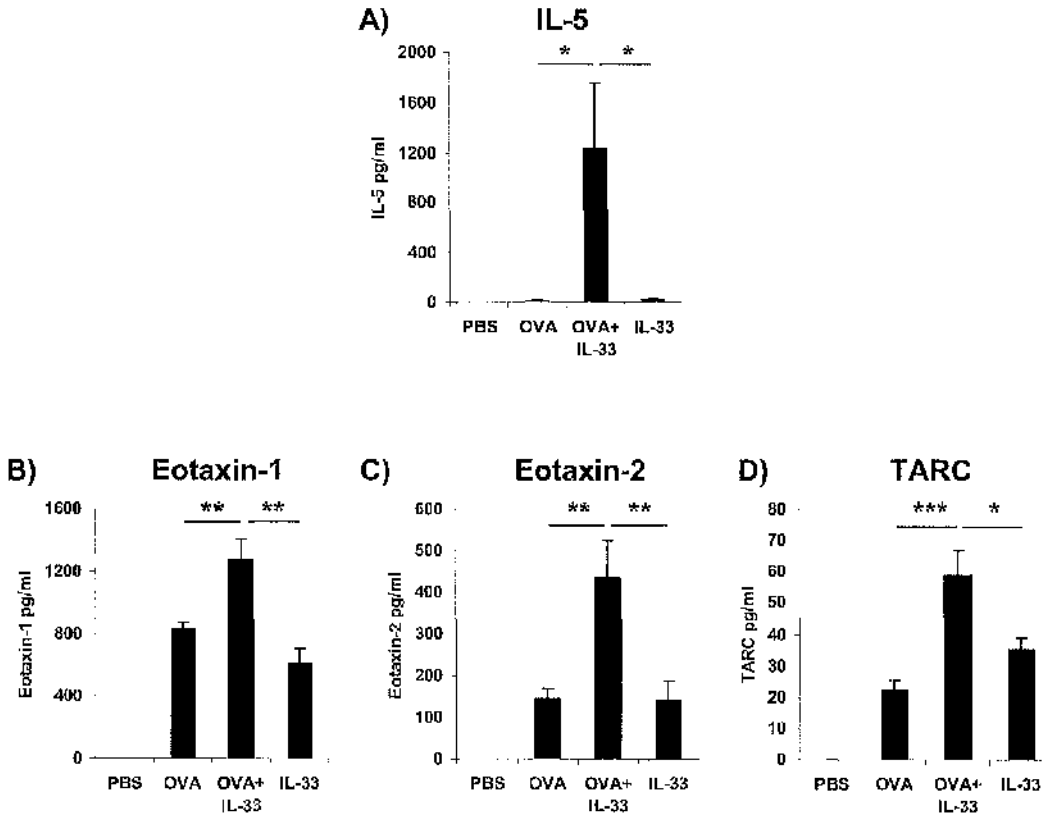


Figure 4.7 IL-33 increases serum levels of cytokines and chemokines

Serum was harvested and stored at -20°C until cytokines and chemokines were measured by sandwich ELISA or multiplex. Airway challenge with OVA and rmIL-33 resulted in increased IL-5 (A), eotaxin-1 (B), eotaxin-2 (C), and TARC (D), compared to challenge with OVA or IL-33 alone. Data are means ± SEM (n=8; *p<0.05, **p<0.01, ***p<0.001).

4.3. Exogenous IL-33 has no effect on airways inflammation in ST2$^{-/-}$ mice

In order to demonstrate that the effect of IL-33 in enhancing OVA-induced airways inflammation was specific, and depended on ST2L, the experiments above were repeated using BALB/c ST2$^{-/-}$ mice. Again pilot experiments were used to optimise the model in terms of the dose of OVA used for airway challenge, and the results shown are representative of two repeat experiments (figure 4.8). The dose of OVA and the number of challenges were higher than those used in examining rmIL-33 in ST2$^{+/+}$ mice in order to generate a similar level of baseline inflammation for comparison, and so any exacerbating or ameliorating effect of rmIL-33 would be apparent. In addition this protocol was used to compare ST2$^{+/+}$ and ST2$^{-/-}$ mice directly, as with lower doses and fewer challenges the ST2$^{-/-}$ mice made very little response, and did not differ significantly from PBS negative controls.

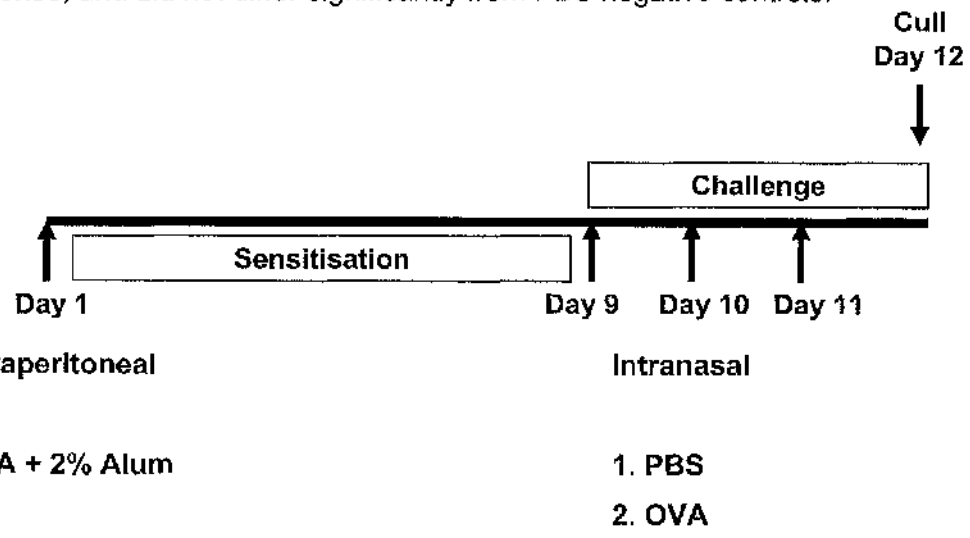


Figure 4.8 Administration of IL-33 in allergic airways inflammation in ST2$^{-/-}$ mice

On day 1 BALB/c mice were sensitised by IP injection of 100µg OVA in 100µl sterile PBS and 100µl of 2% alum. Allergic airway inflammation was induced by IN instillation of 30µl sterile PBS containing 10µg OVA ± 2µg rmIL-33 on days 9, 10 and 11. Mice challenged with PBS or rmIL-33 alone were used as negative controls. Mice were culled 24 hours after the second challenge. Serum, BAL, lungs, and draining lymph nodes were obtained for analysis as described in the materials and methods.

Airway challenge with OVA in ST2$^{-/-}$ mice elicited eosinophilic inflammation and increased BAL IL-5 concentration similar in character to that seen in wildtype mice (Figure 4.9). In the ST2$^{-/-}$ mice IL-33 administration did not enhance any features of airway inflammation, and there was no difference in any cellular or mediator variables from BAL, serum or lymph node cell restimulation with the addition of IL-33. This demonstrated that the presence of ST2L was specific and critical for the effects of IL-33 described in the experiments using wild-type mice above.

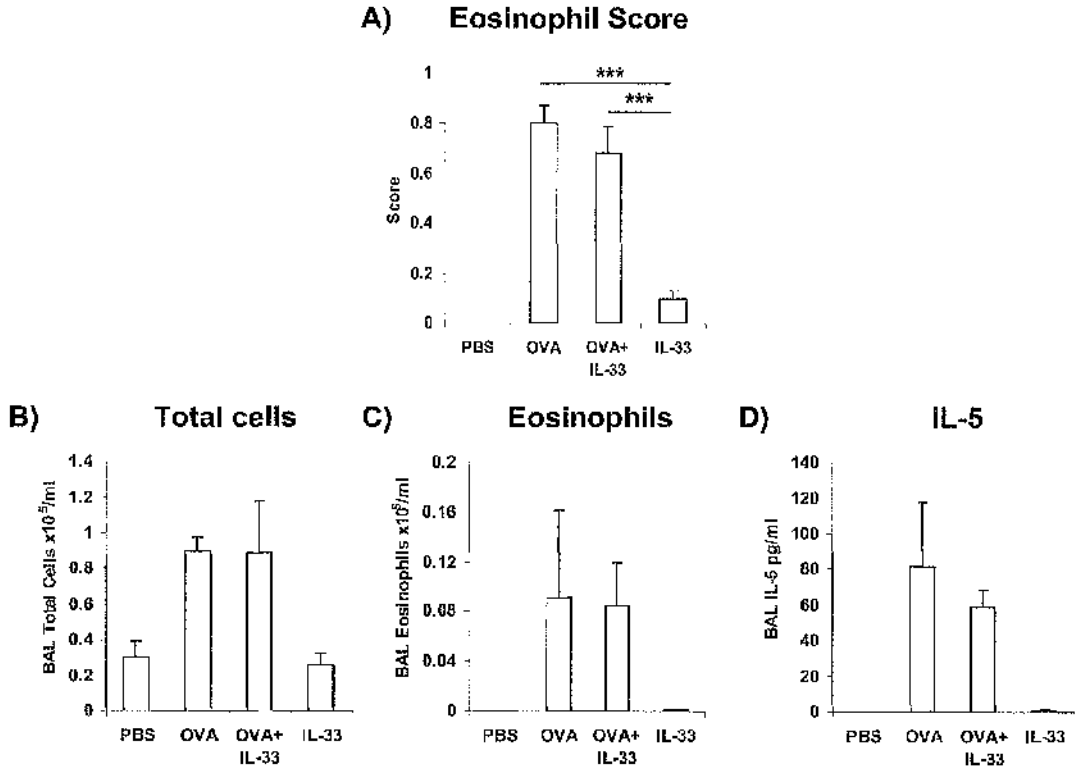


Figure 4.9 IL-33 has no effect on airway inflammation in ST2$^{-/-}$ mice

H&E stained lung sections were examined by light microscopy and eosinophils identified by morphological criteria. The extent of peribronchial and perivascular inflammation was scored as described. BAL total and differential cell counts were performed as described in the materials and methods. BAL supernatants were harvested and stored at -20°C until cytokines were measured by sandwich ELISA Airway challenge with OVA and rmIL-33 resulted in no increase in (A) tissue eosinophil score, (B) total BAL cells, (C) BAL eosinophils, or (D) BAL IL-5 compared to OVA or IL-33 alone. Data are means ± SEM (n=5; ***p<0.001).

4.4. ST2$^{-/-}$ reduces allergic airways inflammation

It was noted that a higher dose of OVA given more often was required to elicit a similar level of inflammation as seen in wildtype mice, suggesting that in contrast to recent findings (329), ST2$^{-/-}$ in BALB/c mice may result in reduced airways inflammation in our model. To test this hypothesis, wildtype and ST2$^{-/-}$ mice were directly compared using the same protocol (figure 4.8). The results shown are representative of 3 repeat experiments.

4.4.1. ST2$^{-/-}$ reduces the inflammation in lung tissue and the airways

ST2$^{-/-}$ mice had a reduced eosinophilic infiltrate compared to wildtype (Figure 4.10A). BAL cellularity was also decreased (Figures 4.10B-D), which was largely due to a reduction in eosinophils, but also some reduction in macrophages. Neutrophils and lymphocytes were present at low levels, but there was no difference between the groups.

There was a small, but not significant, reduction in BAL IL-4, whereas IL-5 was markedly reduced (Figure 4.11A-B). No difference in IL-13 was seen, although basal levels were already quite low. IFNγ and GM-CSF were not detectable.

