The Role of IL-33 and ST2 in Allergic Airways Disease

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ABSTRACT

Asthma is a chronic disease characterised by variable airflow obstruction, bronchial hyperresponsiveness and airways inflammation. At an immunological level Th2 inflammation and the presence of activated eosinophils and mast cells are key features of asthma. ST2, the receptor for the novel cytokine IL-33, is expressed upon Th2 lymphocytes and mast cells but its role in clinical and experimental asthma remains unclear. IL-33 has been shown to induce local and systemic eosinophilia when administered to the peritoneum of mice. In this thesis I have set out to test the hypothesis that the activation of mast cells by IL-33 acting on cell surface ST2 plays a critical role in allergic airways inflammation.

I began by studying the function of ST2 on mast cells *in vitro*. I found that ST2 was expressed at an early stage of development, and correlated closely with the expression of the stem cell factor receptor (c-kit), a marker present on mast cells from a progenitor stage. Despite this mast cells generated form ST2 gene deleted mice proliferated and matured normally. When mast cells were activated by IL-33, acting in an ST2-dependent manner, pro-inflammatory cytokines and chemokines were released that have potential roles in asthma, specifically IL-6, IL-13, MIP-1α and MCP-1.

To extend these findings I looked at the role of ST2 in allergic airways inflammation. I first optimised and validated an ovalbumin and adjuvant based 'short' twelve day model of murine asthma and demonstrated that ST2 gene deletion results in attenuated eosinophilic inflammation. In addition to being ST2 dependent it is possible that this adjuvant based short model is mast cell dependent, unlike longer adjuvant based models which are mast cell and ST2 independent. Therefore I went on to study an adjuvant-free model of asthma which has been demonstrated to be mast cell dependent. In this adjuvant-free model of asthma the airway inflammation was attenuated in ST2 gene deficient mice compared with wild type mice, while AHR was unaffected. There was an associated reduction in IgE production and thoracic lymph node recall Th2 cytokine responses.

I then examined the effect of ST2 activation in the lungs. When IL-33 was administered directly to the airways of naïve mice it induced the features of experimental asthma. There was extensive eosinophilic inflammation within the lung tissue and airspaces. The Th2 cytokines IL-5 and IL-13, and the eosinophil chemoattractant chemokines eotaxin-1 and eotaxin-2 were detected at increased concentrations. Significant airways hyperresponsiveness was also generated. Using ST2 gene deleted mice I demonstrated that these effects were ST2 specific. Although I have shown that mast cells are activated by IL-33 *in vitro*, I used mast cell deficient mice to demonstrate that the eosinophilic inflammation generated by IL-33 is unaffected by the absence of mast cells.

These data show that IL-33 can induce in the lungs the cardinal pathological characteristics of asthma, and that it appears to act upstream of other important mediators such as IL-13 and the eotaxins. Furthermore the IL-33 receptor ST2 is required in an adjuvant free model of asthma, which is more akin to human disease. Placing these findings in the context of recent evidence that IL-33 is released by structural cells in response to damage or injury suggests that IL-33 may play a key role in initiating the immunological features of clinical asthma. As a consequence of this position in the hierarchy of inflammation IL-33 offers a promising direct target for novel biological therapies in asthma.

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DECLARATION

The work presented in this thesis represents original work carried out by the author. Some of the work in this thesis was performed equally and in collaboration with Dr Peter Kewin (4.3.2 to 4.5.1 inclusive) and Dr Grace Murphy (5.2.1 to 5.3.3 inclusive). This thesis has not been submitted in any form to any other University. Where reagents, materials or practical support has been provided by others, due acknowledgement has been made in the text.

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ABBREVIATIONS

-/-	gene deleted mouse
7-AAD	7-amino-actinomycin D
AHR	airways hyperresponsiveness
alum	aluminium hydroxide
ANP	atrial natriuretic peptide
AP-1	activator protein-1
APC	allophycocyanin
BAL	bronchoalveolar lavage
BMMC	bone marrow derived mast cell
BNP	brain natriuretic peptide
Bq	becquerel
BSA	bovine serum albumin
CBMCs	cord blood derived mast cell
CD	clusters of differentiation
(c)DNA	(complementary) deoxyribonucleic acid
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CSF	cerebrospinal fluid
CXCL	CXC chemokine ligand
DAMP	damage-associated molecular patterns
DC	dendritic cell
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
ELISA	enzyme linked immunosorbant assay
EPO	eosinophil peroxidase
FACS	fluorescence-activated cell sorting
Fc	fragment crystallizable [region]
FCS	foetal calf serum
FEV_1	forced expiratory volume in 1 second
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDM	house dust mite
HEV	high endothelial venule

HMGB1	high mobility group box 1
HRP	horseradish peroxidase
HTH	helix-turn-helix
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IN	intranasal
iNKT	invariant natural killer T [cell]
IP	intraperitoneal
IP-10	interferon-gamma-induced protein (CXCL10)
IRAK	IL1 receptor-associated kinase
IT	intratracheal
IV	intravenous
JNK	c-Jun N-terminal kinase
KC	keratinocyte chemoattractant (CXCL1)
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MBP	major basic protein
MCP-1	monocyte chemotactic protein-1 (CCL2)
MI	myocardial infarction
MIG	monokine induced by gamma interferon (CXCL9)
m/hIL-33	murine/human recombinant IL-33
MIP-1	macrophage inflammatory protein-1 (CCL3)
(m)RNA	(messenger) ribonucleic acid
MyD88	myeloid differentiation primary response gene (88)
NAG	*N*-acetyl-ß-d-glucosamine
NBF	neutral buffered formalin
NF-HEV	nuclear factor from high endothelial
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
Ni-NTA	nickel-nitrilotriacetic acid
NK	natural killer [cell]
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
OVA	ovalbumin
$PaCO_2$	partial pressure (arterial) of carbon dioxide
PAMP	pathogen-associated molecular pattern

PBS	phosphate buffered saline
PE	phycoerythrin
Penh	enhanced pause
PMA	phorbol 12-myristate 13-acetate
RAG	recombinase-activating gene
RAGE	receptor for advanced glycation end products
RANTES	regulated upon activation normal T cell expressed and secreted (CCL5)
RPE	R-phycoerythrin
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
SCF	stem cell factor
SCID	severe combined immunodeficiency
SEM	standard error of the mean
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism
sST2	soluble ST2
ST2L	membrane bound 'long' ST2
STAT	signal transducers and activator of transcription
TARC	thymus- and activation-regulated chemokine (CCL17)
TCA3	T-cell activation-3 (CCL1)
TcR	T cell receptor
TGFβ	transforming growth factor beta
Th	T helper [cell]
TLR	toll-like receptor
TMB	tetramethylbenzidine
TNFα	tumor necrosis factor alpha
Tr1	T regulatory cell type 1
TReg	regulatory T cell
VEGF	vascular endothelial growth factor
WT	wild type
w/v	weight/volume

Chapter 1

Introduction

1.1 Asthma

1.1.1 Clinical asthma

Asthma is a cause of significant morbidity and mortality and is estimated to affect 300 million people worldwide, with increasing prevalence(1). Asthma is defined clinically by the presence of variable airflow obstruction and symptoms, namely wheeze, breathlessness, chest tightness or cough. Physiologically airways hyperresponsiveness (AHR) and airway inflammation are cardinal features(2,3). The nature of the immune response in asthma will be discussed in more detail later, but is generally characterised by eosinophilic inflammation. The mainstay of treatment is with bronchodilators plus inhaled, and in more refractory asthma, oral corticosteroids.

While some patients have chronic stable disease of varying severity, exacerbations can also be a feature. Clinically exacerbations present as an acute worsening of symptoms and airflow obstruction for a period of hours or days and represent the commonest reason for hospital admission in asthmatic patients. This airflow obstruction is a result of smooth muscle contraction, airway oedema and mucus plugging(4). While exacerbations can be triggered by a number of factors including allergens, irritants and stress, the commonest cause is viral infection(5). Despite this association, studies have shown that in those with eosinophilic inflammation targeting asthma treatment based on the extent of eosinophilia rather than symptoms reduces exacerbation rates(6).

Asthma can be associated with structural airway changes including smooth muscle hypertrophy and thickening of the epithelial basement membrane(7) and some patients with asthma develop gradually worsening lung function over time(8,9). While it is assumed that the latter is a consequence of the former this is not yet conclusively proven, as disease severity does not always correlate with measures of remodelling(10-14).

Although defined clinically by the presence of variable airflow obstruction and appropriate symptoms, asthma is a heterogeneous disease and there is increasing interest in characterising different phenotypes. There is a wide range of overlapping descriptions, many of which are based on the clinical course and

response to treatment. They include the most widely used severity classifications(2,3) and other subgroups such as difficult asthma, severe asthma, refractory asthma, brittle asthma and steroid-resistant asthma(2,15,16). Another way of classifying asthma is according to pathophysiology, one of the oldest such distinctions being that of atopic (extrinsic) or non-atopic (intrinsic) asthma first described in the 19th century(17). Atopic asthma is characterised by positive skin testing or elevated serum IgE antibody for common allergens. The age of onset is usually earlier than in non-atopic asthma and there is often a positive family history as well as other co-existing atopic disease such as hay fever or eczema. It is thought that 70-90% of asthmatic patients have atopic asthma(18,19). While originally considered as two distinct entities the nature of the airway inflammation in both atopic and non-atopic asthma is almost identical and there is evidence of elevated total serum IgE or local IgE production in non-atopic asthma. This has led to the hypothesis that there may be no fundamental immunopathological distinction between the two subsets(18,19). At present there is considerable clinical interest in the distinction between eosinophilic and non-eosinophilic asthma, the later being characterised by a high proportion of neutrophils in the airways, histological differences and a relative resistance to steroid therapy(20).

1.1.2 Animal models of asthma

In parallel with human studies animal models provide an important tool in the study of mechanisms of asthma(21-23). It is necessary to recognise the limitations of such models and to use them for hypothesis-generating and testing with the clear intention of translational purposes.

Mice [*Mus musculus*] are the species used most frequently to model asthma for a number of reasons including the extensive knowledge of the murine immune system, the availability of reagents and genetically manipulated animals as well as the practical advantages of working with small mammals.

1.1.2.1 Features of animal models

It is generally accepted that the cardinal features required of a model of asthma are eosinophilic inflammation and AHR(21).

The simplest method of measuring eosinophilic inflammation is to quantify the number of eosinophils within the airway lumen, which can be sampled by performing a bronchoalveolar lavage (BAL). Although investigators have used flow cytometry to quantify eosinophils(24), using standard microscopy to identify and count Romanovsky stained cells remains the gold standard. It can also be useful to measure the changes that occur within the lung parenchyma. Most commonly histological lung sections are stained and examined by microscopy. This allows inflammation to be assessed and can demonstrate other abnormalities, such as early perivascular changes and one of the other major features of asthma, mucus productions and associated goblet cell hyperplasia. The study of lung sections remains subjective and can be misleading when the changes are not homogenously distributed throughout the lungs. Scoring systems reduce the subjectivity but can be very labour intensive(25). One alternative is to quantify the eosinophils within the lung tissue by performing digests and counting individual stained cells counted by microscopy(26), however this results in the loss of the other information available on histology and is therefore rarely undertaken.

More problematic than the assessment of inflammation is the measurement of AHR, which relies on assessing bronchoconstriction in response to a provoking agent. The currently accepted best parameter in humans is FEV_1 which is a measure of airflow during a forced expiratory manoeuvre. This is effort dependent and requires patient co-operation and the use of a tight fitting mouthpiece, neither of which are possible in mice. The classical solution, developed in 1988, has been to intubate anaesthetised mice via tracheostomy, artificially ventilate them and then measure pulmonary resistance (R_L) to airflow and dynamic compliance (C_{dyn}), a measure of lung stiffness(27). This method is accepted as the gold standard and gives very reproducible results, but requires considerable time and technical expertise(28). Alternatively lung function can be measured non-invasively using whole body plethysmography(29). Although there have been some reservations regarding this technique(28,30) it remains widely used by those working with asthma models and correlates well with the more technically demanding invasive measurements(29,31-34).

Airway remodelling is a feature of asthma models that becomes evident with increasing duration of antigen challenge. In models of chronic asthma, using

more sustained challenge protocols, it is possible to induce features of airway remodelling, specifically extra-cellular matrix deposition and an increase in airway smooth muscle mass(35-38).

1.1.2.2 Protocols

The most common way of inducing experimental asthma is to employ a method of antigen sensitisation followed by local antigen challenge, although protocols which utilise cell transfer, gene manipulation or viral infection do exist.

The antigen used can range from complex microorganisms, such as *Aspergillus*(39) and *Schistosoma*(40), to simple proteins. However, due to the problems with consistency of dosing, handling and preparation of complex micro-organisms most investigators choose to use soluble purified proteins or extracts.

The majority of antigen based models use chicken egg ovalbumin (OVA), which is a useful experimental antigen available in a highly purified form, with a known epitope structure and available antibody and T cell receptor transgenic mice. Sensitising mice using OVA and alum adjuvant can induce IgE and Th2 responses and has been demonstrated to induce pulmonary eosinophilia and AHR upon subsequent airways challenge with OVA. While less well studied there is also interest in antigens with direct relevance to clinical asthma, such as cockroach antigen(41) and house dust mite extract(38,42). House dust mite antigens have specific properties which may be important in their pathophysiology, including intrinsic protease activity(43) and possibly an ability to modulate TLR signalling(44).

Even among the models that utilise OVA there is considerable variation in the exact protocol used, with differences in dose, timing, route of administration and use of adjuvants(21). While subcutaneous, transcutaneous, intrapulmonary and intravenous sensitisations have been used, generally an intraperitoneal (IP) administration is employed. This is felt to deliver a systemic dose of antigen in a convenient and reproducible manner. The most popular methods of challenge are via direct intratracheal or intranasal administration or via nebulisation. While there are likely to be differences in the actual dose delivered they will all result in the delivery of soluble antigen to the respiratory tract. There is no

evidence that any method is superior and consequently the choice often falls to investigator experience and preference.

Human vaccines generally employ adjuvants to boost the immune response to administered antigens, and the same technique is often used in a wide variety of animal models of allergic and autoimmune disease. Aluminium salts (alum) have been used in human vaccines since 1926(45), and remain by far the most widely used(46). Alum is also the most commonly used adjuvant in murine models of asthma, in part due to the fact that it is a strong inducer of Th2 responses(47,48).