The chemokines eotaxin-1, eotaxin-2 and TARC were all reduced in BAL (Figure 4.11C-E). MIP-1α was also reduced, but not significantly, in ST2$^{-/-}$ mice. Other chemokines were unchanged (KC) or undetectable (IP-10, MCP-1, MIG and TCA-3).

No cytokines or chemokines were detectable in the serum.

4.4.2. ST2$^{-/-}$ has no effect on the antigen-specific recall response

Mixed lymph node cells from ST2$^{-/-}$ mice did not proliferate any less than wildtype in response to OVA restimulation (Figure 4.12A). The production of Th2 cytokines (Figure 4.12B-D) was unaffected by ST2$^{-/-}$, as was chemokine production (eotaxin-1, eotaxin-2 and TARC). IFNγ and GM-CSF were undetectable.

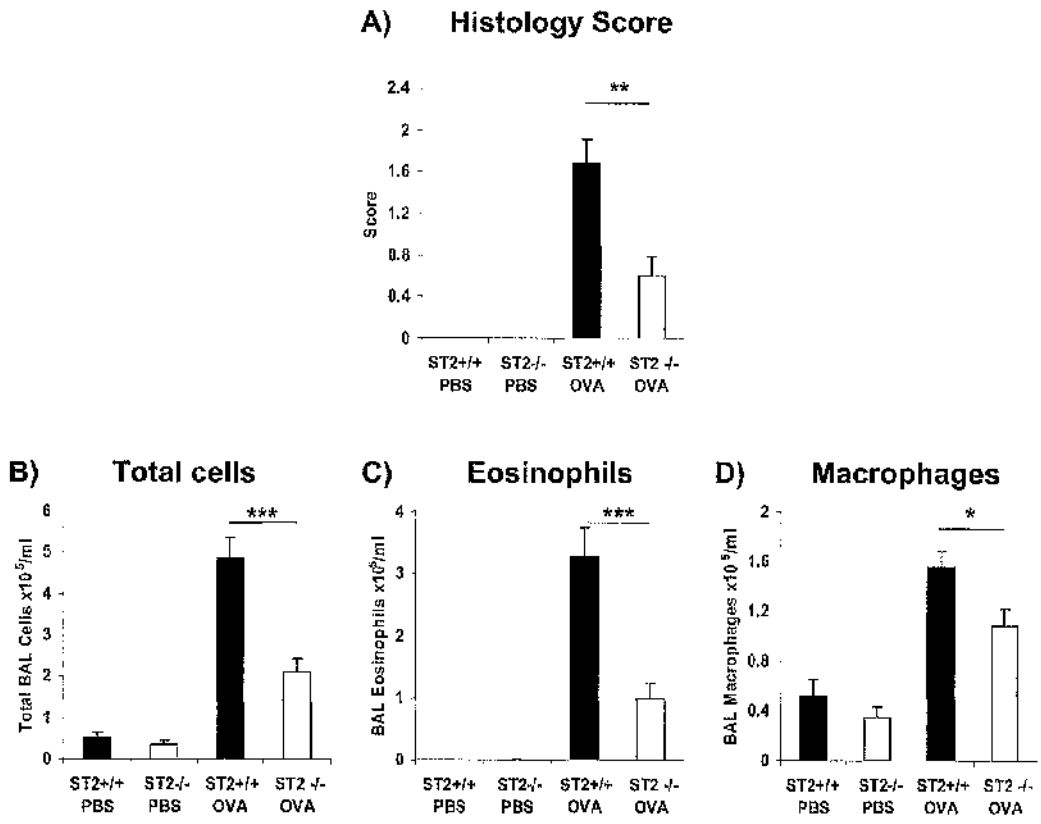


Figure 4.10 Eosinophilic inflammation and BAL cell counts are reduced in ST2$^{-/-}$ mice

(A) H&E stained lung sections were scored for eosinophilic lung inflammation as described. BAL total and differential cell counts were performed as described in the materials and methods. Airway challenge with OVA in ST2$^{-/-}$ mice resulted in decreased (B) total cells, (C) eosinophils, and (D) macrophages compared to challenge with OVA in ST2$^{+/+}$ mice. Data are means ± SEM (n=8; *p<0.05, **p<0.01, ***p<0.001).

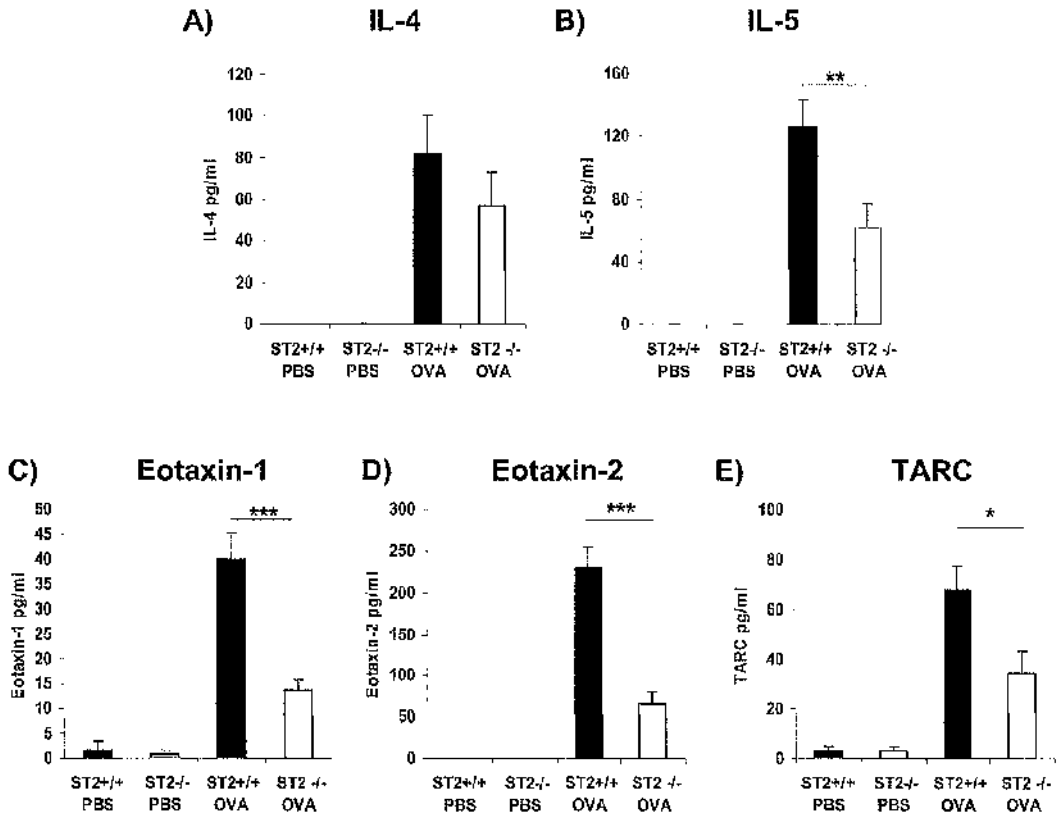


Figure 4.11 BAL cytokines and chemokines are reduced in ST2$^{-/-}$ mice

BAL supernatants were harvested and stored at -20˚C until cytokines were measured by sandwich ELISA. Airway challenge with OVA in ST2$^{-/-}$ mice resulted in decreased (A) eotaxin-1, (B) eotaxin-2, (C) TARC compared to wildtype mice. Data are means ± SEM (n=8; *$<p<0.05$, **$p<0.01$, ***$p<0.001$).

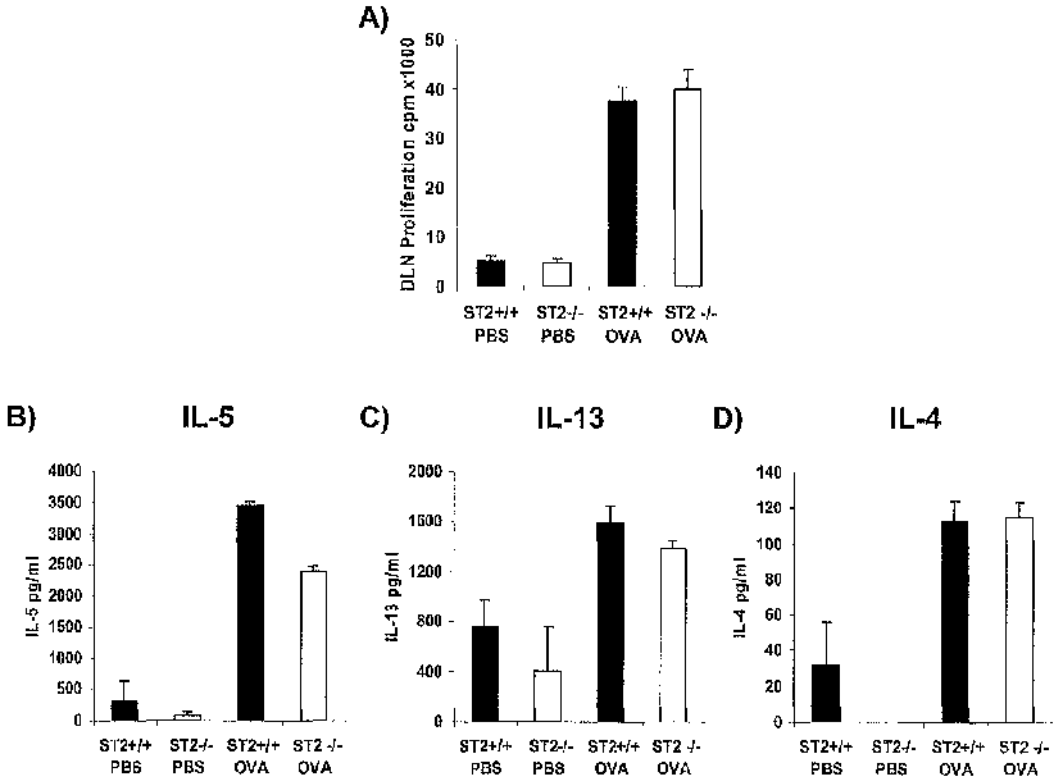


Figure 4.12 No reduction in DLN cytokine production in ST2$^{-/-}$ mice

Thoracic lymph nodes draining the airways (DLN) were harvested and single cell suspensions obtained. Cells were cultured in 24 well plates with 1mg/ml OVA for the recall response. Supernatants were harvested at 72 hours and frozen at -20°C until cytokines were measured by sandwich ELISA or multiplex. Lymph node cells from ST2$^{-/-}$ mice had (A) no difference in proliferation, (B) slightly decreased IL-5 (NS), but no difference in (C) IL-13, or (D) IL-4. Data are means ± SEM (n=8).

4.5. Discussion

In this chapter I have demonstrated that:

1. IL-33 exacerbates OVA-induced allergic airway inflammation
2. IL-33 alone can induce airway inflammation in sensitised mice
3. The effect of IL-33 in airway inflammation is dependent on *ST2* gene expression
4. $ST2^{-/-}$ mice have reduced airway inflammation

I will discuss each of these points below in more detail.