The number of challenges employed and the duration of the model can also have an impact on the pathological changes seen. Models as short as 12 days(49) or models utilising only a single airways challenge(42) have been shown to induce IgE production, pulmonary eosinophilia and AHR. Models designed to demonstrate the features of airway remodelling tend to require multiple repeated challenges over a period of around two months(35-37).

While the various different models used all generate the required features of experimental asthma, these protocol differences can affect the apparent contribution of different cells, cytokines and pathways, as will become clear later in this introduction. It is generally accepted that the best protocols for achieving a robust Th2 dependent model in which pulmonary eosinophilia, antigen-specific IgE and AHR are generated are those using BALB/c mice sensitised with antigen in adjuvant followed by multiple airways challenges over a total period of around four weeks(21).

1.1.2.3 Genetic background

While the majority of investigators use BALB/c mice in the study of asthma others strains are also used, most frequently C57Bl/6 mice which are often the background on which gene knock-out and transgenic animals are generated. It is well established that while experimental asthma can be induced in BALB/c and C57Bl/6 mice there are differences between the strains. While the extent of pulmonary eosinophilia generated does not vary greatly, the magnitude of the IgE is response is reduced in C57Bl/6 mice(50-52). AHR also varies between different strains of mice, with airways responsiveness in naïve BALB/c mice

greater than in C57Bl/6 mice(50,53). There is a similar trend in models of asthma, with some protocols unable to generate AHR in C57Bl/6 mice(21,50,54). It has been suggested that these two strains may rely on different pathways for the generation of AHR, possibly due to functional differences in mast cells(55), including a deficiency in mast cell protease 7 in C57Bl/6 mice(56).

1.1.3 Cellular components of asthma

As early as 1864 Henry Salter recognised the importance of inflammation in asthma; “The inflammation or congestion of the mucous surface appears to be the stimulus that, through the nerves of the air tubes, excites the muscular wall to contract”, as well as ascribing a role to extrinsic “irritants”(17). Since then a great deal has been learnt about immunology and asthma, but the role of different inflammatory cells and mediators in the pathogenesis of asthma remains hotly debated.

1.1.3.1 Eosinophils and IL-5

The eosinophil was one of the first cells to be implicated in the pathophysiology of asthma. Early post-mortem reports identified eosinophils and Charcot-Leyden crystals, later discovered to be eosinophil granule crystals, in the airway lumen of asthmatic patients and in some cases elevated sputum or peripheral blood eosinophil counts were reported(57). A century later the eosinophil remains a focus of interest in the study of asthma, but just how central a role it plays is still unresolved.

The primary evidence implicating eosinophils in the pathogenesis of asthma is the association between increased eosinophil numbers and asthma. Eosinophils in peripheral blood are readily measured and the level of eosinophilia correlates with AHR in response to methacholine(58,59) and in some cases with measurements of airflow resistance(59,60). Sputum eosinophilia is also easily assessed and correlates with asthma severity to the extent that it can be used to direct treatment with corticosteroids(6,61). As initial fears over the safety of bronchoscopy in asthmatics were allayed(62) reports were published showing elevated BAL eosinophils in asthmatic patients(63,64) that correlated with AHR(65-67), lung function(67) and severity(68). Using bronchoscopy it was also possible to take airway wall biopsies for histological studies(69). These

demonstrated increased eosinophil numbers within the airways(70,71) which correlated with AHR in some asthmatics(72) and showed evidence of degranulation and a unique distribution in asthmatics, with intraepithelial eosinophils only found in asthma(68). Despite this close association between airway eosinophilia and asthma it is important to note that this finding is not unique to asthma. Most notably sputum eosinophilia also occurs in eosinophilic bronchitis, a condition which shares the symptom of cough with asthma but without bronchoconstriction or AHR(73).

Not only are eosinophils present in the airways of asthmatics, but they have the potential to contribute to the pathophysiology. Eosinophil granules contain the cytotoxic granule proteins major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN)(74). MBP is the most studied of the granule products in asthma and there is increased MBP in the sputum(75) and lungs(76) of asthmatics. Not only is MBP directly cytotoxic to respiratory epithelial cells(77), but it can also induce bronchoconstriction *in vitro*(78). Eosinophil granule products can stimulate mast cells and basophils to release histamine(79-82). Although when the role of MBP-1 was studied *in vivo* using MBP-1 gene knock-out mice there was no difference in eosinophilic inflammation or AHR(83). In addition to granule products eosinophils are also a potential source of Th2 cytokines(84-86) and may be able to drive Th2 responses by other means(87,88).

Despite the circumstantial evidence implicating eosinophils in the pathology of asthma the definitive proof is lacking. In an attempt to answer the question investigators have tried to study asthma in the absence of eosinophils. Since the discovery of IL-5 in 1985, initially named eosinophil differentiation factor(89), it has been closely linked with eosinophil function, driving differentiation, proliferation, activation and recruitment(90,91). Transgenic mice over-expressing IL-5 display a profound blood eosinophilia with evidence of accumulation in the lungs(92). When IL-5 over-expression is induced in the lungs there is marked eosinophilic inflammation with associated goblet cell hyperplasia and collagen deposition in addition to a degree of spontaneous AHR(93). Consequently IL-5 had been targeted in models of asthma, either by using antibodies against IL-5 or by selective gene deletion (IL-$5^{-/-}$). The results of these experiments are summarised in Table 1.1.

Mice	Model	Method	EØ	AHR	Ref
C57Bl/6	OVA + Alum	IL-5$^{-/-}$	↓	↓	(94)
C57Bl/6	*Aspergillus*	α-IL-5[1]	↓	NC	(95)
C57Bl/6	*Aspergillus*	IL-5$^{-/-}$	↓	NC	(95)
C57Bl/6	*S.mansoni*	α-IL-5	↓	NC	(96)
C57Bl/6	OVA + Alum	α-IL-5	↓	↓	(97)
C57Bl/6	OVA + Alum	IL-5$^{-/-}$	↓	↓[2]	(97)
BALB/c	OVA + Alum	α-IL-5	↓	NC	(54)
BALB/c	OVA + Alum	α-IL-5	↓	↓	(98)
BALB/c	airways OVA[3]	α-IL-5	↓	↓	(99)
BALB/c	OVA + Alum	α-IL-5[4]	↓	NC	(100)
BALB/c	OVA + Alum	IL-5$^{-/-}$	↓	NC	(100)
BALB/c	OVA + Alum	α-IL-5	↓	↓	(101)
BALB/c	OVA	α-IL-5	↓	NC	(102)
BALB/c	OVA + Alum	IL-5$^{-/-}$	↓	NC	(103)
B10.BR	OVA + Alum	α-IL-5	↓	↓	(104)

Table 1.1 - Studies on the requirement for IL-5 in experimental asthma

Key – EØ: effect on lung eosinophil numbers. AHR: effect on airways hyperresponsiveness. α-IL-5: anti-IL-5. ↓: reduced. NC: no change.

Notes - (1) Mice were also B cell deficient. (2) Eosinophil transfer restored AHR. (3) Protocol utilising repeated antigen challenge of naïve mice. (4) Mice were also IL-4-/-

While blocking the function of IL-5 by either method consistently resulted in a significant reduction in lung eosinophils, although notably often not complete loss, the effect on AHR was more variable. All models which utilised systemic sensitisation with OVA alone, or an alternative antigen failed to demonstrate any reduction in AHR. In C57Bl/6 mice blocking IL-5 function in models utilising sensitisation with both OVA and alum invariably resulted in a reduction in AHR. The use of OVA and alum models of asthma in BALB/c mice gave more variable results with AHR unchanged in some and completely abrogated in others, although when IL-5 was targeted in BALB/c mice by gene deletion rather than antibody, AHR was consistently preserved. In the few studies which assessed other changes in lung such as mucus production(97) and subepithelial fibrosis(102), these were shown to be attenuated by blocking IL-5.

In human asthma IL-5 was among the first cytokines to be specifically targeted. The value of this as an immuno-therapeutic target was due not only to the relationship between IL-5, eosinophils and asthma already discussed, but also the finding that IL-5 inhalation induced sputum eosinophilia and AHR in asthmatic patients(105).

In 2000 a randomised double-blind placebo controlled trial of anti-IL-5 involving 24 atopic asthmatics was published(106). Patients were given a single dose of anti-IL-5 antibody and were challenged with antigen one and four weeks later. Treatment resulted in a significant and sustained reduction in blood and sputum eosinophils but failed to demonstrate a difference in pre-challenge AHR or late asthmatic response, as measured by delayed bronchoconstriction in response to allergen challenge. On face value this suggests a dissociation between eosinophilia and AHR. However there have been criticisms of the study, including concerns over the interpretation of the late asthmatic response data and the small number of patients studied(107). Although a subsequent similarly powered study confirmed the reduction of blood eosinophils with no change in measurements of AHR and lung function, it raised questions by demonstrating that anti-IL-5 treatment only reduced eosinophil numbers in the airway wall and bone marrow by around 50%(108). In this study there was a reduction in deposition of extra-cellular matrix proteins and BAL TGFβ(109), both features associated with airways remodelling.

It was not until 2007 that a larger study was conducted in which 362 patients with moderate asthma received 3 monthly doses of anti-IL-5(110). Treatment failed to produce any clinically relevant improvement in measures of airflow obstruction or symptom scores but did show a trend towards a reduction in exacerbations. In light of this trend combined with an increasing understanding of the relationship between eosinophil levels and asthma control two further studies were carried out, the results of which have just been published(111,112). Both recruited subjects with very severe asthma, the majority of whom were on regular oral steroids, and both demonstrated a significant reduction in the primary end-point of exacerbations. Not only do these studies suggest a role for anti-IL-5 therapy in a select group of patients, but they also support a pathophysiological role for IL-5 and eosinophils in asthma exacerbations.

One of the caveats when drawing conclusions regarding the role of eosinophils from any study is the failure to completely eliminate eosinophils from the lung. As discussed above this has been a criticism of the interpretation of clinical studies of anti-IL-5 therapy, but is also a problem in the murine studies with a population of eosinophils persisting in all of them.

In an attempt to address the role of eosinophils more directly eosinophil deficient mice were generated by two different groups using different approaches. The first group genetically engineered C57Bl/6 mice with diphtheria toxin linked to the promoter for the eosinophil granule protein EPO (PHIL mice) which resulted in mice in which eosinophils were almost completely absent(113). When allergic airways inflammation was generated using OVA/Alum in these mice less than 0.5% of BAL cells were eosinophils and no eosinophils were seen in lung sections. Other histological changes, including goblet cell hyperplasia, were attenuated but not lost. AHR however was completely absent in PHIL mice.

The second group, in a paper published simultaneously, used the previously described(114) ΔdblGATA mice on a BALB/c background in OVA/alum driven acute and chronic models of asthma(115). These mice have a deletion in the high affinity GATA-binding site of the GATA-1 transcription factor which results in slightly impaired red cell production and a complete absence of circulating eosinophils, even if an IL-5 transgene is introduced(114). In both the acute and chronic models of asthma there was a complete absence of eosinophils in BAL and lung tissue with goblet cell hyperplasia unaffected(115). Using these ΔdblGATA mice for the chronic asthma model resulted in a reduction in both collagen deposition and airway smooth muscle hypertrophy. In contrast to the findings in PHIL mice, AHR was preserved in ΔdblGATA mice.

These two papers reached contradictory conclusions regarding the role of eosinophils in AHR. In order to reconcile this discrepancy it is necessary to look at the different mice used. As AHR was preserved in the mice that appeared to have the most complete ablation of eosinophils such a difference seems unlikely to be responsible. One possible explanation is the genetic background, the PHIL mice were on a C57Bl/6 background, which appears to be more dependent on eosinophils for AHR in IL-$5^{-/-}$ experiments as discussed above, whereas the ΔdblGATA mice were on a BALB/c background where the effect of eosinophil depletion on AHR is more variable. This is supported by a recent paper in which BALB/c and C57Bl/6 ΔdblGATA mice were compared(116). The BALB/c ΔdblGATA mice had preserved AHR and histological changes as previously reported, whereas the C57Bl/6 ΔdblGATA mice failed to develop AHR or show any histological changes.

Ultimately the role of eosinophils in asthma remains unclear. In mice it appears that, depending on the strain and protocol, AHR can be induced in an eosinophil dependent or independent manner. In humans it appears that eosinophils play a key role in exacerbations of asthma. In addition both murine and human data also suggest a role for eosinophils in airway remodelling.

1.1.3.2 B lymphocytes and IgE

The role of external agents in provoking asthma was identified as far back as the nineteenth century when "extrinsic" asthma was first described (17) and many years later associations were made between asthma and allergy or anaphylaxis(117). Extrinsic asthma came to be viewed as a form of Type I hypersensitivity reaction, which were later found to be mediated by a serum component (118) ultimately identified as a novel immunoglobulin isotype; IgE(119). Mast cells express the high affinity IgE receptor (FcεRI) and Type I hypersensitivity reactions are recognised as being mediated by the degranulation of mast cells due to the cross-linking of surface bound IgE by allergen(120). Following the association between IgE and hypersensitivity elevated levels of serum IgE were detected in some asthmatic patients(121), and atopic asthma is now defined as being associated with elevated serum IgE antibody to common inhaled allergens. Consequently questions have been asked regarding the potential role of IgE in the pathogenesis of asthma.

Initial animal studies showed that the administration of anti-IgE antibodies, which mimicked allergens by cross-linking surface bound IgE, resulted in AHR(122). Similarly the transfer of OVA-specific IgE followed by intranasal OVA challenge also resulted in AHR, in addition to a modest airways eosinophilia(123). When blocking anti-IgE antibodies were used in a house dust mite antigen model of asthma eosinophil accumulation was attenuated, although AHR was not measured(42). More recent studies using mice with a selective IgE deficiency have reached differing conclusions. In an OVA/Alum based model of asthma AHR and inflammation were preserved in IgE deficient mice(124), while in a model where naïve mice were repeatedly challenged with OVA alone IgE deficiency resulted in the loss of AHR and reduced inflammation(125). Taken together these observations fail to provide a clear picture regarding the role of IgE in experimental asthma.