4.5.1. IL-33 exacerbates OVA-induced allergic airway inflammation

When given at the same time as OVA challenge, intranasal IL-33 was able to exacerbate the effector phase of allergic airway inflammation. An increase in all inflammatory cells, but particularly eosinophils, was seen in the airway lumen (BAL) and in the tissues, although the actual pathology was similar to OVA alone. These pathological changes are likely to be mediated through IL-5 and IL-13, and the Th2 chemokines eotaxin-1, eotaxin-2 and TARC, all of which were increased in BAL and/or serum. Of note, however, IL-4 production was not significantly affected. The cells on which IL-33 acts, and therefore the source of these cytokines and chemokines, are unknown. I have already shown that IL-33 can induce an innate type 2 response, and it may be that ST2L expression on cells such as mast cells (288), macrophages (284) or even NKT cells plays a role. However, antigen sensitisation and challenge increases the expression of ST2L on Th2 cells (318), and so IL-33 may increase Th2 cell responses directly.

In keeping with this, mixed lymph node cultures from mice that received OVA and IL-33 proliferated spontaneously, and had increased proliferation after OVA restimulation *in vitro*. Increased levels of IL-5, IL-13 and TARC were seen, and in contrast to BAL, IL-4 and IL-10 production were also increased. Whether these increases were due to increased cell numbers, or increased production from individual cells, is unknown. It is notable that IL-33 was able to exacerbate inflammation despite a large increase in IL-10, which plays an immunosuppressive role in asthma (391).

4.5.2. IL-33 alone can induce airway inflammation in sensitised mice

It was noticeable that IL-33 given to mice previously sensitised to OVA resulted in a very similar response to rechallenging with OVA. I have previously shown that IL-33 can induce an innate type 2 response in the lung. Pilot experiments as part of that work showed that, although a single dose of IN IL-33 could induce detectable levels of eosinophils in the BAL of some mice, at least 3 doses were required to elicit a significant increase in BAL eosinophils in all mice (data not shown). Even then the numbers seen were not as high as in these experiments. Therefore, although the innate effects of IL-33 may be contributing, possibly through the initiation and localisation of the response to the lung, the pre-sensitisation of the mice seems important. Since sensitisation and challenge increases the expression of ST2L on Th2 cells, it is possible that IL-33 could directly activate Th2 cells in the absence of further antigen.

In contrast to the previous study by Schmitz et al (1), who showed systemic IL-33 induces expression of IL-4 and IL-5 mRNA, IN IL-33 alone did not induce the expression of IL-4 protein in the BAL, serum or in DLN cultures, whereas it induced as much IL-5 as OVA did. This is in keeping with previous evidence that IL-33/ST2L plays a particular role in IL-5 expression. However, in combination with OVA, IL-33 did induce an increase in IL-4, particularly in DLN cultures, which suggests this is an indirect mechanism, and relies on enhancement of antigen-specific responses. This echoes the effect of IL-18 discussed in the introduction, where IL-18 can augment IL-4 and IL-13 in the presence of antigen, but only IL-13 in its absence (29).

4.5.3. The effect of IL-33 in airways inflammation is dependent on ST2

As with the innate response, IL-33 had no effect on airway inflammation either alone, or in combination with OVA, in ST2-/- mice. Thus the effects seen are not due to adjuvant effects of contaminants such as LPS, and are dependent on the binding of IL-33 to ST2L.

4.5.4. $ST2^{-/-}$ reduced airway inflammation

Here I have demonstrated that ST2-/- mice have a reduced histology score and a reduction in eosinophils and macrophages in the BAL, mediated by a reduction in BAL IL-5 and Th2 chemokines. Proliferation and cytokine production from DLN was largely unaffected, which is in contrast to the pronounced effect of IL-33 on

DLN cells. Whilst this partly reflects the differences seen when modulating either a receptor or its ligand, it probably also reflects the fact that other compensatory mechanisms may be acting due to the redundancy seen in inflammatory cytokine networks. What it does highlight is the important role of IL-33/ST2L in eosinophil accumulation in the lung, through upregulation of IL-5, eotaxin-1 and eotaxin-2.

These result are in contrast with the recent report from Mangan et al (329), who showed that ST2-/- in BALB/c mice resulted in an increase in eosinophils in BAL, and a reduction in macrophages. They did not find a reduction in BAL IL-5, although they also showed no change in other BAL cytokines, or DLN cytokine production. This may reflect differences in the model, and that other mechanisms may have been able to overcome, or even overcompensate for, the loss of *ST2*.

It is interesting to note that Mangan et al (329) showed that IL-33 mRNA is upregulated in lung tissue after antigen challenge, and IL-33 may be one mechanism by which the Th2 effector response can be promoted. However, other mechanisms must also be important, as IL-33 does not appear to induce IL-4 protein expression, and ST2-/- mice can still develop allergic airways inflammation.

When interpreting this data the presence of confounding factors must not be underestimated. There are many unknown variables in the interactions of IL-33, ST2L and sST2. I have not taken into account the expression of sST2 seen in airways inflammation in mice (319), and in asthma exacerbations in humans (340), and how it may interact with IL-33 and ST2L to modulate inflammation. The drawback of using ST2-/- mice is that both ST2L and sST2 are lost, so it is difficult to distinguish between their relative roles in inflammation.

4.6. Conclusions

In this chapter I have demonstrated that IL-33 exacerbates the effector phase of allergic airways inflammation, and that this is dependent on *ST2* expression. I have also demonstrated that ST2-/- results in a reduction of allergic airways inflammation. Together these results highlight the importance of IL-33/ST2L in eosinophil accumulation in the tissues.

Having examined the role of IL-33 in innate and allergic Th2 inflammation, in the next chapter I will investigate its potential role in autoimmune Th1/Th17 inflammation.

Chapter 5

IL-33 and ST2 in collagen-induced arthritis

5.1. Introduction

In the previous chapters I have shown that IL-33, a novel member of the IL-1 family of cytokines, can induce an innate type 2 response, and can exacerbate the effector phase of an established Th2 response. The other IL-1 family members, IL-1 and IL-18, are pleiotrophic pro-inflammatory cytokines (243,245), and in addition, IL-18 has been shown to enhance both Th1 and Th2 responses, depending on the circumstances (246,392).

Rheumatoid arthritis is an autoimmune inflammatory condition primarily affecting the joints. Initially characterised as a Th1 disease, it is becoming clear that Th17 cells mediate the effector phase of inflammation and joint destruction (205,208,213). TNFα and IL-1 have a prominent role in both inflammation and bone erosion, and inhibiting these is currently being targeted as a therapeutic option in the clinic .(215,221).

I hypothesised that the pro-inflammatory functions of IL-33/ST2 would not be restricted to Th2 type inflammation. I therefore examined the role of IL-33/ST2 in CIA, a model of Th1/Th17 autoimmune arthritis. The data presented in this chapter represents pilot experiments performed to investigate this hypothesis. I induced CIA in the susceptible DBA/1 strain of mice and administered IP rmIL-33 during the effector phase, which exacerbated disease. I also induced CIA in $ST2^{-/-}$ DBA/1 mice, which had been bred in-house, which ameliorated disease.

5.2. IP rmIL-33 exacerbates CIA

CIA was induced as described in the materials and methods. The dose of rmIL-33 used was the same as for inducing an innate type 2 response, and it was felt that 5 subsequent doses after antigen boosting on day 21 would be sufficient, as in previous work even a single IP dose had measurable effects. Mice were culled at early (day 26), middle (day 31) and late (day 38) time points after rmIL-33 administration to investigate the kinetics of the response (figure 5.1).

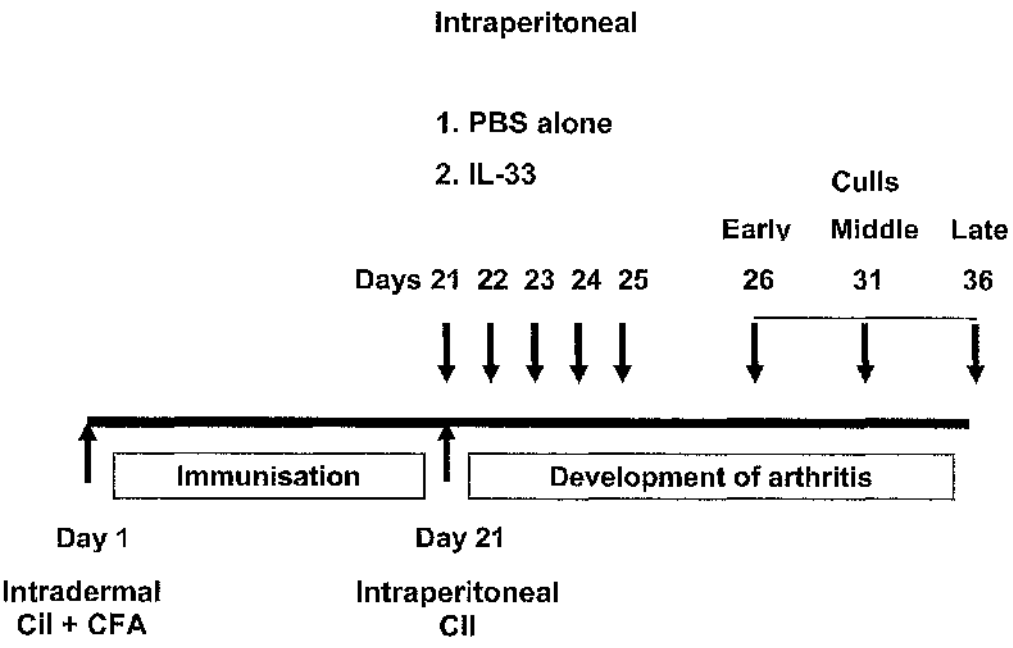


Figure 5.1 A murine model of collagen-induced arthritis

Mice were immunised intradermally at the base of the tail with 50µg bovine collagen type II (CII) in complete Freund's adjuvant (CFA) on day 1, and the response boosted on day 21 with 50µg CII in PBS intraperitoneally. Where indicated 200µl sterile PBS ± 0.8µg mIL-33 was given intraperitoneally for 5 consecutive days from day 21. Footpads were measured using callipers every 1-2 days from day 21, and the clinical score of each paw assessed at the same time. Mice were culled at various times during disease development and draining lymph nodes, spleen and ankle joints harvested to assess the immune response.

5.2.1. IL-33 exacerbates disease severity of CIA

Treatment with rmIL-33 did not increase the number of mice that developed arthritis (Figure 5.2A), which was close to 100% in both groups. However, it did increase the average clinical score per affected mouse (Figure 5.2B). When this was examined further it became apparent that the number of affected paws per mouse was increased (Figure 5.3A), and also the thickness of the swelling (Figure 5.3B). The clinical score per affected joint was not increased as loss of function was rarely seen (Figure 5.3C).

5.2.2. IL-33 increases early spleen proliferation

The proliferation of mixed splenocyte cultures was increased at the early time point, and this was increased in the IL-33 group (Figure 5.4A). At later time points there was very little proliferation in either group. The proliferation of mixed DLN cells gradually increased at each time point, but there was no difference between the groups.

5.2.3. IL-33 increases cytokine production in spleen and DLN

Treatment with IL-33 induced an early burst of cytokine production by mixed splenocyte cultures (Figure 5.4B). IL-17, TNFα, IL-12, and IFNγ were all increased at the early time point compared to the untreated group. IL-17 production was sustained at a higher level at the late time point in the IL-33 group, but there was no difference in the other cytokines at this time point. In contrast, mixed DLN cultures from the IL-33 treated group produced higher concentrations of IL-17, TNFα, and IL-12 at the later time point (Figure 5.4C), but there was little difference at the early time point. IL-5 production was also increased at the early and late time points in spleen and DLN cultures respectively (Figure 5.5).