In clinical asthma the use of the anti-IgE monoclonal antibody omalizumab has provided good evidence for the importance of IgE. Initial studies in asthmatics showed that as well as reducing serum IgE concentrations, omalizumab inhibited bronchoconstriction in response to allergen challenge(126,127). Subsequent clinical trials demonstrated that in patients with moderate to severe allergic asthma, treatment resulted in reduced exacerbations and symptoms and allowed inhaled steroid doses to be reduced(128-130). The greatest benefit appeared to be in patients with the most severe and poorly controlled asthma(131,132). Consequently omalizumab is now recommended for use in patients with uncontrolled atopic asthma both in the UK(133) and elsewhere.

While the need for B cells in asthma is generally considered to be as a source of IgE, their role could potentially include the production of other immunoglobulins, antigen presentation and cytokine production. By using B cell deficient mice it has been possible to study the contribution of these cells to asthma. As expected these mice consistently fail to generate any immunoglobulins, but no study has shown any difference in the development of typical inflammation and eosinophil accumulation within the lungs(104,134-136). As in IgE deficient mice, the requirement for B cells in the generation of AHR depends on the model used. AHR in response to OVA challenge was unaffected in B cell deficient mice immunised with OVA and alum(104,136). However, when allergic airways inflammation was generated by the repeated challenge of naïve mice with OVA, AHR was B cell dependent and restored by the transfer of OVA-sepcific IgE, although it is important to note that in this report AHR was measured by studying tracheal segments *ex vivo*(134).

1.1.3.3 Mast cells

While mast cells were first identified by Ehrlich in his doctoral thesis in 1878(137) their function remained unclear for many years, although they were described as sentinel cells because of their location in mucosal surfaces of the gut, lung and skin. Since histamine was already associated with anaphylaxis(138) and AHR(139), the mast cell began to attract attention in the study of allergy and asthma with the discovery in 1952 that mast cells could release histamine(140,141). By the early 1970s this had led to the hypothesis that asthma was a form of Type I hypersensitivity reaction, with inflammation a

result of allergen triggered release of mast cell mediators via surface IgE. While the importance of IgE in atopic asthma discussed earlier lends support to this theory, IgE can interact with a number of other cells, for example to mediate antigen presentation by dendritic cells and B cells, or to activate eosinophils(120). Therefore more direct evidence is required for a pathogenic role of mast cells in asthma.

Bronchoscopic lavage has consistently found elevated mast cell numbers from the airway lumen of asthmatic patients(66,70,142-144), with some evidence of correlation with airflow obstruction(64). Biopsy studies of mast cell numbers within the wall of the airways themselves have sometimes yielded conflicting data. Within the epithelium there are reports of increased(145-147) or unaltered(143,148) mast cells numbers, while within the submucosa mast cell numbers have been found to be unchanged(72,148,149) or reduced(150). Within the airway smooth muscle mast cell numbers are consistently elevated(151,152). The number of mast cells within the smooth muscle layer correlates with AHR in both asthmatic(151) and allergic non-asthmatic patients(153). Further evidence for the importance of mast cells in smooth muscle bundles in asthma is the comparison with eosinophilic bronchitis. In this disease there is eosinophilic inflammation and cough as in asthma but no AHR, and mast cells are not found within the smooth muscle bundles(151).

Support for mast cell activation comes from the findings there is histological evidence of mast cell degranulation in the airways of asthmatic patients(70,145,148,150,152) and that BAL histamine levels are elevated in symptomatic asthmatics(154).

These reports that mast cells, which are capable of releasing a wide array of pro-inflammatory mediators, are present in increased numbers within asthmatic airways and show evidence of degranulation suggests they may play a role in asthma. Using W/W^v or W-sh mast cell deficient mice investigators have tried to establish whether or not there is a requirement for mast cells in allergic airways inflammation.

While there had been a previous experiment showing no change in *Ascaris* induced pulmonary eosinophilia in W/W^v mice(155), the earliest report on the requirement for mast cells in experimental asthma was published in 1994. Wild

type and W/W^v mice were immunised with OVA and alum on day 0 before being challenged with nebulised OVA on days 14 to 21. There was no change in histological inflammation but a small, non-significant reduction in BAL eosinophils(156). Three subsequent papers also failed to show any requirement for mast cells in the generation of airways eosinophilia. All three models utilised alum at sensitisation, with house dust mite(42) or OVA(157,158) as the antigen, and used protocols between 20 and 30 days long. When AHR was assessed results were inconsistent with one study demonstrating no requirement for mast cells(157) and another mast cell dependence(158).

Contrasting results were obtained utilising protocols which differed from these more traditional models of asthma. In a short model utilising OVA and alum with the final challenge on day 12, BAL and histological eosinophilia was significantly reduced in W/W^v mice and was restored when these mice were reconstituted with mast cells(159). When mice were immunised with OVA and alum on days 0 and 14 before receiving a single nebulised OVA challenge on day 28, lung eosinophil accumulation was significantly reduced in W/W^v mice 48 hours later, although not at any of the four other time points studied(160). In another variant of the model mice were immunised with OVA and alum on day 0 followed by OVA alone on day 10 after which they were challenge with nebulised OVA on days 17 and 24, resulting in a significant reduction in eosinophilia in W/W^v mice(161). Once again the effect on AHR was variable with some showin depence on mast cells(161) and others independence(160).

In 2000 Williams and Galli made a significant contribution to our understanding of the role of mast cells in experimental asthma when they compared the requirement for mast cells of two different models(162). As with most previous studies they found that when mice immunised with OVA and alum were challenged lung eosinophilia was generated equally well in wild type and W/W^v mice. However when immunised with OVA without adjuvant eosinophilic inflammation was significantly reduced in W/W^v mice. The same was also true of AHR, which was attenuated in mast cell deficient mice only when immunised with OVA alone. Subsequent studies have confirmed the requirement for mast cells in alum-free models of asthma using W-sh as well as in W/W^v mice(33,37,163). Interestingly in mast cell reconstitution experiments one of the essential roles of mast cells in these models is as a source of TNFα (33,163). The

data regarding experimental asthma in mast cell deficient mice is summarised in Table 1.2.

Mice	Model	Length	EØ	AHR	Ref
W/W^v	OVA + Alum	21 days	NC	-	(156)
W/W^v	OVA + Alum	12 days	↓	-	(159)
W/W^v	HDM + Alum	21 days	NC	-	(42)
W/W^v	OVA + Alum	30 days	NC	NC	(157)
W/W^v	OVA + Alum	20 days	NC	↓	(158)
W/W^v	OVA + Alum	30 days	NC	NC	(162)
W/W^v	OVA	46 days	↓	↓	(162)
W/W^v	OVA + Alum	28 days	↓[1]	NC	(160)
W/W^v + W-sh	OVA	75 days	↓	↓	(37)
W/W^v	OVA + Alum[2]	24 days	↓	↓	(161)
W-sh	OVA	46 days	↓	↓	(33)
W/W^v + W-sh	OVA	30 days	↓	↓	(163)

Table 1.2 – The effect of mast cell deficiency on the outcomes of experimental asthma.

Key – Length: duration of model used. EØ: effect on lung eosinophil numbers. AHR: effect on airways hyperresponsiveness. ↓: reduced. NC: no change. HDM: house dust mite antigen

Notes - (1) Of five timepoints studied eosinophils only reduced at 48 hours. (2) Alum was used with first sensitisation only.

The clinical data certainly suggest a role for mast cells in asthma, perhaps related to smooth muscle function. The data from asthma models is more difficult to interpret, with a clear requirement for mast cells when alum is omitted, although the exact role they play is not clear.

1.1.3.4 T lymphocytes

Initial histological studies in asthma focused on the eosinophils and mast cells(57,150) and T cells were not much studied until their central role in the immune response became understood. In particular their capacity to recruit cells of the innate immune system, including eosinophils, and their ability to provide help to B cells in antibody production and isotype switching(164). It was also at this time that T cell biology was revolutionised by the discovery of the Th1 and Th2 subsets of helper T cells(165).

In humans there are reports of increased T cell numbers within the airway wall(166) and lumen(67,167), but not peripheral blood(167,168) of asthmatics. Regardless of the location sampled, the proportion of activated T cells is consistently elevated(71,168-171) and this rise correlates with eosinophil

numbers(67,166,172) and AHR(67). A study using anti-CD4 to target helper T cells in severe asthma demonstrated an improvement in peak expiratory flow but no significant change in FEV_1 or symptoms(173). While this raised the possibility that T cells could be targeted in asthma there has been no further progress in this area.

Antibodies against CD4 have also been used in murine studies of allergic airways inflammation, showing that helper T cell depletion before or after sensitisation results in markedly reduced eosinophilia, AHR and remodelling(100,174-176), but has no effect on AHR or remodelling when given after challenge in a chronic model(177,178). Similarly B and T cell deficient SCID or $RAG^{-/-}$ mice fail to develop airways eosinophilia and AHR(54,95,179,180). In $RAG^{-/-}$ mice both could be restored by reconstitution with CD4 cells(95). The corollary of this is that eosinophilic inflammation and AHR can be induced in naïve mice by the transfer of antigen-specific helper T cells(180,181). The requirement for CD8 cells is less clearly defined; two adjuvant free models have found conflicting results with one showing that CD8 cells were required for eosinophilic inflammation and AHR(182) and another that eosinophilic inflammation was unaffected in CD8 deficient mice(179).

Th2 inflammation

Shortly after the identification of Th1 and Th2 lymphocyte subsets investigators began to look for a link between Th2 cells and atopy due to the established role of the Th2 cytokine IL-4 in IgE synthesis(183). BAL obtained from asthmatic patients contains elevated levels of Th2 cytokines(170,171) and cells expressing Th2 cytokines(184,185). Similar results have been obtained from bronchial biopsies (184,186-188), sputum(189,190) and peripheral blood(170). In one study there was a direct correlation between the number of IL-5+ cells within the BAL and clinical measures of bronchoconstriction(191), and while expression of IL-5 is not necessarily restricted to T cells, other have shown that in asthma the majority of IL-4 and IL-5 positive cells are T cells(184).

Further support for Th2 inflammation in asthma comes from studies looking at the Th2 associated transcription factors STAT6(192) and GATA-3(193) which are expressed at elevated levels in BAL and biopsy samples from

asthmatics(194,195). Conversely the Th1 associated transcription factor T-bet(196) is expressed at significantly reduced levels(197).

Data from murine models concur with those from humans. Transfer of polarised antigen-specific Th1 cells followed by airways challenge results in neutrophilic rather than eosinophilic inflammation and fails to induce AHR(180,198,199). However when antigen-specific Th2 cells are transferred into naïve wild type or SCID mice then airways challenge induces eosinophilic inflammation, mucus hyper-secretion and AHR(180,198,199).

Th2 cytokines

The defining cytokines of the Th2 subset are IL-4, IL-5, IL-13 and variably IL-9(165,200,201). The contribution of IL-5 has already been discussed when considering the role of eosinophils in asthma.

In 1986 the name IL-4 was proposed for what had previously been variously known as B-cell stimulating factor 1, B-cell growth factor 1, B-cell IgG differentiation factor and IgG1 induction factor(202). As the names imply, IL-4 was already recognised for being able to induce B cell growth and isotype switching to the Th2 associated isotypes IgG1(203) and IgE(204). Two months later IL-4 was associated with the Th2 subset of T cells(165) and was subsequently shown to be a crucial factor in driving Th2 development(205).

When IL-13 was discovered in 1993 it was found to be closely linked to the IL-4 gene on chromosome 5 and expressed by Th2 but not Th1 cells(206,207). The association with Th2 inflammation and atopy was strengthened by the finding that IL-13 could induce IgE production, independently of IL-4. The functional overlap between IL-4 and IL-13 is due to the fact that they share a common receptor subunit. The Type I IL-4R consists of the IL-4Rα and γ_c subunits and the Type II IL-4R consists of the same IL-4Rα subunit plus an IL-13Rα1 subunit. IL-4 signals through both receptors whereas IL-13 signals only through the Type II receptor(208).

The finding that IL-4 can induce the features of asthma in mice, including eosinophilia, AHR and goblet cell hyperplasia(209,210), is perhaps not surprising given the ability of IL-4 to induce Th2 cytokine production and inflammation. In

order to assess whether or not experimental asthma is IL-4 dependent IL-4 deficient mice have been used, which have impaired Th2 responses(211) and are unable to produce IgG1 or IgE(212). As summarised in Table 1.3 these have consistently shown a requirement for IL-4 in the development of airways eosinophilia, regardless of strain, model or adjuvant use. The only exception was the finding that epicutaneous sensitisation with OVA results in IL-4 independent BAL eosinophilia in response to OVA challenge(213). Also shown in Table 1.3 are the effects of using anti-IL-4 antibodies on airways eosinophilia, which are less consistent, perhaps due to an inability to completely block IL-4 signalling. AHR is also variable with the suggestion of a strain effect, being preserved in IL-$4^{-/-}$ BALB/c mice but reduced in C57Bl/6 IL-$4^{-/-}$ mice, similar to the strain difference seen in IL-$5^{-/-}$ mice.

Mice	Model	Method	EØ	AHR	Ref
C57Bl/6	OVA + Alum	IL-$4^{-/-}$	↓	-	(156)
C57Bl/6	OVA + Alum	IL-$4^{-/-}$	↓	-	(214)
C57Bl/6	OVA + Alum	α-IL-4	↓	-	(214)
C57Bl/6	OVA + Alum	IL-$4^{-/-}$	↓	↓	(215)
C57Bl/6	OVA + Alum	IL-$4^{-/-}$	↓	↓	(216)
BALB/c	OVA + Alum	α-IL-4	NC	↓	(54)
C57Bl/6	OVA + Alum	IL-$4^{-/-}$	↓	NC	(217)
BALB/c	OVA + Alum	IL-$4^{-/-}$	↓	NC	(100)
BALB/c	TF-OVA	IL-$4^{-/-}$	↓	NC	(198,199)
C57Bl/6	airways OVA[1]	IL-$4^{-/-}$	↓		(213)
C57Bl/6	epicutaneous OVA[2]	IL-$4^{-/-}$	NC		(213)
BALB/c	GM-CSF + OVA[3]	IL-$4^{-/-}$	↓		(218)

Table 1.3 – Studies on the requirement for IL-4 in experimental asthma

Key – EØ: effect on lung eosinophil numbers. AHR: effect on airways hyperresponsiveness. α-IL-4: anti-IL-4. ↓: reduced. NC: no change. TF-OVA: model employing adoptive transfer of OVA-specific T cells followed by antigen challenge.