Of the other cytokines measured in culture supernatants, IL-1β, IL-4 and IL-10 were undetectable, and IL-6 was unaffected by IL-33 treatment. No difference was seen between IL-33 treated and untreated mice in serum levels of cytokines or collagen specific IgG1 and IgG2a.

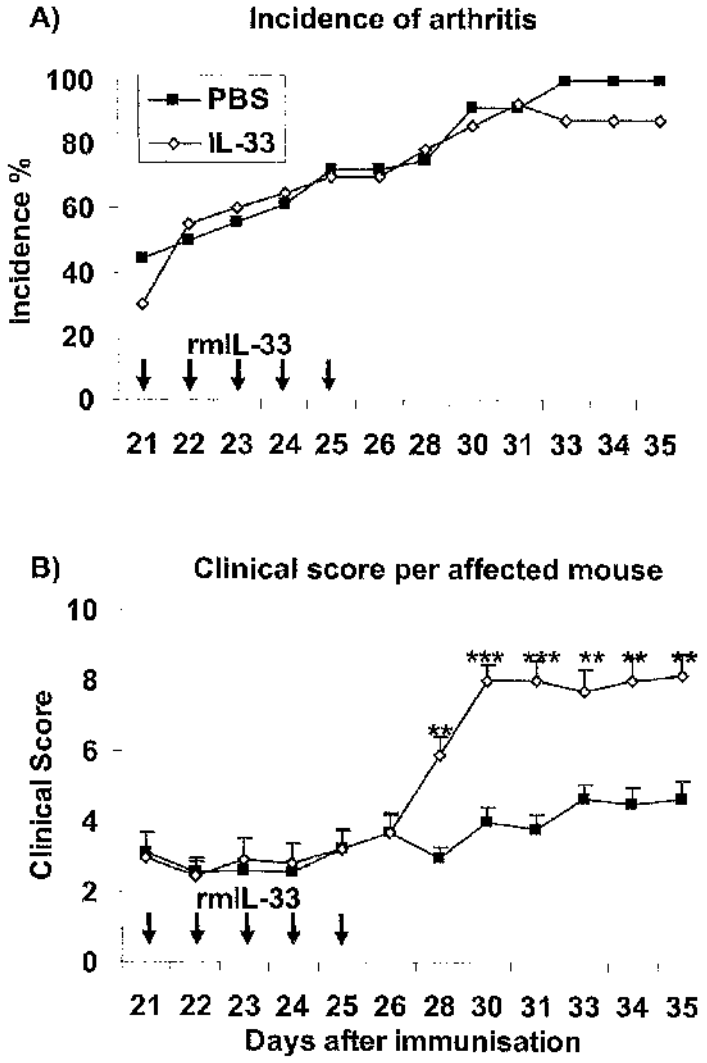


Figure 5.2 IL-33 increases the severity of CIA

Arthritis was induced in mice as described. (A) The incidence of arthritis was determined as the percentage of the group which had developed arthritis in at least one paw, and was unaffected by treatment with IL-33 (n = up to 20 mice per group). (B) The total clinical score of each mouse with disease was assessed, with the maximum being 12. Treatment with IL-33 increased disease severity. Results are expressed as mean ± SEM (n = up to 20; **p<0.01, ***p<0.001).

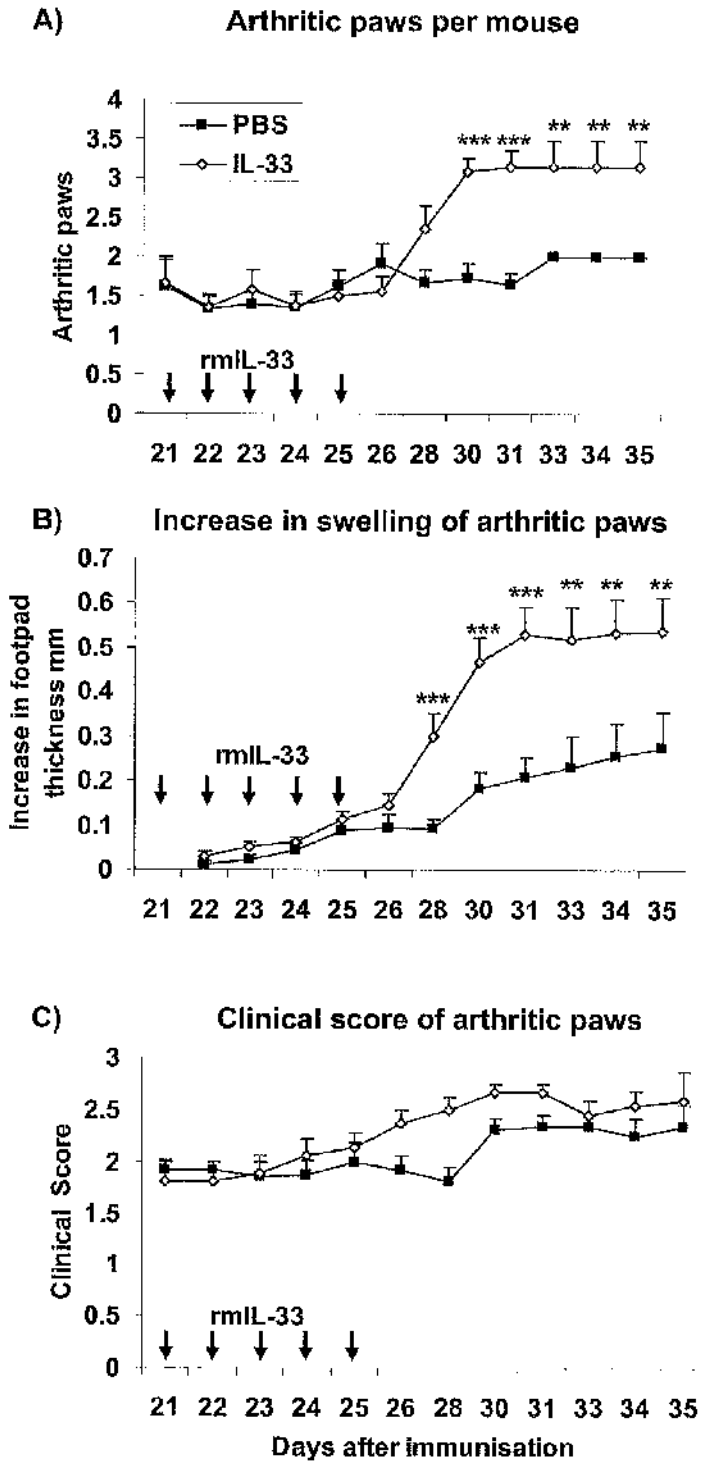


Figure 5.3 IL-33 increases the number of diseased paws

Arthritis was induced in mice as described. Treatment with IL-33 increased (A) the number of arthritic paws per mouse and (B) the amount of swelling in arthritic paws. (C) The clinical score per individual paw was not significantly affected (n= up to 20; **p<0.01, ***p<0.001).

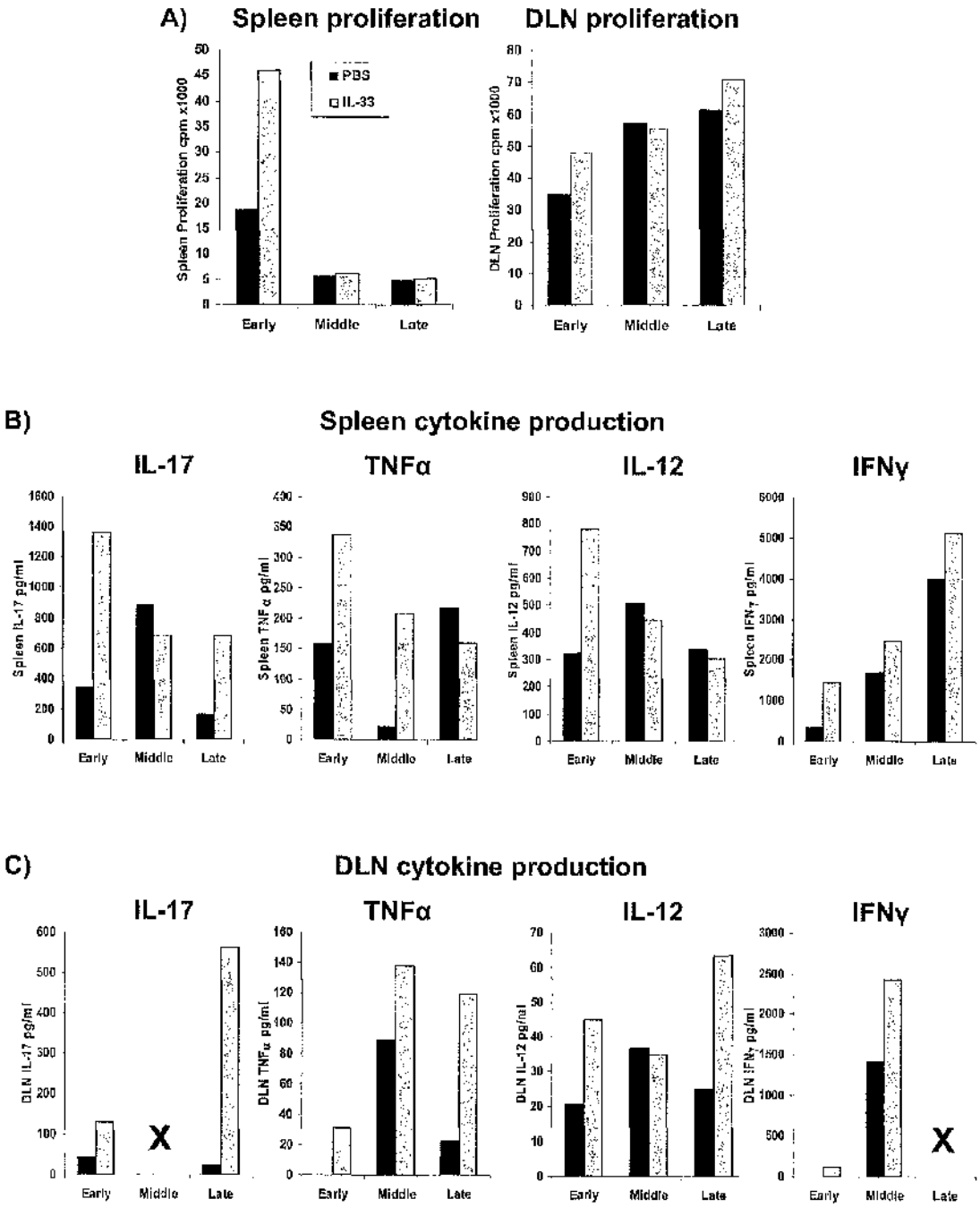


Figure 5.4 IL-33 increases proliferation and cytokine production

Arthritis was induced in mice as described. Mice were culled at day 26 (early), day 31 (middle) and day 36 (late) and DLN and spleens harvested. Single cell suspensions were restimulated with CII and proliferation and cytokine production measured. Treatment with IL-33 increased (A) early splenocyte proliferation (B) early splenocyte cytokine production and (C) late DLN cytokine production (**X** = not analysed). Statistics were not performed on *in vitro* analyses as organs from each group were pooled prior to preparation for cell culture.

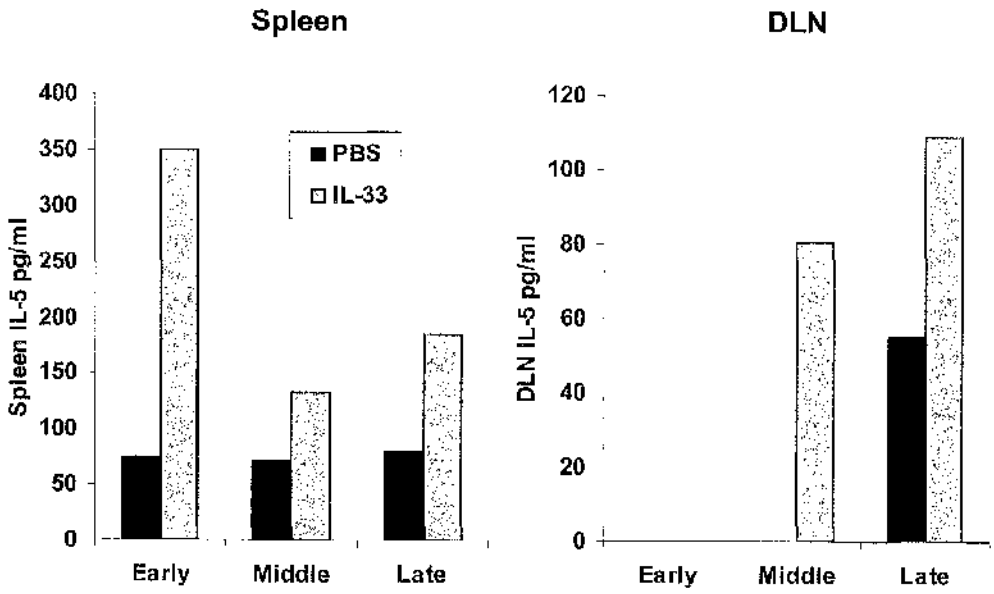


Figure 5.5 IL-33 increases IL-5 production

Arthritis was induced in mice as described. Mice were culled at day 26 (early), day 31 (middle) and day 36 (late) and DLN and spleens harvested. Single cell suspensions were restimulated with CII and IL-5 measured by sandwich ELISA. Treatment with IL-33 increased IL-5 production in (A) spleen and (B) DLN cultures. Statistics were not performed on *in vitro* analyses as organs from each group were pooled prior to preparation for cell culture.

5.3. $ST2^{-/-}$ mice have reduced severity of CIA

CIA was induced in DBA/1 $ST2^{-/-}$ mice using the same protocol as before (Figure 5.1). $ST2^{+/+}$ littermates were used as the control group. Unfortunately, the number of mice available from the breeding programme was below the number required to reach significance according to the power calculation, but there was a definite trend towards a reduction in disease severity. The experiment was repeated and the same results obtained.

$ST2^{+/+}$ and $ST2^{-/-}$ littermates developed a similar incidence of arthritis (2/3 and 3/4) respectively (Figure 5.6A). However, $ST2^{-/-}$ mice developed less severe disease, with a reduction in clinical score per affected mouse (Figure 5.6B). The number of arthritic paws per mouse was reduced (Figure 5.7A), and also the thickness of the swelling (Figure 5.7B), but the clinical score per arthritic joint was not (Figure 5.7C).

No significant difference was seen in DLN or spleen proliferation, or in TNFα, IL-6, or IL-12 production (Figure 5.8A and B). IL-1β and IFNγ were not detected, and IL-17 was not measured.

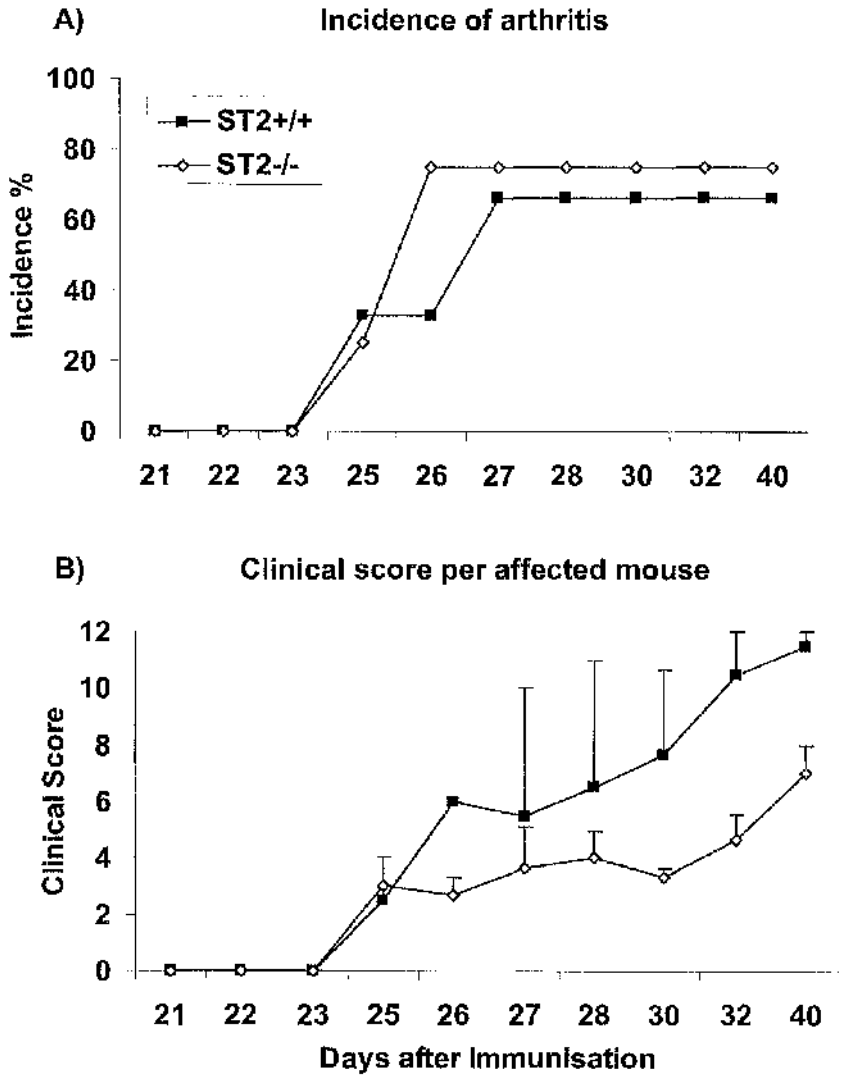


Figure 5.6 $ST2^{-/-}$ mice have reduced severity of CIA

Arthritis was induced in mice as described. (A) $ST2^{-/-}$ did not affect the incidence of arthritis (n=3-4). (B) The total clinical score of each mouse with disease was assessed, with the maximum being 12. $ST2^{-/-}$ reduced disease severity. Results are expressed as mean ± SEM (n=3-4). No statistics were performed due to the small number of mice available.

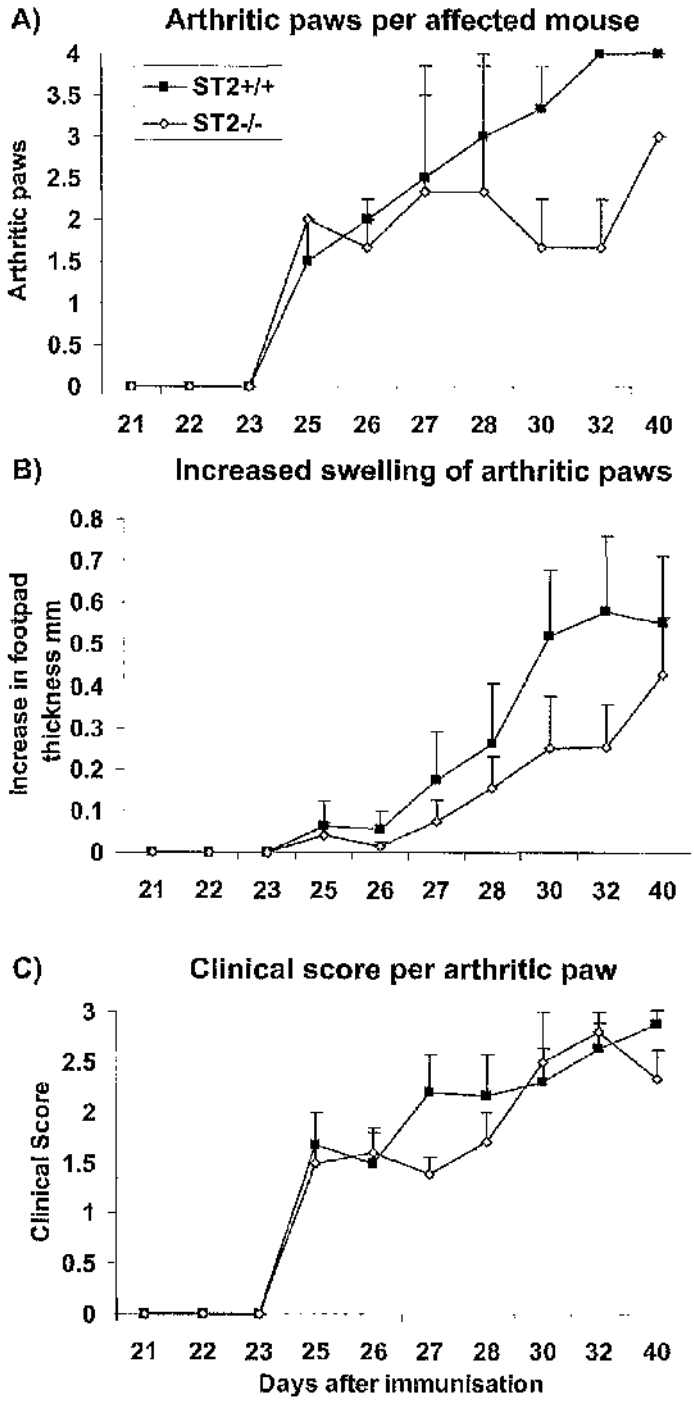


Figure 5.7 ST2$^{-/-}$ mice have fewer diseased paws

Arthritis was induced in mice as described. ST2-/- mice had (A) decreased arthritic paws per mouse and (B) reduced swelling in arthritic paws. (C) The clinical score per individual paw was not significantly affected (n=3-4). No statistics were performed due to the small number of mice available.