Notes (1) Protocol utilising repeated antigen challenge of naïve mice. (2) Protocol utilising epicutaneous sensitisation followed by airways challenge. (3) Protocol utilising airways GM-CSF over-expression and OVA challenge.

A number of studies have also been carried out in mice deficient in STAT-6, an essential element in both IL-4 and IL-13 signalling. These have shown loss of AHR and mucus production with reduced or absent airways eosinophilia(219-221). Studies in mice deficient in the common IL-4Rα chain used for both IL-4 and IL-13 signalling have shown that eosinophilic inflammation and mucus production

are attenuated while AHR is preserved(209,221), perhaps due to the existence of an IL-4Rα independent signalling pathway for IL-13(222).

A number of approaches have been applied to address the specific role of IL-13, independently of IL-4. When IL-13 is administered to, or expressed in, the lungs IgE production, AHR and local eosinophilia and goblet cell hyperplasia are induced(209,221,223-225). Blocking the function of IL-13 has been achieved with soluble anti-IL-13 and IL-13Rα2 (IL-13Rα2-Fc) or by generating mice lacking IL-13 or IL-13Rα1. Results of these experiments are summarised in Table 1.4 and show a fairly consistent failure to generate AHR or goblet cell hyperplasia with preserved airways eosinophilia.

Mice	Model	Method	EØ	AHR	Goblet	Ref
A/J	OVA	IL-13Rα2-Fc	NC	↓↓	↓	(223)
BALB/c	OVA + Alum	IL-13Rα2-Fc	↓	↓	↓	(209)
BALB/c	OVA + Alum	IL-13$^{-/-}$	NC	NC	↓	(226)
BALB/c	OVA + Alum	IL-13$^{-/-}$	NC	↓↓	↓↓	(225)
BALB/c	TF-OVA	IL-13$^{-/-}$	NC	↓↓	↓↓	(225)
BALB/c	OVA + Alum	α-IL-13	NC	↓↓	-	(227)
BALB/c	*S. mansoni*	IL-13Rα1$^{-/-}$	NC	↓↓	↓↓	(228)
NS	OVA + Alum	IL-13Rα1$^{-/-}$	↓	↓↓	↓↓	(229)

Table 1.4 - Studies on the requirement for IL-13 in experimental asthma

KEY – EØ: effect on lung eosinophil numbers. AHR: effect on airways hyperresponsiveness. Goblet: goblet cell hyperplasia. ↓: reduced. ↓↓: absent. NC: no change. NS: Not stated.

Overall it would appear that IL-4 is crucial in the generation of IgE and the development of Th2 inflammation and consequent eosinophil accumulation, IL-5 plays a key role in eosinophilic inflammation and IL-13 is responsible for AHR and goblet cell hyperplasia.

Other cytokines in asthma

While evidence of Th2 inflammation is consistently found in clinical asthma there are reports of increased IFNγ producing cells within the airways(230-232), particularly in those with more severe disease(233,234). As discussed above the transfer of Th1 cells alone fails to generate experimental asthma in mice, but when co-transferred with Th2 cells results have been conflicting. Two independent groups found that when polarised antigen-specific Th1 and Th2 cells were co-transferred before airways challenge the extent of BAL eosinophilia was

attenuated as compared with the transfer of Th2 cells alone(180,235), although histological inflammation and AHR were unaffected(180). However another group utilising a similar model, but with only a single antigen challenge, found that BAL eosinophilia was not generated following the transfer of Th1 or Th2 cells alone, but only with the co-transfer of both populations(236). While it is hard to draw firm conclusions it would certainly seem that asthma is not simply due to changes in the Th1-Th2 balance.

Drugs targeting TNFα are now widely used in Crohn's disease and rheumatoid arthritis, and are the only anti-cytokine therapies in regular clinical use for inflammatory disease. TNFα is found at elevated levels in the airways of asthmatics, particularly in mast cells(237,238). Murine models also support a role for mast cell derived TNFα(33,163). Following on from the apparent efficacy of anti-TNFα for severe asthma in a small uncontrolled study(239) there have been a number of studies addressing the use of anti-TNFα therapy in asthma. An initial randomised controlled study of 10 patients with severe asthma also looked promising with improvements in AHR, lung function and quality of life(240). Unfortunately subsequent small trials in both patients with severe(241) and more mild(242,243) asthma failed to show any convincing benefit. A much larger trial of 309 patients with severe asthma also failed to demonstrate any benefit(244). In light of these results and concerns over safety it seems unlikely that anti-TNFα therapies will become widely used in asthma, although the possibility remains that a specific subset of patients may be identified who will benefit.

There has recently been considerable interest in the novel Th17 subset of helper T cells. They are characterised by the production of IL-17 and play a key role in certain autoimmune diseases(245,246). While IL-17 has been detected at elevated levels in the sputum and BAL of asthmatic patients(247,248), the role it plays in disease is unclear. In rodent models IL-17 expression or administration to the lungs results in non-eosinophilic inflammation(249-251). However in murine models of asthma IL-17 deficiency attenuates eosinophil accumulation in some studies(251,252) but not others(253), and IL-17 administered at challenge blocks AHR and eosinophil recruitment(252). Antibody studies with anti-IL-17 have yielded similarly conflicting results(254,255)

The subset of chemotactic cytokines, or chemokines, play an important role in asthma due to their function as inducers of leucocyte migration and activation. In asthma there has been particular interest in the chemokines which act on Th2 cells and eosinophils. Th2 cells preferentially express the chemokine receptors CCR3 and CCR4(256,257), the ligands for which include eotaxin-1 (CCL11), eotaxin-2 (CCL24), RANTES (CCL5) and TARC (CCL17). While RANTES and TARC are both elevated in asthma (258,259), the eotaxin chemokines have attracted particular interest due their potent and relatively selective effect on eosinophil migration(260), mediated via CCR3. In clinical asthma CCR3 and eotaxin-1 are detected at increased levels within the lungs and serum(261-264), and increased pulmonary or serum eotaxin-1 correlates increased AHR and airflow obstruction(263,264). Epithelial cells, fibroblasts and macrophages appear to be the source of eotaxin-1 in asthma(261,262,265). Murine models have shown that blocking eotaxin-1 or eotaxin-2 alone by antibodies or gene deletion partially reduces eosinophil recruitment(179,260,266-268), whereas elimination of all eotaxin signalling by CCR3 knock-out or eotaxin-1/eotaxin-2 double knock-out, has a much more dramatic effect on lung eosinophilia(268-270). Effects on AHR have been much more variable(266,269,270), in keeping with the dissociation between eosinophilic inflammation and AHR discussed earlier. CCR3 antagonists have yielded promising results in animal studies(271,272) and there is currently interest from pharmaceutical companies in developing such molecules for therapeutic use in asthma.

Regulatory T lymphocytes

There are at least three major types of regulatory T (TReg) cells: $CD4^+CD25^+$, Tr1 and Th3 cells with overlapping functions(273-275). $CD25^+$ T cells are arguably the best characterized so far. This subset of CD4+ T cells constitutively express the IL-2R α-chain (CD25) and a role in immune regulation was postulated following the observation that depletion of $CD25^+$ T cells results in the development of organ-specific auto-immunity and the production of auto-antibodies (275,275). $CD25^+$ T cells can suppress both Th1 and Th2 cell development and the effector functions of committed Th1/Th2 cells (276,276). In humans CD25+ TReg numbers are reduced in asthma(277), and may be functionally impaired (277,278). In experimental asthma there is evidence that $CD25^+$(26,279-282) and Tr1(283) cells can suppress IgE production, pulmonary eosinophilia and AHR.

1.1.3.5 Macrophages and dendritic cells

While there is some debate as to the relationship between them(284), macrophages and dendritic cells (DCs) are derived from circulating monocytes(285,286) and are both involved in the uptake of foreign material within the lung, although probably have distinct functions.

Macrophages are the most abundant inflammatory cells within the lung, making up the majority of cells within the normal BAL(285). There are both interstitial macrophages within the lung parenchyma, and alveolar macrophages that line the alveoli and airways and are therefore directly exposed to the air. While traditionally considered as phagocytic effector cells, macrophages can also direct the immune response by antigen presentation and cytokine release. Despite the presence of high numbers of macrophages at the interface between the lung and the environment, little is known about the role they play in asthma. When macrophages are adoptively transferred into murine lungs they attenuate models of allergic airways disease(287,288). Initial studies using macrophage depletion demonstrated a worsening of experimental asthma, with the suggestion that the mechanism is the promotion of a protective Th1 response(289,290). However more recent studies have been contradictory, with a subset of $CD11b^{+}$ $CD11c^{int}$ macrophages capable of inducing allergic airways inflammation(291), and a macrophage depletion model showing abrogated eosinophilia and AHR(255). All these results must be interpreted with caution as adoptively transferred macrophages may not be representative of the normal lung macrophage population, and depletion techniques are incomplete and not necessarily clearly targeted.

DCs are professional antigen presenting cells and play a key role in directing the adaptive immune response. Within the lungs immature mucosal DCs sitting beneath the epithelium, as well as alveolar DCs within the air-spaces, are constantly sampling inhaled antigens(292). In the context of inflammation, particularly associated with TLR ligands(293), DCs are activated and migrate to the draining lymph nodes to present their antigens, thereby activating helper T cells(292). Under other circumstances DCs can promote tolerance(294) and the generation of regulatory T cells(295). The development of Th cells into a Th1, Th2 or Th17 phenotype is probably determined at the time of antigen

presentation by DCs and is strongly influenced by co-stimulatory molecules and the cytokine milieu present(296). In addition to the role DCs play at sensitisation they are also found at increased numbers following antigen challenge in both mice and humans(297). Evidence for the importance of DCs in experimental asthma comes from the findings that challenge of mice which have received antigen pulsed DCs results in eosinophilic airways inflammation(298,299) and depletion of DCs attenuates the inflammation and AHR in models of asthma(300,301).

1.1.3.6 Stromal cells

The most important structural cells in the lung are epithelial cells, endothelial cells, fibroblasts and smooth muscle cells. While initially considered as target cells in asthma which were bystanders affected by the inflammatory process, it is now becoming clear that they are active participants in the immune response.

Epithelial abnormalities, particularly shedding, have long been recognised as features of asthma (57,69) and were thought to be an end result of inflammation. It is now accepted that epithelial cells are far from inert and can release a number of mediators including TGFβ, eotaxin, IL-5 and IL-13 in response to pathogens, toxins or direct injury(302). There is also evidence that epithelial cells can drive myofibroblast proliferation, and collagen deposition, thereby contributing to airway remodelling. TGFβ appears to be central in this process(303) and can be induced in epithelial cells in response to IL-4 and IL-13(304).

While the function of smooth muscle in healthy lungs is debated(305), the role it plays in bronchoconstriction and AHR is well established. Smooth muscle cells also contribute to remodelling, both in terms of increased smooth muscle mass(306) and influences on the extracellular matrix(307,308). Some of these effects may be due to fundamental differences in smooth muscle cells of asthmatics, while others may be due to the response of smooth muscle cells to inflammatory mediators(308). In addition to these downstream effects, it is now becoming clear that smooth muscle cells play a role in the inflammatory response by releasing a number of pro-inflammatory mediators including IL-6, IL-8, TGFβ and eotaxin(308,309).

1.1.3.7 Neutrophils

In healthy controls the cellular content of sputum is comprised of around two thirds macrophages and one third neutrophils, with eosinophils makingup less than 1%(310-312). As discussed previously, clinical asthma is characterised by an increase in eosinophil numbers within the sputum, BAL and airway walls. However reports began to emerge in the 1990s suggesting that airways eosinophilia is not universal in asthmatic patients. Not only did a significant proportion of patients experiencing a mild exacerbation have a normal eosinophil count(313), but patients with sudden onset fatal asthma(314), severe exacerbations(315), and severe asthma on oral corticosteroids(316) often had significantly elevated numbers of neutrophils within the airways and lung tissue. Since then there has been considerable interest in trying to characterise this subgroup of patients. While around 70-80% of asthmatic patients have elevated sputum eosinophil numbers the remainder have “non-eosinophilic” asthma, with either normal or elevated neutrophils(20). Not only is a neutrophilic asthma associated with more severe asthma, but also with a failure to respond to corticosteroids(317). Interestingly mast cells are also present within the smooth muscle bundles in neutrophilic asthma(318), a characteristic finding in eosinophilic asthma(151). One of the histological features that appears to be unique to eosinophilic asthma is basement membrane thickening, suggesting that airway remodelling may not be a feature of neutrophilic asthma.

The role of neutrophils in the pathophysiology of asthma remains unknown. In patients with neutrophilic asthma there is evidence of activation of the innate immune system, with elevated sputum IL-8, TLR mRNA and LPS as compared with eosinophilic asthma(319). The role of neutrophils in experimental asthma has not been addressed and almost all current animal models reproduce eosinophilic asthma. There is one report describing a model of non-eosinophilic asthma in which AHR was generated(320). However neutrophils were only modestly elevated within the BAL at 8% with eosinophils still detectable at 1%, and within the lung tissue eosinophils were more abundant than neutrophils.

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Despite significant advances in our understanding of asthma over the past one hundred years the exact role of different cells and cytokines remains unclear.

While eosinophils are perhaps not as central as once thought, particularly in AHR, they appear to be important in asthma exacerbations and airways remodelling. This implicates IL-5 and the eotaxins, which are key mediators in the activation and recruitment of eosinophils, in these processes. IL-4 and T cells are likely to be crucial in the development of Th2 inflammation which results in the production of IL-5 as well as IL-13, which appears to be important for AHR and mucus production. B and T lymphocytes as well as IL-4 are essential for the generation of IgE which is central in atopic asthma and may play a role in non-atopic asthma. The function of mast cells remains undefined, and while animal models suggest they are an essential source of TNFα, in clinical asthma TNFα does not appear to be an important therapeutic target. However the location of mast cells within the smooth muscle bundles in asthma but not eosinophilic bronchitis suggests they may play a role in AHR. Macrophages, DCs and neutrophils are also likely to have their roles to play. In addition to the cells of the immune system stromal cells are increasingly recognised as contributing to the inflammatory process.