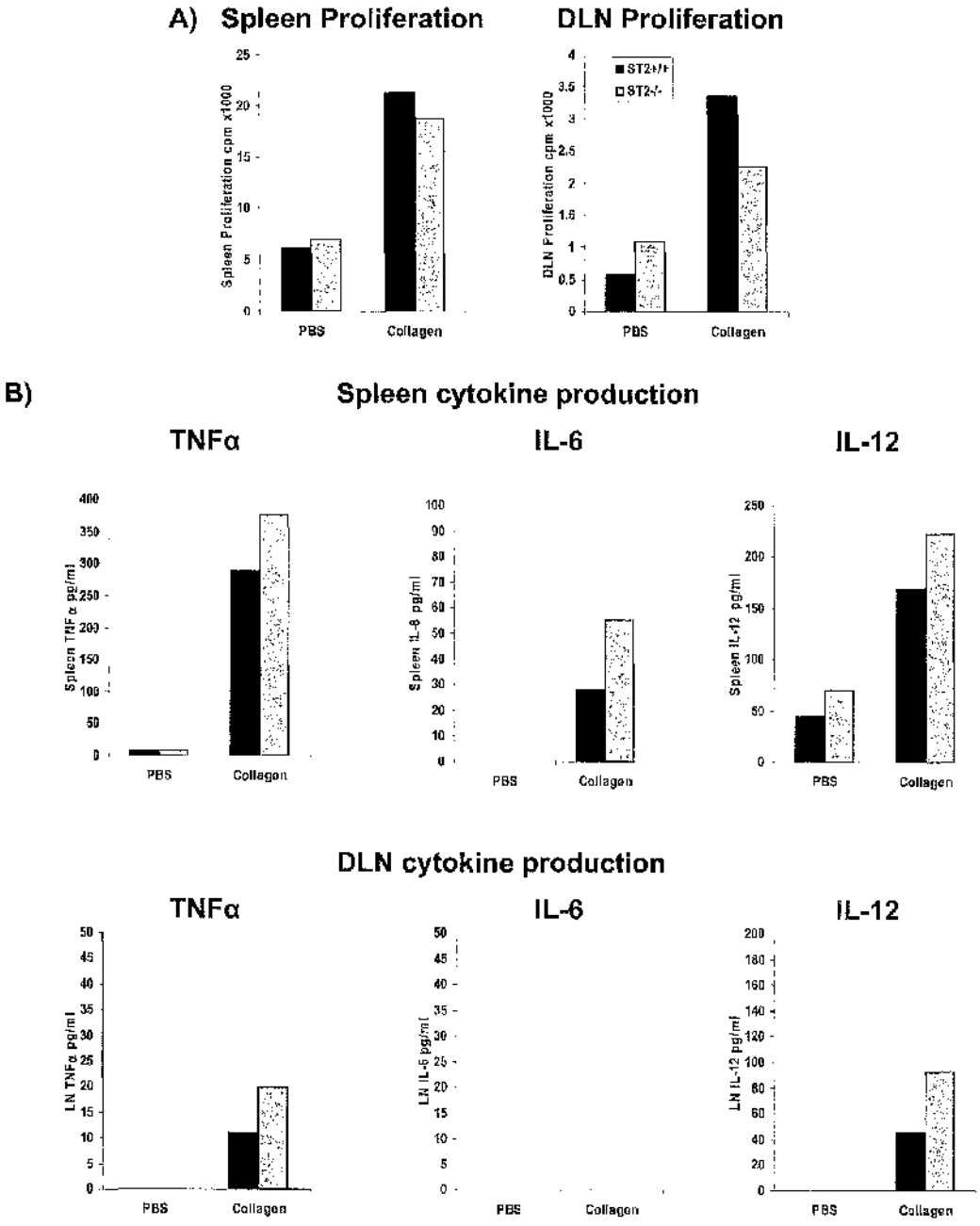


Figure 5.8 IL-33 increases proliferation and cytokine production

Arthritis was induced in mice as described. Mice were culled at the peak of disease severity (day 40) and DLN and spleens harvested. Single cell suspensions were restimulated with collagen and (A) proliferation and (B) cytokine production measured. Statistics were not performed on *in vitro* analyses as organs from each group were pooled prior to preparation for cell culture.

5.4. Discussion

The pilot data in this chapter indicates that IL-33 may exacerbate CIA, whilst $ST2^{-/-}$ may ameliorate disease. Specifically, neither IL-33 nor $ST2^{-/-}$ affected disease incidence, but IL-33 increased the number of arthritic joints per affected mouse, and the amount of joint swelling per arthritic paw, whilst $ST2^{-/-}$ reduced these measures.

An increase in cytokines known to play important roles in arthritis was seen. Whilst some of the increased cytokine production seen in splenocytes could be due to increased proliferation and numbers of cytokine producing cells, there was no increased proliferation in DLN. Of particular interest were the marked increase in IL-17 and TNFα from the DLN cells. Therefore IL-33 could exacerbate arthritis indirectly through the induction of TNFα and IL-17. Both of these play crucial roles in the initiation and persistence of joint inflammation, and in cartilage and bone erosion. However, it is unknown exactly where the inflammatory cytokine came from and whether IL-33 induces these cytokines directly or indirectly. It was also interesting to note that, in contrast to IL-33, $ST2^{-/-}$ seemed to have little effect on T cell function. This is similar to the results seen with allergic airway inflammation.

IL-5 was elevated by IL-33 treatment, which is in keeping with previous data in the allergic airway model, and in the literature, that IL-33/ST2 modulates IL-5 production. It is of note that IL-33 can induce IL-5 production even despite the ongoing Th1/Th17 response. The possible functional relevance of this unknown, although elevated Th2 cytokines (IL-4 and IL-13) are part of the pattern of cytokines found in the synovial fluid in very early synovitis (393) which predict progression to RA.

5.5. Conclusions

Overall, although this preliminary data suggests that the function of IL-33/ST2 plays a role in CIA, and therefore may be important in diseases other than those mediated by Th2 type inflammation, these studies need to be repeated with sufficient numbers to ensure robust biological and statistical significance, and the analysis extended to elucidate possible mechanisms of action.

Chapter 6

General Discussion

6.1 Summary of Results

The hypothesis on which the work in this thesis is based was that the novel cytokine IL-33 and ST2, its receptor, have an important pro-inflammatory role in regulating innate and adaptive inflammation. The data generated demonstrates that this hypothesis is valid.

In Chapter 3 I established the use of rmIL-33 as a useful reagent for investigating the *in vivo* functions of IL-33 and ST2. I went on to show that IL-33 induces local and systemic type 2 inflammation dependent on the expression of ST2. Direct administration of IL-33 to the airways resulted in eosinophilic airway inflammation, and pathological changes similar to asthma, mediated by elevated IL-5, IL-13, eotaxin-1, eotaxin-2 and TARC. Experiments with SCID mice revealed that innate and/or structural cells are sufficient to initiate this response, but lymphocytes greatly enhance the response. The exact cell types involved were not identified.

In Chapter 4 I showed that IL-33 administered with antigen at the time of airway challenge exacerbated Th2 mediated allergic airway inflammation, including both local tissue effector responses and DLN T cell responses. In addition, IL-33 induced allergic airway inflammation in pre-sensitised mice in the absence of further antigen challenge. Both of these effects were dependent on ST2 expression. $ST2^{-/-}$ mice developed less severe inflammation in the airways despite having normal T cell recall responses.

In Chapter 5 I presented pilot data that raise the intriguing possibility that IL-33 and ST2 do not just modulate type 2 responses. IL-33 exacerbated the severity of CIA, and enhanced DLN T cell responses, whilst $ST2^{-/-}$ mice had reduced disease severity, without affecting DLN T cell responses. However, these data need to be confirmed using appropriate numbers of mice to ensure robust biological and statistical significance.

This series of experiments clearly demonstrate that IL-33 and ST2 play an important role in inflammation. Figure 6.1 summarises some of the known and hypothetical interactions that could be involved in the IL-33/ST2 axis, and the possible outcomes.

6.2 Unanswered questions

A number of key questions arise from this work, which are discussed in more detail below:

1. Which cells express IL-33 and ST2 expressed *in vivo*
2. Is IL-33 a cytokine, a nuclear factor, or both?
3. How do IL-33 and ST2 interact?
4. How can ST2L both enhance and suppress inflammation?
5. What role do IL-33 and ST2 play in Th2 cell function?
6. What role might IL-33 and ST2 play in inflammatory diseases?

6.2.1. Which cells express IL-33 and ST2 *in vivo*?

In vitro studies and cDNA libraries have shown IL-33 and sST2 are expressed in epithelial and dendritic cells, and also in activated fibroblasts and macrophages, with sST2 also released from Th2 cells. *In vitro* studies and animal models have shown ST2L is expressed on mast cells and some macrophages, and is inducible on Th2 and other type 2 lymphoid cells. However, very little is known about which cells express IL-33, ST2L and sST2 at the protein level in humans. Identifying the cellular source of IL-33 and sST2 and the target cells expressing ST2L will be of paramount importance in characterising the *in vivo* roles of each. A number of techniques could be employed to do this. Surgically removed tissues could be examined for mRNA expression by real-time quantitative PCR or in-situ hybridisation, and protein expression analysed using immunohistochemistry, and in particular laser scanning cytometry, to label IL-33 and ST2L/sST2 in tissues using monoclonal antibodies. Expression in specific cell types could be analysed by digesting tissues, co-staining with antibodies to cell-specific markers, and sorting cells by flow cytometry into purified populations for further analysis and functional studies .

I have not directly examined ST2L expression and response to IL-33 at the cellular level, but the data in SCID mice suggest that lymphoid cells, possibly NKT cells, are the key cell in the response triggered by IL-33. However, even though SCID mice lack lymphoid cells, there remains a population of non-lymphoid cells, probably mast cells and/or macrophages, that can mount a response after IL-33

stimulation. The precise contribution of each remains to be confirmed by repeating these experiments in mice specifically lacking NKT cells (eg $J\alpha18^{-/-}$ mice) or mast cells (eg Kit-W-sh mice). In the context of adaptive inflammation, IL-33 may play a role in the effector response, also through mast cells and macrophages, but it may also be able to directly activate pre-sensitised Th2 cells in the absence of further antigen, and enhance antigen-specific activation.