By studying novel cytokines which fit into this network of cells and mediators we can not only improve our understanding of the pathophysiology of asthma, but may also discover new therapeutic targets. IL-33 and the receptor ST2 represent one such cytokine.

1.2 ST2

1.2.1 ST2 gene and protein

1.2.1.1 The discovery of ST2

The gene for the protein referred to herein as ST2 was first described in 1989 by two independent groups. Initially referred to as T1, it was identified by screening of a cDNA library obtained from the murine 3T3 fibroblast cell line under mitogenic stimulation by over-expression of the *ras* oncogene(321). In these cells the T1 gene was also induced by dexamethasone and by re-feeding following serum starvation(321). At around the same time an identical gene,

referred to as ST2, was identified in 3T3 fibroblasts following serum re-feeding, which was absent in serum starved fibroblasts(322).

Although ST2 would remain an orphan receptor until the discovery of the ligand IL-33 in 2005(323), a great deal was learnt about the biology of ST2 in the intervening sixteen years.

1.2.1.2 The ST2 gene and its products

The first described sequence for ST2 was 2.7kb long with two potential initiation sites encoding a 337 amino acid protein with a predicted size of 37.7 or 38.5 kDa(322,324). It has significant homology to the IL-1 receptor (IL-1R1) and is a member of the immunoglobulin superfamily, although it appears to lack transmembrane or cytoplasmic domains(322,324). Consequently it is referred to as soluble ST2 or sST2(325). The human cDNA for sST2 was then isolated from a granulocyte DNA library by screening with a probe from the murine sST2 sequence before being sequenced from a helper T cell clone(326), the final protein having 68% homology to murine sST2(327). The murine *St2* gene is located on chromosome 1 and is closely related to the *IL-1r1* locus(328). The human *St2* gene is also found closely related to *Il-1r*, in this case on chromosome 2q12-13(329). Cells transfected with soluble ST2 produce an intracellular protein of 45-60 kDa and an extracellular protein of 60-70 kDa when glycosylation is permitted, the differences in size presumably representing different degrees of glycosylation (330,331).

A third ST2 gene product has been described in human cells, referred to as ST2V, which was initially identified in a helper T-cell line(332). It resembles sST2, but due to alternative splicing, one of the three extracellular immunoglobulin domains is replaced by a hydrophobic tail. Although a homolog exists in chickens there is no evidence for the existence of ST2V in mice. In chickens there also exists a fourth product, ST2LV, which is identical to ST2L but due to alternative splicing lacks the exon encoding the transmembrane domain(333). No data exists regarding their function. Figure 1.1 shows the three transcription products from the human ST2 gene.

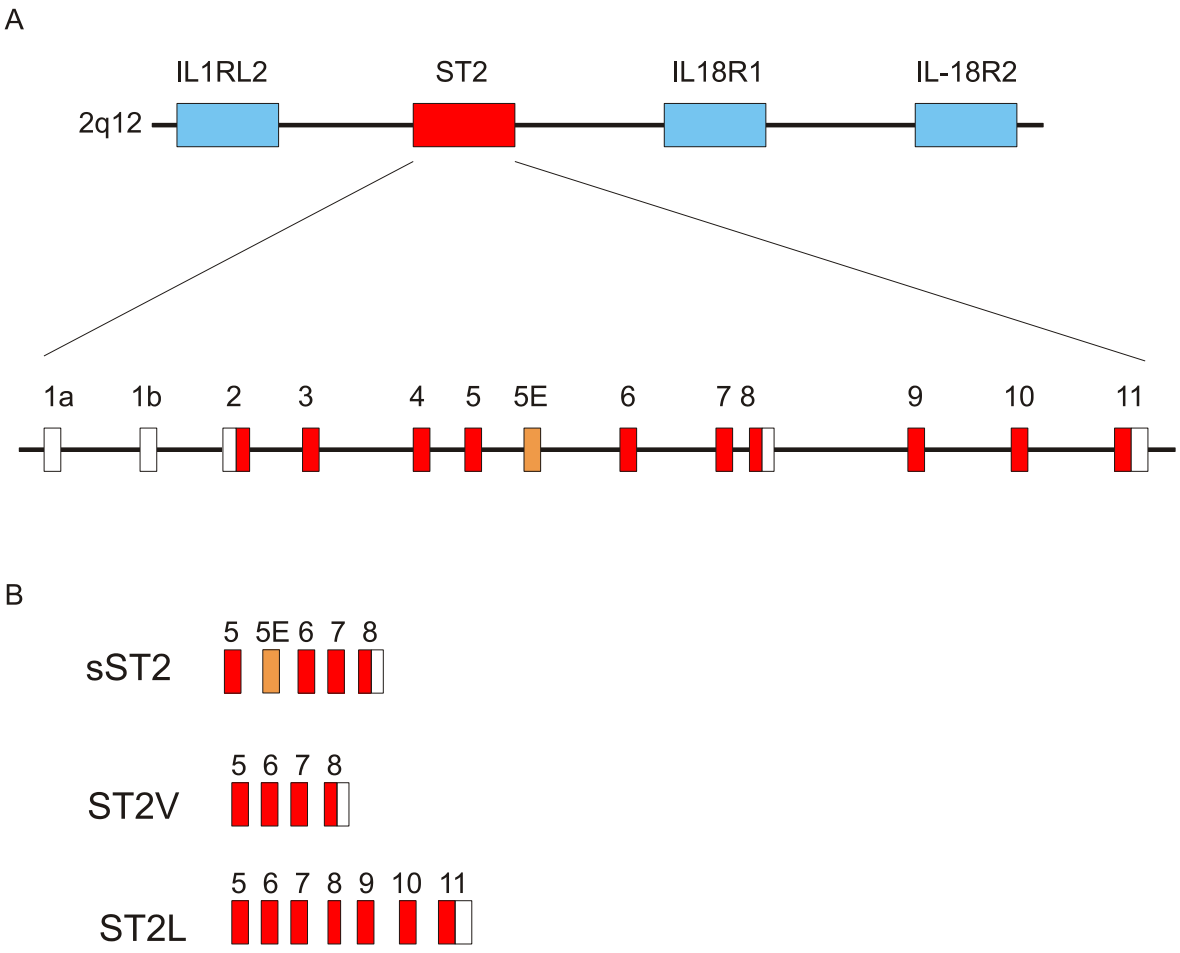


Figure 1.1 - Schematic representation of the human ST2 gene

(**A**) The top line shows the location of ST2 in the human genome. The bottom line indicates the expanded region for the human ST2 gene. Exons are shown as boxes and numbered from 1a to 11 with coding regions in red, and non-coding regions in white except for exon 5E in orange, which is specific to ST2V. (**B**) The exon usage starting from exon 5 for three spliced isoforms is shown. All the spliced isoforms thus far found commonly utilize from exon 2 to exon 5. The entire exon 8 is integrated for ST2 or ST2V cDNA, while part of exon 8 is utilized for ST2L cDNA. Adapted from Li *et al* (334)

1.2.1.3 ST2 regulation and expression

As previously discussed ST2 was initially identified in murine fibroblast lines and much of the understanding of ST2 gene regulation is from early studies in fibroblasts. Due to the fact that ST2 was induced by oncongene transfection or feeding following serum starvation(321,322), it was associated with the transition between the G0 and G1 state.

Immediate early genes, many of which are transcription factors, are induced rapidly in resting cells without the need for synthesis of other proteins. Delayed early genes require the synthesis of other proteins, such as immediate early transcription factors, for activation and therefore such activation occurs later. The initial finding that ST2 induction peaked at 6-10 hours after serum stimulation of fibroblasts suggested that it might be a delayed early gene(321,322,335,336).

Activator Protein-1 (AP-1) is a prototypical transcription factor and is a heterodimer made up of two variable immediate early gene products, often Jun and Fos proteins(337). Support for ST2 as a delayed early gene comes from the finding that the expression of AP-1 subunits can drive sST2, and to some extent ST2L, in both mouse and rat fibroblasts(335,338-340). Further evidence for ST2 as a delayed early response gene is the finding that ST2 could not be induced by serum stimulation of fibroblasts in the presence of the protein synthesis inhibitor cycloheximide(335,336,339), although there is one report contradicting this(325).

Ultimately it appears that ST2 can be induced via Fos/AP-1 as a protein synthesis dependent delayed early response gene (as is the case with serum stimulation), or via activation of p38 MAP kinase as a protein synthesis independent immediate early gene(341).

Two potential promoter regions for ST2 have been identified in fibroblasts (342,343), one of which was found to be activated by AP-1 and contained a classical AP-1 site(343). Subsequently it has been shown that inflammatory stimuli can also upregulate ST2. In human fibroblasts sST2, and to a much lesser degree ST2L, are induced by PMA, TNFα, IL-1α and IL-1ß(344).

Since the initial identification of ST2 in fibroblasts ST2 expression has been detected in a number of other cells types under different conditions as shown in Table 1.5. These studies highlighted that sST2 is predominantly found in structural cells such as epithelial cells and fibroblasts while ST2L expression is more restricted to lympoid tissue and inflammatory cells, with particularly good evidence of expression by T cells and mast cells as discussed below. As in fibroblasts ST2 can be upregulated by inflammatory stimuli. TNFα similarly upregulates sST2 and ST2L in human vascular endothelial cells(341,344). In macrophages and monocytes ST2 expression is induced by LPS (345), and other TLR ligands can upregulate ST2 in spleen preparations(346).

	murine		human		
Tissues	**sST2**	**ST2L**	**sST2**	**ST2L**	**Ref**
Liver		R, P	R	R	(339,344,347,348)
Spleen		R			(334,339,344,347)
Lung		R	R	R	(334,339,344)
Lymph node		R			(339)
Bone marrow	R	R, P			(339,347,348)
Bone	R, P				(347,349,350)
Skin	R, P				(344,347)
Bowel			R		(334)
Placenta			R	R	(334,344)
Kidney			R	R	(334,344)
Cells					
Fibroblasts	R, P	R, P	R[1], P[1]		(324,327,331,344,348,351-354)
Epithelial	R		R[1], P	R[1]	(339,353-356)
Endothelial			R, P	R	(344,351,353)
Smooth muscle			R	R	(353,357)
Mast cell		R, P		R, P	(347,348,350,352,358)
T cells[2]		R, P	R	R, P	(344,347,348,359-364)
B cells		P			(348,360)
PBMCs			R	P	(365,366)
Mono/MO		R, P	R[1]	R[1]	(339,348,353)
Erythroid	R	R	R		(339,344,347,350)

Table 1.5 – Tissues and cells in which ST2 expression has been detected

Whole tissues or cells, including cell lines, where sST2 or ST2L have been identified at the RNA (R) or protein (P) level. PBMCs = Peripheral blood mononuclear cells. Mono/MO = monocytes and macrophages. (1) Includes detection in cells specifically of lung origin (2) Preferentially detected in Th2 cells(359,360,362,364,365).

The expression of ST2 in different cells is controlled by the use of differing promoters. Mice and rat fibroblasts use a 4kb upstream promoter for both forms of ST2(339,343,350). While ST2 and ST2L from mast cells have an alternative exon 1 which is 10.5kb(367) upstream of the transcription start site in fibroblasts and has a separate promoter which is independent of serum growth factors(350) and contains several GATA elements, with GATA-1 over-expression enhancing ST2 expression (367,368). Like mast cells, Th2 cells utilise the distal promoter for sST2 and ST2L(368,369) and GATA-3 can bind to the distal promoter and activate ST2 in these cells(368). In humans the situation is less clear cut, although fibroblasts predominantly use the proximal promoter and leucocytes the distal promoter for both ST2 and ST2L(357,370). There is once again a GATA-1 site in the distal promoter(370). However in human mast cells while ST2L is expressed utilising the distal promoter, sST2 appears to utilise both(357).

T lymphocytes

The report that ST2L RNA was expressed in T cell lines(347) was followed by the finding that this expression was limited to Th0 and Th2 lines and was enhanced under conditions favouring Th2 development(365). Subsequently two independent groups identified ST2L by differential display PCR as being expressed by murine Th2 and not Th1 cells(359,360), with similar findings from human Th2 cells(362,364).

The frequency of ST2+ cells in naïve mice(360) and human peripheral blood(364,371) is low, at around 1-3% of CD4+ cells. However surface ST2L is upregulated under Th2 polarising conditions *in vitro* and *in vivo*. When naïve T cells are driven towards a Th2 phenotype *in vitro* 41-77% have been reported to express ST2(359,372,373). This upregulation of ST2 under Th2 polarising conditions is further enhanced by the addition of IL-6(373). Established Th2 cells also upregulate surface ST2L in response to TcR stimulation in the presence of co-stimulation(369). While IL-4 enhances ST2L expression IFNγ results in a net reduction in ST2L(369). In the context of disease ST2 can also be significantly upregulated, with expression on between 10% and 60% of murine CD4+ cells in *Schistomsoma mansoni* egg responses(374,375), *Leishmania major* infection(375,376) and experimental asthma(218,298,377,378). Although ST2L is restricted to Th2 T cells, IL-4 is not required for the expression of ST2L *in vitro*(360) or *in vivo*(376). Similarly IL-4 is not required for the production of IL-5

by ST2+ T cells, while the absence of IL-5 results in reduced ST2 expression (360). As would be expected ST2L+ T cells produce Th2 cytokines and are IL-4+ and IL-5+ on intra-cellular staining(359,360,373,374), and while under conditions favouring Th2 cells development CD4+ST2- cells expressed IL-4 and IL-10, albeit at lower levels than the CD4+ST2+ cells, none of them expressed IL-5(379).

Mast cells

The other main cell line of the haematopoetic lineage found to express ST2L are mast cells. Not only are they strong expressers of ST2L RNA(347,350,367), but they also express the protein on the cell surface(347,350). When compared with other leukocytes mast cells are all $ST2L^{+}$ and have the highest concentration of surface ST2L(348). Following the finding that ST2L is expressed on foetal mast cell progenitors(348), a search for mast cell lineage cells in adults using an *in vitro* culture system found that ST2 appeared to be a marker of committed mast cell progenitors(380). Whereas the ß7+CD27-ST2- fraction of bone marrow cells was able to generate mast cells, macrophages, erythrocytes and megakaryocytes, the ß7+CD27-ST2+ fraction gave rise to mast cells alone. This fraction was the only one able to generate significant numbers of mast cells and also reconstituted irradiated W-sh mice with mast cells but not B cells, macrophages or granulocytes.

Despite this, ST2 is not essential for mast cell production as $ST2^{-/-}$ mice have normal mast cell numbers in the skin and stomach and mast cells can be cultured from the bone marrow of these mice(381). These cultured $ST2^{-/-}$ mast cells degranulate normally in response to IgE cross-linking(381).

1.2.2 ST2 function

1.2.2.1 The function of ST2 *in vitro*

The first studies investigating the role of ST2 *in vitro* used either antibodies against ST2 or recombinant sST2, often fused with the Fc portion of human IgG1 (sST2-Fc) as experimental blocking agents. In the presence of ST2-Fc there is a reduction in IL-4 and IL-5 and an increase in IFNγ production by CD4+ T cells under both neutral and Th2 polarising conditions(372). When measuring Th2 cell proliferation and Th2 cytokine production not only is anti-ST2 antibody able to

enhance the effect of TcR signalling, but anti-ST2 alone can mediate these effects. This is dependent on the anti-ST2 being plate bound, presumably to mediate ST2 crosslinking, and is blocked by recombinant sST2(373).

The development of ST2 gene knockout mice made it possible to study the effect of ST2 deficiency on T cells. $ST2^{-/-}$ and wild type T cells stimulated with anti-CD3 produce identical levels of IL-4, IL-5 and IL-10 or IFNγ under Th2 or Th1 polarising conditions respectively(381-383). However when $ST2^{-/-}$ OVA-specific T cells are activated by OVA peptide they produce significantly more IL-5 than wild type cells(383).

When murine macrophages were studied it was shown that pre-treatment with sST2-Fc attenuates LPS mediated release of IL-6, IL-12 and TNFα(353,384-386). Similarly, sST2 was shown to inhibit LPS induced IL-1ß, IL-6 and TNFα production by a human monocytic cell line and was associated with reduced I*κ*B degradation and reduced binding of NF*κ*B to the IL-6 promoter(387). In addition sST2-Fc induces macrophage NF*κ*B(384,388) and down-regulates TLR-4 and TLR-1 mRNA(384). Conversely $ST2^{-/-}$ macrophages produce increased quantities of IL-6 and IL-12 in response to treatment with TLR ligands or IL-1(345).

ST2 also appeared to be inhibitory in mast cells, with transfection reducing IL-1 or LPS mediated NF*κ*B activation and anti-ST2 antibody inhibiting IL-1 mediated NF*κ*B activation in an ST2+ mast cell line(345). One possible mechanism for these inhibitory effects is the downregulation of NF*κ*B by ST2 sequestration of MyD88 and Mal(345).

1.2.2.2 The function of ST2 *in vivo*

The first studies investigating the role of ST2 *in vivo* used antibodies against ST2. One such polyclonal anti-ST2 antibody was shown to induce complement mediated lysis *in vitro*. When administered to *Leishmania major* susceptible Th2 prone BALB/c mice they developed less severe disease with a reduced parasite load and evidence of switching to a protective Th1 response(359). However a later study using a different, non-lytic antibody or ST2-Fc found no difference in the course of *L. major* infection in BALB/c mice(385). In collagen induced arthritis the use of depleting anti-ST2 antibody exacerbated disease with evidence of enhanced Th1 responses(359). Conversely blocking ST2 function with

ST2-Fc at the time of disease onset reduces the severity of collagen-induced arthritis(389).

sST2-Fc reduces mortality and cytokine production in LPS induced shock, whereas blocking anti-ST2 antibody has the opposite effect(384). While $ST2^{-/-}$ mice are as susceptible to LPS induced shock as wild type mice, pre-treatment with LPS protects wild type but not $ST2^{-/-}$ mice from subsequent challenge with a lethal dose of LPS(345). This effect may be mediated by LPS induced upregulation of ST2 on macrophages and subsequent MyD88 sequestration inhibiting TLR-4 signalling, phenomena which have been observed *in vitro*(345).

Transfection with sST2-Fc results in reduced liver damage in a liver ischaemia-reperfusion model. This was associated with a reduction in liver macrophage number and serum IL-6 and TNFα(386). Similar results were seen in an intestinal ischaemia-reperfusion model where treatment with sST2-Fc reduced neutrophil influx, histological damage and vascular permeability with an associated reduction in TNFα, CCL2(MCP-1) and CXCL1. Interestingly this effect was IL-10 dependent, not only was there a rise in local IL-10, but the protective effect of sST2-Fc was lost in $IL\text{-}10^{-/-}$ mice(390). In a model of hepatic injury sST2-Fc treatment did not alter the extent of liver damage, but did enhance subsequent fibrosis with an associated increase in *ex vivo* Th2 cytokine production. This effect was abrogated by CD4 depletion and attenuated in $IL\text{-}4^{-/-}$ mice(388). Taken together these data suggest that ST2 can play a role the inflammatory response to tissue injury.

The development of ST2 gene knockout mice(381,382,391) made it possible to ask further questions regarding the role of ST2. Initial phenotyping showed that naïve $ST2^{-/-}$ mice have normal serum immunoglobulin levels and a normal population of immune cells(382).

In $ST2^{-/-}$ mice infected with *Nippostrongylus brasiliensis* serum immunoglobulin levels and *ex vivo* IL-4 and IFNγ production by CD4 T cells are unaffected(381). Although a later study suggested that T cell IL-5 was reduced in the lungs of $ST2^{-/-}$ and ST2-Fc transgenic mice during *N. brasiliensis* infection, peripheral and lung eosinophilia, serum immunoglobulin induction and parasite clearance was unaffected(391).

A study of *Schistosoma mansoni* egg induced granuloma formation, a Th2 dominated response(392), yielded intriguing results(382). When *S. mansoni* eggs were administered intravenously (IV) to naïve mice there was a dramatic impairment of eosinophil accumulation and pulmonary granuloma formation in $ST2^{-/-}$ mice. Conversely, when the eggs were administered IV to mice that were first immunised IP with eggs, granuloma formation and antibody production was unaffected in $ST2^{-/-}$ mice, although there was a reduction in *ex vivo* Th2 cytokine production(382).

$ST2^{-/-}$ mice developed more severe pancreatic inflammation in a streptozotocin induced diabetes model(393). Observational studies have also reported elevated ST2 in models such as LPS induced acute lung injury, where BAL sST2 protein was elevated(353), an allergic model of contact dermatitis, where ST2 RNA was elevated in the skin(394) and bleomycin induced pulmonary fibrosis where ST2 RNA was induced locally(354),

ST2 in asthma

Given the expression of ST2 on mast cells and Th2 cells it seemed reasonable that ST2 may be involved in the pathophysiology of asthma. A hypothesis that was later strengthened by the finding that lung ST2 RNA and serum sST2 protein were elevated during an OVA/Alum driven murine model of asthma(395).

Initial studies into the role of ST2 in allergic airways inflammation relied on the administration of antibodies or exogenous sST2, and consistently favoured a role for ST2 in asthma models. The first of these studies used anti-ST2 or sST2-Fc, both at a dose of 20 to 100 µg per mouse. Allergic airways inflammation was induced by the adoptive transfer of OVA specific T cells which had been polarised to a Th2 phenotype, followed one day later by two challenges of nebulised OVA on two consecutive days. When anti-ST2 or sST2-Fc was administered one hour prior to antigen challenge there was a substantial reduction in BAL eosinophils and Th2 cytokines, histological inflammation in the lung and AHR(360,372). A similar effect was seen in a 21 day OVA sensitisation and challenge model where mice were sensitised with IP OVA/Alum on two occasions before being twice challenged with nebulised OVA. Administration of the same anti-ST2 antibody 1 hour before each sensitisation and challenge resulted in a significant reduction in BAL eosinophils and IL-5 as well as serum

OVA-specific IgE(372). In a model of asthma mediated by the adoptive transfer of OVA pulsed DCs followed by seven nebulised doses of OVA, treatment with anti-ST2 or ST2-Fc at the time of DC transfer reduced BAL eosinophilia and histological inflammation(298). In respiratory syncytial (RSV) virus induced Th2 mediated eosinophilic lung disease in mice administration of anti-ST2 around the time of RSV challenge attenuated disease severity, as measured by BAL eosinophilia and weight loss, although viral clearance was delayed(396). Using the alternative technique of intravenous ST2 gene transfer resulted in reduced BAL eosinophils, IL-4 and IL-5 in a 14 day OVA/Alum model of asthma(395).

Subsequent studies in ST2 gene knockout mice yielded results contrary to the data from anti-ST2 and sST2 experiments. In a 35 day model of asthma with OVA/Alum sensitisation followed by OVA challenge C57Bl/6 wild type and $ST2^{-/-}$ mice showed identical levels of BAL eosinophilia, histological inflammation and serum immunoglobulins(381). There were comparable results in BALB/c mice using a similar OVA/Alum model, this time over 30 days(383). Wild type and $ST2^{-/-}$ mice had a similar of degree of AHR, histological inflammation and *ex vivo* cytokine production. However BAL eosinophils were elevated and macrophages were reduced in $ST2^{-/-}$ mice. An adoptive transfer model was then used to focus on the role of T cells. OVA specific wild type or $ST2^{-/-}$ Th2 cells were generated *in vitro* and transferred into wild type mice which had been primed with OVA/Alum, mice were challenged daily with nebulised OVA over the following five days. When compared with mice that received wild type Th2 cells those that received $ST2^{-/-}$ Th2 cells had elevated BAL and lung eosinophilia, increased inflammation and mucus production on lung histology and dramatically enhanced AHR, leading to increased mortality. The BAL, lungs and lymph nodes were enriched for antigen specific T cells, but total macrophage numbers in the BAL were reduced. BAL and lung cytokine and chemokine levels were all low and there was no difference between groups. There was no evidence of a mast cell or basophil contribution as serum mast cell protease-1 and lung mast cell numbers were unaffected. Allergic inflammation was also independent of B-cells or recipient T cells as lung and BAL inflammation was unaffected if $RAG^{-/-}$ mice were used as recipients(383).

1.2.2.3 ST2 in clinical disease

Although sST2 and ST2L had been detected in human cells and samples at both the RNA and protein level, it was the development of an ELISA for sST2(397) which made it possible to identify and study the role of ST2 in clinical disease. The first of these studies suggested that serum sST2 was elevated in a small cohort of six asthmatics as compared with patients with lung cancer or healthy controls(397). A subsequent larger study of 56 patients also found that serum sST2 was slightly elevated in asthma, but was further elevated during exacerbations when levels correlated with severity of exacerbation, peak expiratory flow rate and $PaCO_2$(398).

While sample sizes were often very small, serum sST2 was also found to be elevated in a number of other conditions including eosinophilic pneumonia(399), autoimmune diseases such as rheumatoid arthritis, SLE, Wegener's granulomatosis and Bechet's disease (400), idiopathic pulmonary fibrosis (401), sepsis and trauma where ST2 correlated with mortality (402), in patients treated with H2 antagonists(403,404), and in the CSF of patients with subarachnoid haemorrhage(405).

A large Japanese cohort study found a number of Single-nucleotide polymorphisms (SNPs) in the ST2 gene and went on to study seven of them in patients with atopic dermatitis and normal controls. One SNP, located in exon 1a, showed a significant association with atopic dermatitis, and among patients with atopic dermatitis those with the associated genotype had higher levels of serum sST2 and IgE. Of the haplotypes incorporating all seven loci one was associated with atopic dermatitis and this haplotype was associated with stronger ST2 promoter activity in a reporter system(406).

ST2 in heart disease

The possibility that ST2 might be involved in heart disease was raised after the finding that sST2, and to a lesser extent ST2L, were up-regulated in cardiac myocytes and fibroblasts under mechanical strain(406,407). This observation was extended to the whole organism with data showing ST2 RNA was upregulated in the left ventricle and sST2 was elevated in the serum of mice following myocardial infarction (MI)(406). In a small cohort of patients serum ST2 was

elevated immediately post MI and levels correlated with creatinine kinase, a marker of cardiac damage, and with poor left ventricular function post MI(406). A later study of 810 patients with acute MI found that serum ST2 at presentation correlated with troponin, a marker of cardiac damage, and was a predictor of subsequent mortality or heart failure(408). However sST2 appeared to be a poor predictor of acute coronary syndrome in an emergency setting, although measurements were made very early and there was no comparison with gold standard biomarkers(409). Serum sST2 was also found to rise following coronary artery bypass grafting(410). A study of 161 patients with severe heart failure found that serum sST2 was elevated, correlated with other biomarkers of heart failure, BNP and proANP, and was a predictor of mortality (411). A study of 593 breathless patients, of whom 208 had acute heart failure, found that serum sST2 was elevated in those with heart failure and correlated with NT-proBNP, left ventricular systolic dysfunction and mortality. Interestingly, among patients with elevated sST2 without heart failure the commonest cause of death was lung disease, including obstructive lung disease, pneumonia and lung cancer(412).

1.2.2.4 The search for a ligand for ST2

Given the structure of ST2 and it's homology to the IL-1 receptor it was immediately ascribed the function of a receptor(347,351,365,413). IL-1 was quickly excluded as a ligand given that it did not bind to, or signal via, ST2 (414). However the hypothesis that ST2 could function as a receptor was supported by the findings that IL-1 could induce reporter activation, NFκB activation, kinase activity and IL-4 produvction from T cells via a fusion protein with the extracellular portion of IL-1R1 and the intracellular portion of ST2(351,415,416). While a number of candidate ligands were proposed, they failed to show any functional activity(351,415).

1.3 Interleukin-33

1.3.1 IL-33 gene and protein

In late 2005 the discovery of IL-33 was reported, a novel IL-1 family cytokine and a functional ligand for ST2(323). It was discovered using a computational database search for IL-1 family members. Human IL-33 is found on chromosome 9p24.1 and murine IL-33 on 19qC1. They encode proteins of 270 and 266 amino acids respectively, both with a molecular weight of 30kDa. Human and murine IL-33 share 55% homology at the amino acid level. Within the IL-1 family IL-33 most closely resembles IL-18. It later became clear that IL-33 is identical to the previously reported nuclear factor from high endothelial venules (NF-HEV) which was found by subtractive hybridization to be preferentially expressed in high endothelial venule (HEV) cells as compared with other endothelial cells(417).