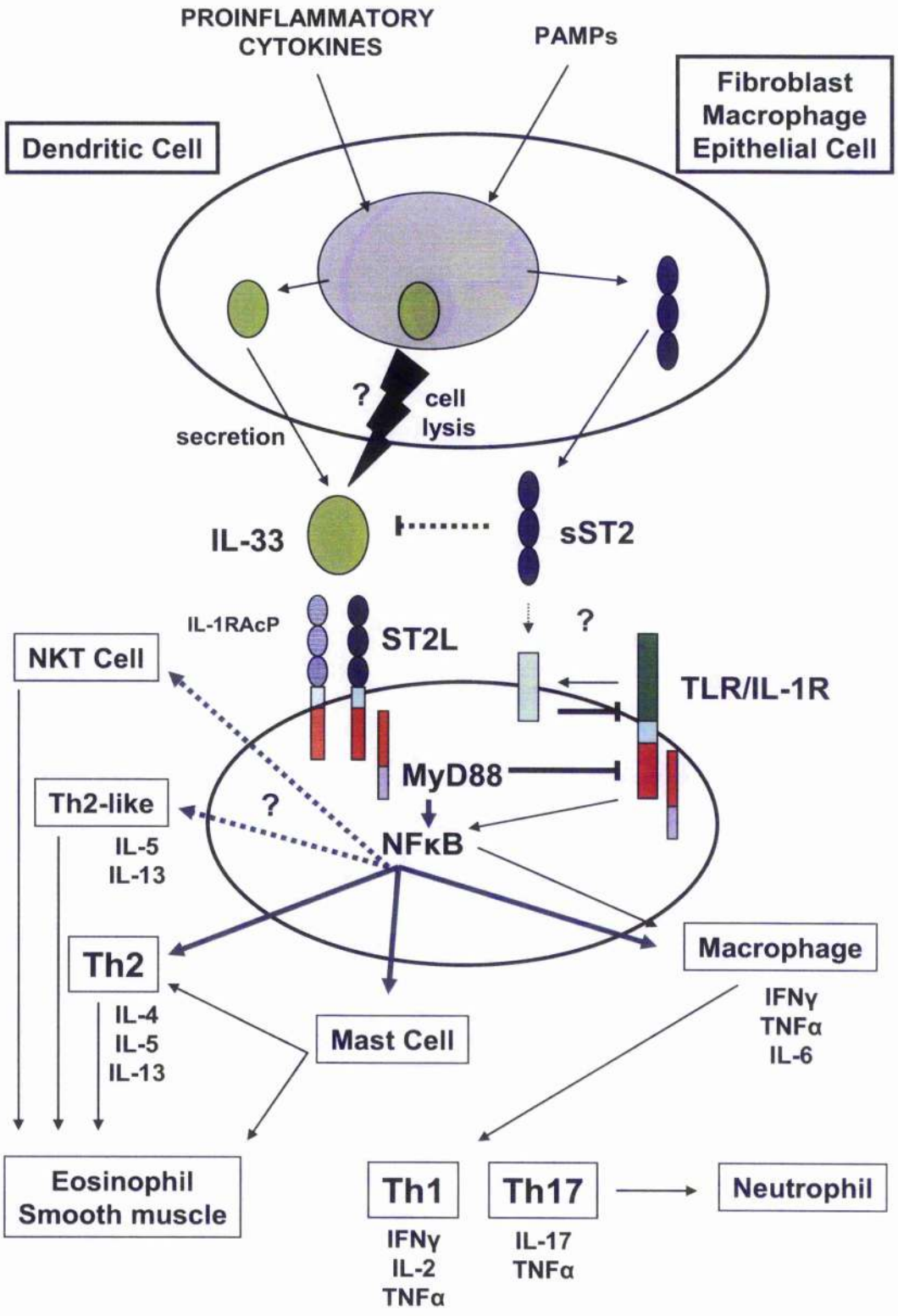


Figure 6.1 The possible role of IL-33 and ST2 in inflammation

This diagram illustrates some of the possible sources and downstream effects of IL-33 and ST2 signalling. IL-33 mRNA is expressed in many cells types, and is upregulated by pro-inflammatory

cytokines and PAMPs. The protein has thus far only been identified in the nucleus of endothelial cells, so in order to act as a cytokine it may be released from the cell when it is damaged, or it may be actively secreted by an as yet unidentified mechanism. ST2L and IL-1RAcP comprise the cell surface receptor for IL-33, which activates NF-κB through a MyD88 dependent mechanism. ST2L can also sequester MyD88 to downregulate TLR and IL-1R signalling. Soluble (s)ST2 is also released from many cells following activation by cytokines or PAMPs, and has been shown to bind to IL-33 and reduce the subsequent inflammatory response by preventing its interaction with ST2L/IL-1RAcP. sST2 can also bind to an unknown factor on the cell surface which is induced by TLR signalling, and can then downregulate TLR signalling. The overall outcome of IL-33 signalling or ST2L/sST2 modulation of TLR signalling is a shift towards Th2 type responses. The exact downstream effects depend on which cell the IL-33/ST2 axis is acting. IL-33 may act on Th2 cells to potentiate differentiation and increased Th2 cytokine release. In addition, it may be that IL-33 can induce the differentiation of Th2-like cells that preferentially produce IL-5 and IL-13. Il-33 may activate mast cells to release cytokines, and there may also be a role in activating NKT cells to produce Th2 cytokines. The effector cytokines released (IL-4, IL-5 and IL-13) can then have pro-allergic effects. Thus IL-33 may play a role in modulating immune responses by tipping the balance in favour of Th2 responses. ST2L/sST2 may also play a direct role in innate, Th1 and Th17 responses in the absence of IL-33 by modulating the activation of macrophages and other cells through TLRs and IL-1R.

6.2.2. IL-33: cytokine, nuclear factor or both?

One of the features of the members of the IL-1 family is that they do not have a leading sequence. Therefore, most proteins of the family are located in the cytosol or nucleus. However, the proteins can be processed via caspase I digestion and released through a non-classical pathway upon inflammatory stimulation (257). IL-33 has many structural and functional elements in common with IL-1α, which is usually present in the nucleus and acts as a nuclear factor, but can act as a cytokine by binding to IL-1R when released from the cell. This can occur if cells are damaged or lysed, and also by presentation at the cell surface or secretion. Due to the lack of an effective antibody and ELISA, the expression and location of IL-33 at protein level has not yet determined. IL-33 protein expression has currently only been seen in the nucleus of HEVECs from human lymphoid and chronically inflamed tissue (255), where it may have a role in transcriptional regulation, although its precise function is unknown. In contrast, although IL-33 has not yet been directly identified extracellularly *in vivo*, functional data in this

thesis and from Schmitz et al (1) clearly define a role for IL-33 as a cytokine promoting type 2 inflammation. Under resting conditions IL-33 may function as a nuclear factor, but at epithelial surfaces may act as a "danger signal" released either after cell damage or stimulation by pro-inflammatory signals. Cells expressing ST2L (macrophages and mast cells) would then be ideally placed to initiate an immediate innate response. The pro-inflammatory cytokines and chemokines released could then attract other immune cells and induce further expression of ST2L, and IL-33 may subsequently play a role in Th2 differentiation and effector function.

The downstream effects of IL-33 in these experiments involve the induction of pro-inflammatory cytokines and chemokines, and the influx of immune cells. Indirect effects as a result of initiating inflammatory cascades may explain how IL-33 can modulate Th2 and other types of inflammation. Many of these effects of IL-33 are also shared by Th2 cytokines, and this redundancy makes it difficult to ascertain which are the most important factors *in vivo*. Investigation of the functions of IL-33 and ST2 in gene knockout mice, particularly IL-4$^{-/-}$, IL-5$^{-/-}$ and IL-13$^{-/-}$, and in cell deficient mice, particularly mast cells and NKT cells, will be important in identifying which cells and mediators are vital at which stage. It is possible that some of the effects of Th2 cytokines are mediated through IL-33 expression.

6.2.3. Interactions between IL-33 and ST2

Like the most members of the family (236), IL-33's function could be regulated by its own soluble receptor, and it is interesting to note that sST2 has been upregulated in several inflammatory conditions, including sepsis (334), tissue ischaemia (336,338), Th2 diseases (340,341) and autoimmunity (344). IL-33 and sST2 are often expressed by the same cell types after the same stimuli. Although it has not yet been shown, sST2 is likely to bind to IL-33 and act as a soluble decoy receptor. Thus, as with IL-1 and IL-1Ra (220), the activity of IL-33 may be regulated by release of IL-33 or sST2 in different amounts or at different times, even by the same cell. IL-33 binds to ST2L *in vitro* (1), and I have shown here that both the innate and adaptive *in vivo* functions of exogenous IL-33 are dependent on ST2 expression. As ST2$^{-/-}$ mice lack both ST2L and sST2, I cannot exclude a role for sST2 in these explaining these data, as it has been shown to bind to an unknown surface ligand, and have downstream effects on TLR and cytokine expression (284,301,303,308). However, this also raises the possibility that, like

IL-1α, IL-33 may be associated with the cell surface and bind to ST2L through cell-cell contact, and sST2 could also interfere in this interaction.

6.2.4. ST2L has pro- and anti-inflammatory effects

One of the paradoxes of ST2L function has been that on mast cells and Th2 cells it is involved in activation, whereas in macrophages it is suppressive of TLR function. The molecular mechanism involved in these disparate effects of ST2L is currently unknown. It may be explained through the common use of MyD88 in signalling. When IL-33 binds to ST2L it can then signal through MyD88 to have pro-inflammatory actions via NFκB (1). However, the suppressive function on TLRs and IL-1R is mediated by ST2L sequestering MyD88 and preventing signalling (306). Thus the role of ST2L in the resting state, in the absence of IL-33, may be to limit TLR activation. ST2L expression is increased by TLR and pro-inflammatory cytokine signals, and could represent a mechanism to regulate inflammation. Interestingly TLR signalling generally favours Th1 type responses, whereas previous studies and the data here demonstrates that IL-33/ST2L generally results in Th2 type inflammation, so crosstalk between Th1 and Th2 driving signals can occur in the same cell. This field requires further investigation.

6.2.5. IL-33 and ST2 in Th2 cell function

6.2.5.1. T cell activation

ST2L is established as a marker for some Th2 cells (291,292,328), but its function in Th2 responses is unknown. It has been noted that ST2L $CD4^+$ cells are most closely related to the site of infection or inflammation (141,291,292,314,317,318), and are less frequent at distant sites such as lymph nodes, perhaps suggesting they are important effector cells. In Chapter 4 I demonstrated that IL-33 induces allergic airway inflammation in pre-sensitised mice without further antigen stimulation, so ST2L expressing Th2 cells may be directly activated through ST2L. However, as has recently been shown in asthma, it cannot be excluded that at least some of these cells are NKT cells, which have also been shown to express ST2L. One way of distinguishing the relative roles of NKT and Th2 cells would be to give IL-33 to mice which lack lymphocytes (SCID mice) and have been selectively reconstituted with NKT and/or Th2 cells from OVA sensitised mice.

A recent report (329) showed that *in vitro* polarised Th2 cells express more IL-5 than wildtype Th2 cells, suggesting ST2L plays a suppressive role in Th2 cells.

Apparently in keeping with this $ST2^{-/-}$ mice with allergic airways disease had greater numbers of eosinophils in the BAL, despite normal DLN T cell responses *in vivo*. This data is in contrast to the observation that ST2L expression is associated with increased IL-5 expression both *in vitro* and *in vivo* (291,314,325,328), and with my data which shows $ST2^{-/-}$ mice have fewer eosinophils in BAL, mediated by lower IL-5 and chemokine levels. The reason for the discrepancy is unknown. As we were both using the same strain of $ST2^{-/-}$ mice, with the same ST2 gene disruption, the difference may lie in the model or the technique for counting eosinophils.

6.2.5.2. T cell differentiation

IL-33 may be ideally placed to be an important factor in influencing Th2 cell differentiation. It is particularly expressed in tissue cells at or near epithelial surfaces, where Th2 responses are important in parasite responses. It may be released early in the response as a danger signal which alerts the immune system to mount a Th2 response. It is also expressed in dendritic cells, so could also directly influence T cell differentiation at the time of antigen presentation. Naïve T cells express very little ST2L, but as the Th2 phenotype becomes more established ST2L expression increases. Also, as ST2 deficiency has no effect on the IL-4-dependent Th2 cell differentiation (325-327), IL-33 is perhaps more important in maintaining the Th2 cell phenotype rather than their development.

Another intriguing possibility is that IL-33/ST2L could influence T cells to differentiate along a non-classical Th2 pathway. Effective Th2 type responses are usually generated via an IL-4-dependent pathway (394), although they can occur in the absence of IL-4 (46,395) by undefined mechanisms. Subsequently GM-CSF was found to generate Th2 responses in the airway in the absence of IL-4 (48). In the data presented here IL-33 preferentially induced IL-5 and IL-13 and was unable to directly induce IL-4 expression, even in pre-sensitised mice. The existence of Th2-like cells, expressing IL-5 and IL-13, and not IL-4, has been shown in various models of parasite infection and allergy (45-47,145,318,395), and it may be that ST2L is a marker for these cells, and IL-33 a factor in their development. Thus IL-33 may be a differentiating factor for a specific subset of Th2 cells. It remains to be seen whether these cells represent a novel subset with unique effects.