1.3.1.1 IL-33 expression

In the mouse IL-33 RNA is detected at high levels in the stomach, lung, spinal cord, brain, and skin and at lower levels in lymph nodes, resting DCs, spleen, pancreas, kidney, and heart. In the human IL-33 RNA is detected in resting bronchial epithelial cells and smooth muscle cells, including those from bronchi, pulmonary arteries and coronary arteries, and in cytokine activated fibroblasts. Activated murine bone marrow macrophages, and to a much lesser extent activated human macrophages and DCs also express IL-33(323). Using in-situ hybridization and RT-PCR IL-33 RNA was detected in endothelial cells from human tonsils, rheumatoid arthritis synovium and Crohn's disease intestine(418). As mentioned previously, IL-33 protein is also detected in endothelial cells, specifically those of HEVs, and in a few scattered cells in the T- and B-cell zones, of human tonsils(417).

In an adoptive transfer model of asthma OVA/Alum priming alone induced IL-33 RNA in the lungs, and expression was further enhanced following challenge(383). Within the rat heart IL-33 RNA and protein has been detected from cardiac fibroblasts, and to a much lesser extent cardiac myocytes, and is upregulated by biomechanical strain(407). A similar result was seen *in vivo* where cardiac overload in mice led to increased IL-33 in cardiac fibroblasts(407).

1.3.1.2 IL-33 processing

In keeping with the finding that within the first 65 amino acids of IL-33 there is a putative Helix-Turn-Helix (HTH) DNA-Binding Domain(417), IL-33 has been found to localise to the nucleus(417). In human tonsillar HEV endothelial cells an exclusively nuclear distribution of naturally occurring IL-33 was seen, and specifically in heterochromatin (tightly packed transcriptionally inactive DNA) rich areas(418). This association was demonstrated in other cell types using transfection with GFP tagged IL-33(418). Using truncated proteins it was confirmed that the 65 amino acids containing the HTH domain at the N-terminal end of IL-33 were responsible for this nuclear localisation(418). These data would be in keeping with the suggestion that IL-33 has a pro-domain(323), and the evidence that it can be cleaved from the 30kDa product to an 18kDa "mature" form by caspase-1(323). However IL-33 does not contain a classical caspase-1 cleavage site and there is no evidence of cleavage *in vivo*. Nuclear localisation was observed with endogenous IL-33 in HEV endothelial cells, or with transfected IL-33 in other cells, even when specific antibodies against the IL-1 like C-terminal end of "mature" IL-33 were used(418).

1.3.2 IL-33 function

1.3.2.1 IL-33 signalling

The binding of IL-33 to ST2 was demonstrated by the fact that ST2 co-precipitates IL-33, and vice versa(323,407), and that binding of anti-ST2 to the surface of mast cells is blocked by IL-33(323). Cells transfected with ST2 showed NFκB activation using a GFP reporter system(323). ST2 transfected cells stimulated with IL-33 were also used to show that ST2 recruits MyD88, IRAK, IRAK4, and to a lesser extent TRAF6 and that IκBα, Erk1/2, p38, and JNK are phosphorylated. This was confirmed using bone marrow derived mast cells (BMMCs), previously shown to express surface ST2L(347,350), where IL-33 treatment resulted in NFκB, Erk1/2, p38, and JNK phosphorylation, which could be blocked with anti-ST2 antibody(323).

1.3.2.2 IL-33 function

At the time of undertaking this research there was very little data on the effect of IL-33, all of which was in the paper by Schmitz *et al*(323).

The only functional *in vitro* data was of the effect of IL-33 on T cells. In keeping with earlier studies which had activated ST2 by antibody cross-linking(373), IL-33 enhanced IL-5 and IL-13 production by Th2 cells stimulated via the TcR with CD28 co-stimulation, although IL-4 production was unaffected(323). While there was evidence of IL-33 induced signalling in mast cells there was no data on the functional effects of this(323).

To study the effects *in vivo*, recombinant IL-33 was administered IP to C57Bl/6 mice at a dose of 0.4 or 4 μg each day for a total of seven days(323). This resulted in a marked increase in peripheral blood eosinophils, and to a lesser extent lymphocytes and neutrophils, with elevated serum IgE and IgA. There was splenomegaly with increased monocytes, eosinophils and plasma cells within the spleen. Histological studies revealed changes within the gastro-intestinal tract and lungs. There was epithelial hyperplasia and inflammatory infiltrates, including eosinophils, in the oesophagus and goblet cell hypertrophy with excess mucus in the intestines. In the lungs there was epithelial hypertrophy and mucus hypersecretion in addition to vascular changes, namely perivascular mononuclear and myeloid infiltrates round medium and small vessels with medial hypertrophy. There was evidence of Th2 activation with elevated serum IL-5 and IL-13 and increased IL-4, IL-5 and IL-13 RNA in the thymus, spleen, liver and lung. When IL-33 was administered to IL-$13^{-/-}$ mice the epithelial changes seen in the lungs of wild type mice were absent, but the vascular changes remained.

1.4 Objectives

In the preceding Introduction I have highlighted the current understanding of the immunological basis of asthma and some of the key questions that remain. While the importance of Th2 inflammation is well established the exact role of different cells and cytokines remains unclear. There is still uncertainty over

what mechanisms are involved in the initiation of airways inflammation, the place of different cytokines within the hierarchy and sequence of events that occurs and the relationship between structural cells and inflammatory cells.

IL-33 is a novel cytokine about which very little is known, but which has the ability to induce Th2 inflammation and eosinophilia *in vivo*. The receptor for IL-33, ST2 is expressed at very high levels on mast cells, and there is a body of evidence that suggests ST2 plays an important role in Th2 inflammation, although the importance of the IL-33 – ST2 axis in experimental asthma is unresolved.

My hypothesis is that 'The activation of ST2 on mast cells by IL-33 plays an important role in allergic airways inflammation'.

To address this question I have set out three objectives.

2. To establish if mast cells can be activated by IL-33, acting via ST2 to produce pro-inflammatory cytokines and chemokines *in vitro*.

3. To optimise a murine model of experimental asthma and establish whether there is a need for ST2 in this model.

4. To assess whether or not IL-33 can activate mast cells within the airways to induce the features of experimental asthma *in vivo*.

By achieving these aims I hope to show that IL-33 is a key cytokine in asthma and hence potentially a novel therapeutic target.

Chapter 2

Materials and Methods

2.1 Reagents and buffers

Reagents and chemicals were purchased from Sigma-Aldrich (Poole, UK) and culture media was purchased from Invitrogen Ltd (Paisley, UK) unless stated otherwise. Commonly used buffers and solutions are listed in Table 2.1.

IL-33 used was from one of two sources. For all *in vitro* experiments commercial IL-33 (ALEXIS Corporation, Switzerland) was used. For *in vivo* experiments IL-33 produced within the laboratory was used. The methods for the production of IL-33 have been published elsewhere(419). Briefly, mouse IL-33 cDNA was cloned from IL-1-stimulated fibroblasts, and the cDNA sequence was transformed into host *Escherichia coli*. IL-33 protein was induced by IPTG and purified by Ni-NTA affinity chromatography followed by polymyxin B column to remove endotoxin. The purity of IL-33 was more than 95% and endotoxin levels were less than 0.01 EU/μg of protein by the QCL-1000 pyrogen test (Bio-Whittaker). Bioactivity and specificity of recombinant IL-33 were confirmed by its ability to induce IL-5 production from wild type but not $ST2^{-/-}$ Th2 cells.

Phosphate buffered saline (PBS)	8g NaCl, 1.16g Na_2HPO_4, 0.2g KCl, 0.2g KH_2PO_4 in 1 litre distilled water, pH 7.4
Avertin stock	1:1 w/v solution of 2,2,2-tribromoethanol in *tert*-amyl alcohol
Complete medium	500 ml RPMI 1640, 50 ml inactivated FBS, 5 ml pen / strep, 5 ml L-glutamine
Complete IMDM	500 ml Iscove's Modified Dulbecco's Medium (IMDM), 50 ml inactivated FBS, 5 ml pen / strep, 5 ml L-glutamine
FACS buffer	2% FCS in PBS
BD ELISA Coating buffer	0.1M NaHCO3 pH 8.4
ELISA Wash buffer	0.05% Tween-20 in PBS pH 7.4
BD ELISA assay buffer	10% FCS in PBS
R&D ELISA assay buffer	1% BSA in PBS
Biousource ELISA assay buffer	0.5% BSA and 0.1% Tween in PBS
OptEIA Coating Buffer	8.4g $NaHCO_3$, 3.56g Na_2CO_3 in 1 litre distilled water, pH 9.5
0.05M Citrate buffer	24.8 ml 0.05M citric acid 25.2 ml 0.05M trisodium citrate
Sodium Carbonate Buffer	25 ml 0.2M Na_2CO_3 25 ml 0.2M $NaHCO_3$

Table 2.1 – List of commonly used buffers and solutions

2.2 Animals

Wild type (WT) BALB/c and C57Bl/6 mice were obtained from Harlan Olac (Bicester, Oxon, UK). ST2 gene knockout BALB/c mice have a deletion within the ST2 gene which involves the majority of exons 4 and 5 (382). These mice had previously been obtained from Professor Andrew Mackenzie, BBRC, University of Cambridge and were bred in in-house by Biological Services, University of Glasgow.

W-sh mice are among the mice which have a mutation of the white spotting (W) locus. This locus was first described nearly a century ago when a mutation was found to result in abnormal haematopoiesis and pigmentation(420) and the gene responsible was subsequently identified as the *Kit* gene which encodes the stem cell factor receptor(421). Kit^{W}/Kit^{W-v} (W/W^{v}) mice are a heterozygous cross between the generally non-viable Kit^{W}/Kit^{W} mice and the modestly mast cell deficient Kit^{W-v}/Kit^{W-v} mice. These W/W^{v} mice are profoundly mast cell deficient, with mast cells only detected in the skin, and even there at <1% of the level found in normal mice(422). They are also profoundly anaemic, relatively neutropenic and sterile(423,424). W/W^{v} mice were the most utilised strain in the investigation of mast cell deficiency until the Kit^{W-sh}/Kit^{W-sh} (W-sh) mutation was identified, named after the white "sash" coat colouring which was observed on heterozygotes(425). W-sh mice have a markedly reduced expression of Kit upon mast cells and consequently a dramatic mast cell deficiency with no detectable mast cells in any organs including the lung(426,427), with the exception of the skin where mast cells have been reported as absent(426) or detected from birth, falling to around 1% of the level found in wild type mice by 12 weeks of age(427). In addition these mice are fertile and have no evidence of any other haematopoietic defect(427,428). For the purposes of this work W-sh mice, on a C57Bl/6 background (B6.Cg-Kit^{W-sh}/HNihrJaeBsmJ, stock number 005051) were ordered directly from The Jackson Laboratory (Bar Harbor, Maine, USA) and a colony established with the help of Biological Services, University of Glasgow.

All animals were housed in pathogen-free conditions in facilities run by Biological Services, University of Glasgow in strict accordance with United Kingdom Home Office regulations under the Animals (Scientific Procedures) Act 1986. Animals were given access *ad libitum* to standard feed and water. All

procedures were carried under Project Licence Number 60/3119, Procedure 9, or Project Licence Number 60/3791, Procedure 5.

2.3 *In vivo* procedures

2.3.1 Antigen dosing procedures

2.3.1.1 Intranasal dosing

Mice were anaesthetised by the intraperitoneal injection of 250 µl of avertin solution (1:40 dilution of avertin stock in PBS). Using a 200 µl pipette, 30 µl of reagent in sterile PBS was administered to the nostrils. Mice recovered from anaesthesia in a warmed box with supplemental oxygen.

2.3.1.2 Intratracheal dosing

Mice were anaesthetised by the intraperitoneal injection of 250 µl of avertin before being placed upon a custom made support which separated the upper and lower incisors. This allowed direct insertion of a blunt-tipped 25G needle (Becton Dickinson, Franklin Lakes, USA; blunted by the IBLS Workshop, University of Glasgow) into the trachea and instillation of 50 µl of reagent in sterile PBS. Mice were removed from the apparatus and recovered from anaesthesia in a warmed box with supplemental oxygen.

2.3.2 Induction of allergic airways inflammation

Initially a model of experimental asthma was employed that was already in use within the laboratory(429-431), and had been adapted from a previously published model(432). Female BALB/c mice aged 6-8 weeks were immunised by IP injection of 100 µg chicken ovalbumin (OVA, Grade V, Sigma-Aldrich) adsorbed to 2mg aluminium hydroxide (alum, Brenntag Biosector, Frederlkssund, Denmark) on days 0 and 14. On days 14, 25, 26 and 27 mice were challenged with 30 µg OVA by intranasal administration. Mice were culled on day 28 by IP injection of avertin 500 µl before exsanguination. Samples were collected as detailed below. In some experiments airway AHR to methacholine was measured

by Penh on day 28 prior to culling. As detailed in Chapter 4, modifications were made to this model during the course of this work.

2.3.3 IL-33 administration

As detailed in Chapter 5, a number of different dosing protocols were used in the study of the effect of IL-33 upon the lungs. Female wild type or $ST2^{-/-}$ BALB/c, or wild type or W-sh C57Bl/6 mice aged 6-8 weeks received 2 µg of murine IL-33 using the intranasal dosing technique. In the administration of multiple doses the interval was 24 hours between dosing. Mice were culled 24 or more hours after the final dose by the method detailed in Section 2.3.2. Samples were collected as detailed below. In some experiments AHR was measured by Penh prior to culling.

2.3.4 Measure of airways hyperresponsiveness

The gold standard for the assessment of AHR is to directly measure airflow resistance and lung compliance in anaesthetised mice via tracheostomy. However this technique is not only technically demanding and time consuming, but also requires considerable expertise and investment in equipment. Due to local experience the decision was made to use whole body plethysmograph, the equipment for which was available. This technique is used to measure Enhanced Pause (Penh) as an indicator of airways responsiveness to broncho-provocation with methacholine(29). Animals are placed in a sealed chamber with a transducer which measures the pressure changes within it. These changes are driven by the respiratory cycle of the mouse within the chamber, partly as a direct result of airflow and partly due to rapid warming and cooling of air entering and leaving the lungs. Penh is a dimensionless parameter which is derived from the relationship between inspiratory and expiratory pressure as well as the ‘pause’ which occurs at the end of expiration. Penh correlates well with the more technically demanding invasive measurements and remains widely used in murine models of asthma(29,31-34).