6.2.6. What role might IL-33 and ST2 play in inflammatory disease?

6.2.6.1. Asthma

The current results suggest that IL-33 plays a critical role in asthma, since all the key cells involved in asthma pathogenesis express ST2L. Therefore, the increased inflammatory response in allergic airway inflammation induced by IL-33 is likely to be due to multiple mechanisms. As discussed above, IL-33 may play a role in directly and indirectly activating Th2 cells and effector cells in the tissues or DLNs. However, $ST2^{-/-}$ had little effect on DLN T cell recall responses, suggesting ST2L, and therefore IL-33, plays a more important role in the local response. Whether this represents ST2L on Th2 cells or effector cells is unknown. Although the role of IL-33 in T cell differentiation was not studied, it is possible that the increased inflammation is due to increased presence of a subset of Th2-like cells expressing increased IL-5 and IL-13.

Eosinophilia plays an important role in asthma pathogenesis. I found that the main effect of IL-33 is inducing eosinophilia by at least two mechanisms. It may have a role in the generation of eosinophils via enhancing IL-5 production. In support of this, $ST2^{-/-}$ mice produced less IL-5 and developed a reduced eosinophilia in allergic airway inflammation. IL-33 may also promote the recruitment of eosinophils by inducing chemokine expression. Mast cells are another key cell in asthma which release pro-inflammatory cytokines and mediators. Importantly, ST2L is highly expressed by most mast cells, our preliminary data suggest that IL-33 is capable of inducing inflammatory cytokine secretion and degranulation of mast cells (unpublished result). Thus, IL-33 is a novel pathogenic factor and should be a new therapeutic target.

6.2.6.2. Rheumatoid arthritis

IL-33 exacerbated footpad disease in CIA, and $ST2^{-/-}$ reduced it. Similar to allergic airways disease, T cell recall responses were normal in $ST2^{-/-}$ mice, suggesting that loss of ST2, and therefore IL-33, is more important in the tissues. CIA is thought to be a Th1 and/or Th17 mediated disease, so it seems unlikely this reflects a loss of ST2 from Th2 cells. Therefore innate effector cells are probably the more important target for IL-33 in rheumatoid arthritis. The pro-inflammatory effect of IL-33 in CIA is likely to be mediated indirectly through macrophages, and perhaps even mast cells, which have been shown to play an important role in initiating joint pathology (225).

6.3 Conclusions

In conclusion, I have presented data in this thesis that suggests IL-33 and ST2 play crucial roles in initiating and regulating innate, allergic and autoimmune inflammation, and is therefore a valid target for further research into the mechanisms by which it can diverse modes of inflammation. It also identifies it as a potential therapeutic target in a wide range of human diseases.

6.3.1. Future Work

IL-33 biology

Initial investigations into the biology of IL-33 in the lung were constrained by the rate at which rmIL-33 could be produced, purified and tested in the lab during the project. If more rmIL-33 and more time had been available it would have been possible to extend the studies of the effect of IL-33 in the airway. In particular a more detailed analysis of the effects of single and multiple applications over a range of doses in various compartments, such as BAL, lung parenchyma and serum, would allow elucidation of possible downstream mechanisms of action of IL-33. In order to determine how relevant these doses are it will be necessary to detect physiological levels of human IL-33 *in vivo* in tissues such as BAL, lung and serum.

Lung biology

In this work I have made a detailed analysis of airway (BAL), systemic (serum) and lymph node responses involved in lung inflammation. However, had the timescale of this project allowed I would like to have extended the analysis of the parenchymal compartment, thus linking the systemic and airway responses. This could include lung digests to examine the levels of cytokines and chemokines within the lung itself, and levels of eosinophil cationic protein (ECP) would also give an indirect measure of the degree of eosinophilic inflammation. Flow cytometry would allow a more accurate direct quantification of the cells comprising the inflammatory infiltrate. It would also be useful to further characterise the pathology by using histological stains specific for goblet cells and mast cells, as these were difficult to confidently identify just using the H&E stain. In addition, indicators of mast cell function, such as tryptase levels in tissues, could be measured. Using laser scanning cytometry would also have allowed identification of specific lymphocyte subsets (T cells, NKT cells) in the lung.

In the studies presented in this thesis I have focussed on the inflammatory response in the lung. However, it is also relevant to study the changes in airway physiology that may also occur in the presence of allergic type inflammation. I have performed preliminary studies measuring Penh, an indirect measure of changes in airway resistance (and thus AHR) in $ST2^{+/+}$ and $ST2^{-/-}$ mice. These studies were performed in a different model of airway inflammation carried out over a longer period of time, and so the results may not be applicable here. However, they do demonstrate that $ST2^{-/-}$ mice develop the same level of AHR as $ST2^{+/+}$ mice, despite having reduced eosinophilic inflammation (Kewin P et al, unpublished data). This dissociation of inflammation from AHR is in keeping with previous data that showed anti-IL-5 can profoundly reduce eosinophils numbers, but has no effect on AHR (95).

Joint biology

The data presented in Chapter 5 largely represents pilot data, partly because there were many difficulties encountered in the breeding programme, resulting in a long delay in mice being available. Had time allowed I would have liked to have repeated these studies with larger groups of mice to confirm the results seen. This would also allow a more detailed analysis of the response by looking at different time points, and also by giving IL-33 at different times to see when it is most effective. It would also be important to analyse the pathology in the joints in detail to see which cells and mediators are involved in the inflammatory lesion.

Priority areas for further work

I have already alluded to possible avenues of further investigation, but outlined below are some specific areas of research that are current priorites for furthering our understanding of the biology of IL-33 and ST2:

- Development of further reagents to enable analysis of IL-33, such as anti-IL-33 antibodies for neutralisation or detection, IL-33 transgenic and knockout mice, and specific sST2 and ST2L knockout mice
- Identification of the *in vivo* source of IL-33 as a cytokine, and its mechanism of secretion from the cell
- Identification of the cellular target of sST2 and the mechanism of its downstream effects

- Elaboration of the signalling mechanisms used by ST2L and how they interact with and influence other IL-1R family member and TLR function
- Investigation of the role of IL-33 in T cell differentiation by *in vitro* studies of the factors required to differentiate IL-5 and IL-13 producing Th2 like cells
- Confirmation of these cells as a distinct entity by adoptive transfer into immunodeficient mice and demonstrating they can initiate immune responses.

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Appendix 1: Buffers and solutions

All buffers stored at room temperature unless otherwise stated.

2% Agarose gel:
2g agarose
100ml TAE buffer
Heat in microwave until clear and boiling
Add 5µl ethidium bromide (0.005%) and mix gently
Pour into gel tray with comb and allow to cool

Avertin:
3g 1,1,1 tribromoethanol
3ml amyl alcohol
Store at 4˚C
Dilute 1:40 in PBS before use

Complete medium:
500ml RPMI medium
50ml heat-inactivated foetal calf serum
5ml penicillin/streptomycin
5ml L-glutamine
0.5ml 2-mercaptoethanol

+ 25mM HEPES for cultures from CIA experiments

ELISA assay buffer A:
10% heat-inactivated foetal calf in PBS
pH 7.4
Made fresh and stored at 4˚C

ELISA assay buffer B:
1% bovine serum albumin in PBS
pH 7.4
Made fresh and stored at 4˚C

ELISA coating buffer A:
500ml distilled water
4.2g $NaHCO_3$
pH to 8.4

ELISA coating buffer B:
500ml distilled water
4.2g $NaHCO_3$
1.78g Na_2CO_3
pH 9.5
Stored at 4˚C for up to 1 month

ELISA blocking buffer:
1% bovine serum albumin in PBS
5% sucrose
0.05% NaN_3
made up in PBS

70% Ethanol:
7 parts 100% ethanol
3 parts distilled water

FACS buffer:
2% foetal calf serum in PBS
Store at 4˚C

PCR loading buffer:

PBS x10:

PCR reaction buffer

	Per sample
10x PCR buffer	2µl
$MgCl_2$ (50mM)	1µl
dNTPs (10mM)	0.5µl
Taq DNA polymerase	0.5µl
Pure water	13µl
Primer 1 (10OD)	0.5µl
Primer 2 (10OD)	0.5µl
Primer 3 (10OD)	1µl

TAE buffer:

Tail lysis buffer:

10mM Tris pH 8.0
50mM EDTA
100mM NaCl
0.5% SDS

+ 500µg/ml proteinase K added at the time of use

^{3}H-Thymidine:

37 MBq ^{3}H-Thymidine (in 1ml sterile saline)
Dilute to 25ml with sterile wash medium
Store sterile at 4˚C in an approved area

1% Trypan blue stock:

0.2g trypan blue crystals
20ml distilled water
Dilute 1:10 and filter through Nitex before use

Wash medium:

500ml RPMI Medium
5ml penicillin/streptomycin
Store at 4˚C

Appendix 2: Reagents

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

Table A1 Primers used for ST2 PCR

All primers were obtained from Sigma, diluted to 10OD with pure water, and stored at -20°C until use

Primer	*5'.....3'*
Human	CGGATCCATCACAGGAATTTCACCTAT
	GAGATCTCTAAGTTTCAGAGAGCTTAA
Mouse	CGGATCCACTTCACTTTTAACACAGTC
	GAGATCTTTAGATTTTCGAGAGCTTA

Table A2 Primers used for IL-33 RT-PCR

Reagent	Supplier	Use
All inorganic salts	Sigma-Aldrich	Buffers and solutions
All antibodies and standards	Becton-Dickinson (BD) R&D Systems	ELISA
Agarose		DNA detection
2% Alhydrogel (alum)	Brentag Biosector	Mouse sensitisation
Amyl alcohol	Sigma-Aldrich	Mouse anaesthesia
Bovine collagen type II	Sigma-Aldrich	Cell culture
Bovine collagen type II + Complete Freund's Adjuvant	Chondrex	Induction of CIA
Bovine serum albumin	Sigma-Aldrich	ELISA
DPX	Sigma-Aldrich	Coverslip fixation
EDTA	Sigma-Aldrich	Tail lysis buffer
Ethidium bromide	Sigma-Aldrich	DNA detection
Ethanol (100% molecular biology grade)	Sigma-Aldrich	DNA extraction
Extravidin-peroxidase	Sigma-Aldrich	ELISA
Foetal calf serum (FCS)	Harlan	Cell culture, ELISA
L-glutamine	Invitrogen	Cell culture
Goat serum	Invitrogen	ELISA
^{3}H-thymidine	Amersham Life Sciences	Proliferation assay
2-Mercaptoethanol	Sigma-Aldrich	T cell culture
dNTPs (10mM)		PCR reaction mix
Ovalbumin (OVA; fraction V)	Sigma-Aldrich	Sensitisation and challenge in allergic airways disease Cell culture
PCR buffer (10x)		PCR reaction mix
Phosphate buffered saline (PBS)	Invitrogen	Cell culture
Penicillin	Invitrogen	Cell culture
Phenol/chloroform/isoamyl alcohol (25:24:1)	Sigma-Aldrich	DNA extraction
Proteinase K	Sigma-Aldrich	Tail lysis buffer
Pure water for injection		DNA extraction
Rapi Diff II		Cytoprep staining
Red cell lysis buffer	Sigma	Cell culture
RPMI medium	Invitrogen	Cell culture
SDS		Tail lysis buffer
Streptavidin-peroxidase		ELISA
Streptomycin	Invitrogen	Cell culture
Sucrose	Sigma-Aldrich	ELISA
Taq DNA polymerase		PCR reaction mix
TMB	Insight (KPL)	ELISA
1,1,1-tribromoethanol	Sigma-Aldrich	Mouse anaesthesia
Tris		Tail lysis buffer
Trypan blue		Cell counting
Tween-20	Sigma-Aldrich	ELISA washing

Table A3 Reagents and suppliers