Penh was measured at doubling doses, from 12.5 to 50 mg/ml of methacholine, as previously described(29). Briefly, individual mice were placed in each chamber of a 4 chamber plethysmography unit (EMMS, England, UK), and left

undisturbed for a 30 minute acclimatisation period. Mice were then challenged for 2 minute periods with nebulised saline (control) or graded doses of methacholine chloride. Penh was continually measured and an average reading for the challenge period calculated using EMMS EDAQ® software.

2.3.5 Sample collection and preparation

2.3.5.1 Serum

Mice were injected with a lethal dose (500 µg) of avertin and killed by exsanguinations following the onset of full anaesthesia. The heart was exposed and blood withdrawn using a 1 ml syringe and 23G needle. The blood was stored in 1.5 ml centrifuge tubes for 2-3 hours to allow clotting, before being centrifuged at 14,000 rpm at 4°C for 30 mins (Jouan BR4i centrifuge). Serum was then decanted and stored at -20°C until analysed.

2.3.5.2 Bronchoalveolar lavage

After termination by cardiac puncture, BAL was performed. The trachea was exposed and a small incision made at the proximal end to allow cannulation with a 1 ml syringe and 23G needle sheathed with polythene tubing (0.58mm ID, 0.78mm OD; VWR International). The needle was held in place and a seal formed using forceps, before instilling 0.8 ml of PBS into the lungs. After 10 seconds the fluid was aspirated and the process repeated with a second 0.8 ml aliquot. All the aspirated fluid was pooled in a 1.5 ml centrifuge tube and stored on ice until further processing. Live cell counts were performed in a Neubauer haemocytometer (Weber Scientific International Ltd, Teddington, UK) using a 1:2 dilution with 0.4% trypan blue solution. $1x10^5$ cells were spun onto glass slides using a Shandon Cytospin3 (ThermoShandon, Runcorn, UK) at 350rpm for 6 minutes. Slides were air dried before fixing in methanol at room temperature for 10 minutes. Fixed slides were stained by the Romanovsky method using Rapi-Diff II (BIOS Europe Limited, Lancashire, UK) and coverslips secured in place with DPX (BDH Laboratory Supplies, Poole, UK).

2.3.5.3 Lung and lymph node collection

Once BAL had been performed the lungs were carefully removed from the thorax, *en bloc* with the heart. In some experiments the lymph nodes draining the lungs were harvested to study the response of local T cells. For these purposes mediastinal lymph nodes were collected and placed in 1.5 ml centrifuge tubes containing complete medium, where they were stored on ice until further processing. In experiments where lung histology was required lungs were inflated with 10% neutral buffered formalin (NBF, Sigma) via the trachea using a 1 ml syringe and 23G needle sheathed with polythene tubing, as used for BAL. The trachea was then tied off with thread and the lungs immersed in 10% NBF for later analysis.

2.3.5.4 Bone marrow collection

Mice aged 6-8 weeks were killed by cervical dislocation and the femur and tibia aseptically removed from both legs and placed in complete medium on ice until further processing. Under sterile conditions the proximal and distal ends were removed and the shaft flushed with RPMI 1640 medium using a 0.5 ml syringe with a prefitted 29G needle (MONOJECT insulin syringe, Covidien, Ireland). The cells were then dispersed by passage through a 100 µm nylon monofilament gauze (Cadisch Precision Meshes, London) and washed twice in RPMI 1640 medium by centrifugation at 1400 rpm for 5 minutes at 4°C (Jouan CR3i centrifuge). Cells were resuspended in complete medium before being counted in a haemocytometer.

2.3.6 In vitro *culture*

All cell culture work was carried out under sterile conditions and wash steps were performed using sterile RPMI 1640 medium followed by centrifuge at 1400 rpm for 5 minutes at 4°C (Jouan CR3i centrifuge) unless otherwise stated. Live cell counts were performed in a haemocytometer using a between a 1:2 and 1:10 dilution with 0.4% trypan blue solution. Cultures were incubated at 37°C in a humidified incubator supplemented with 5% CO_2.

2.3.7 Cell lines

The human HMC-1 mast cell line was kindly donated by Dr J.H. Butterfield (Mayo Clinic, USA) and maintained in tissue culture flasks in complete IMDM. The murine P815 mast cell line was obtained from American Type Culture Collection, USA and maintained in tissue culture flasks in standard complete medium. Cells were split approximately twice per week.

2.3.8 Bone marrow mast cell culture

Bone marrow cells were prepared at a concentration of $1x10^6$ cells/ml in complete medium and cultured in tissue culture flasks. Medium was supplemented with stem cell factor (PeproTech, London, UK) at 50 ng/ml and IL-3 (PeproTech, London, UK) at 1 ng/ml. Twice per week, one third of the culture medium was aspirated and centrifuged at 1400 rpm for 5 minutes at 4°C (Jouan CR3i centrifuge). The medium was discarded and the cells resuspened in the same volume of complete medium containing stem cell factor (SCF) at 50 ng/ml and IL-3 at 1 ng/ml. Once a week cell counts were performed and the volume in each flask adjusted to maintain a concentration of between $0.5x10^6$ and $1.5x10^6$ cells/ml.

2.3.9 IL-33 stimulation

Mast cells were cultured at a concentration of $1x10^6$ cells/ml in 24, 48 or 96 well culture plates. Recombinant murine IL-33 was added to give the final desired concentration, medium alone was used in control cultures. Each condition was replicated in duplicate or triplicate. After the desired culture period medium was aspirated. In some cases the entire plate was centrifuged at 1400 rpm for 5 minutes at 4°C (Jouan CR3i centrifuge) before supernatants were aspirated, in other cases the medium was aspirated into separate 1.5 ml tubes which were then centrifuged prior to supernatants being aspirated. All samples were stored frozen until analysed.

2.3.10 Lymph node culture

Lymph nodes were prepared by crushing under 100 µm nylon monofilament gauze (Cadisch) with a 5 ml syringe plunger in RPMI 1640 medium. Single cell suspensions were made by passing through nylon gauze into 25 ml centrifuge tubes before being washed twice. Cells were resuspended in complete medium and counted. Cells were placed in 24 well culture plates at a final concentration of $1x10^6$ cells/ml and stimulated with medium alone or sterile OVA at final concentration of 1 mg/ml. After 72 hours of incubation supernatants were aspirated frozen for later analysis.

To asses proliferation, lymph nodes cells were prepared and stimulated as above in triplicate for 48 hrs in round-bottomed 96 well tissue culture plates (200 µl per well). 15kBq of Tritiated [^{3}H]-thymidine (West of Scotland Radionuclide Dispensary, Glasgow, UK) in 10µl sterile wash medium was added for the last 8 hours of culture. Cells were harvested onto glass fibre filter paper (PerkinElmer, Beaconsfield, UK) using a 295-0054 Betaplate 96 well harvester (PerkinElmer). [^{3}H]-thymidine incorporation was measured using a MicroBeta TriLux scintillation counter (PerkinElmer). All procedures were performed according to local radiation safety guidelines.

2.4 Assays and analysis

2.4.1 Flow cytometry [FACS]

Prior to analysis mast cell numbers were counted and $1x10^5$ cells added to FACS tubes (BD Biosciences, Oxford, UK) and kept on ice. Cells were centrifuged at 1400 rpm for 5 minutes at 4°C (Jouan CR3i centrifuge) and washed in 1 ml FACS buffer before being suspended in a final volume of 40 µl FACS buffer. 1 µl of Fc block (anti- CD16/CD32, BD Biosciences) was added to each tube and incubated for 5 minutes. Fluorochrome labelled anti-ST2, anti-FcεRIα and anti-c-kit antibodies were added as detailed in Table 2.2. Full compensation was carried out before any FACS analysis and therefore a number of tubes were prepared with isotype controls as shown in Table 2.3 using mature mast cells. Antibodies

were incubated for 30 minutes on ice in the dark, after which cells were washed in 1 ml of FACS buffer before being resuspended in 400 µl of FACS buffer. Prior to analysis 3 µl of 7-Amino-actinomycin D (7-AAD, Via-Probe, BD Biosciences) was added to each tube to enable exclusion of dead cells.

Samples were analysed using a FACSCalibur flow cytometer (BD Biosciences) and data acquired using CellQuest software (BD Biosciences). Live cells were gated according to FSC and SSC using the sample "cells". 7-AAD positive dead cells were then excluding by gating using samples "cells" and "via". Four quadrant plots were then made with each combination of FL1 (FITC), FL2 (PE) and FL4 (APC). Using sample "iso" gates were drawn so that less than 2% of total cells were falsely positive on any axis. Using the single stained samples "ST2", "Fcε" and "c-kit" compensation was carried out for emission overlap. The sample "no ST2" was run as a final isotype control for detailed analysis of ST2 expression. All data analysis was carried out using the FlowJo package (Tree Star Inc., Oregon, USA).

Specifcity	Label	Volume	Supplier	Cat No.	Clone	Isotype
ST2	FITC	2 ul	MDB	101001F	DJ8	Rat IgG1
isotype	FITC	4 ul	BD	BD553924	R3-34	Rat IgG_1
FcεRIα	PE	1 ul	EB	12-5898-81	MAR-1	Hamster IgG
isotype	PE	1 ul	BD	BD553972	A19-3	Hamster IgG1
c-kit	APC	1 ul	BD	BD553356	2B8	Rat IgG2b, κ
isotype	APC	1 ul	BD	BD556924	A95-1	Rat IgG_{2b}

Table 2.2 – FACS antibodies

Key - MDB = MD Biosciences, Zürich, Switzerland. BD = BD Biosciences, Oxford, UK.
EB = EBioscience, Hatfield, UK.

Label	FITC Antibody	PE Antibody	APC Antibody	7AAD
cells	-	-	-	-
via	-	-	-	3 µl
iso	isotype	isotype	isotype	-
ST2	anti-ST2	isotype	isotype	-
Fcε	isotype	anti-FcεRIα	isotype	-
c-kit	isotype	isotype	anti-c-kit	-
no ST2	isotype	anti-FcεRIα	anti-c-kit	3 µl

Table 2.3 – Samples used in FACS compensation

2.4.2 Cytokine immunoassay

Enzyme linked immunosorbant assays (ELISAs) were performed using paired antibodies or kits. Protocols and reagents differed depending on manufacturer. Table 2.4 details antibodies used. Buffers used are as detailed in Table 2.1.

Analyte	Supplier	Capture (ug/ml)	Detection (ug/ml)	Sensitivity (pg/ml)
Murine				
IL-4	BD	2	2	20 - 20,000
IL-5	BD	4	4	10 - 10,000
IL-6	BD	1	1	10 - 10,000
IL-13	R&D	Kit	Kit	5 - 5,000
TNFα	BD	Kit	Kit	4 - 4,000
IFNγ	BD	1	1	40 – 40,000
MIP-1α	R&D	Kit	Kit	5 - 5,000
Eotaxin-1	R&D	0.8	0.4	5 - 500
Eotaxin-2	R&D	2	0.075	4 - 4,000
TARC	R&D	2	0.2	5 - 1,000
RANTES	R&D	2	0.4	5 - 1,000
OVA-IgE	BD	-	2	-
OVA-IgG1	BD	-	0.5	-
OVA-IgG2a	BD	-	0.5	-
Human				
TNFα	Invitrogen	Kit	Kit	4 - 4,000
IL-5	R&D	Kit	Kit	3 - 3,000
MIP-1α	R&D	Kit	Kit	5 - 2,000
Eotaxin-1	R&D	Kit	Kit	5 - 5,000

Table 2.4 – ELISA antibodies and concentrations

The protocol that follows is for antibody pairs from BD Biosciences. Unless stated otherwise all reagents and samples were added at a final volume of 50 µl/well. Immulon 4 HBX plates (Thermo Labsystems, Franklin, USA) were coated with detection antibody in BD ELISA coating buffer and incubated overnight. Plates were washed three times with ELISA wash buffer then incubated with 200 µl/well of assay buffer for 1 hour at 37°C. After washing three times samples and standards were added. An eleven point doubling dilution standard curve and a blank were employed, both in duplicate and using assay buffer. After incubation for 2 hours at room temperature plates were washed five times before biotin-conjugated detection antibody in assay buffer was added to each well. After 1 hour at room temperature plates were washed three times and Streptavidin-HRP conjugate (Extravidin, Sigma), diluted 1:1000 with assay

buffer, was added. After 30 mintes plates were washed three times and 100 µl/well of TMB substrate (KPL, Gaithersburg, USA) added. Plates were incubated in the dark for up to 30 minutes or until sufficient blue colour had developed. Optical density was read on an MRX II microplate reader (Dynex Technologies, Worthing, UK) at 630 nm running Revelation (Dynex Technologies) software, and sample values calculated from the standard curves generated.

The protocol for antibodies from R&D (Abingdon, UK) was as above but the R&D ELISA assay buffer (Table 2.1) and Streptavidin-HRP conjugate provided by R&D and diluted to 1:200 were used. In addition the detection antibody was incubated for 2 hours rather than 1. The Eotaxin-2 detection antibody was diluted in the R&D ELISA assay buffer with the addition of 2% heat-inactivated normal goat serum.

The protocol for antibodies from Invitrogen was as for the BD ELISA but the following modifications. Invitrogen ELISA assay buffer was used. The sample and detection steps were combined, with 50 µl/well of sample or standard added to each well followed immediately by 25 µl/well of detection antibody in assay buffer and then a 2 hour incubation. Streptavidin-HRP conjugate (Invitrogen) was used at a dilution of 1:200 in assay buffer.

2.4.3 Ovalbumin-specific immunoassay

OVA-specific IgE ELISA was carried out using BD ELISA assay buffer and volumes of 50 µl/well. Immulon 4 HBX plates (Thermo) were coated with OVA 10 µg/ml in OptEIA buffer and incubated overnight. Plates were washed three times with ELISA wash buffer then incubated with 200 µl/well of assay buffer for 1 hour at 37°C before being washed three times again. In the absence of any standard for OVA specific immunoglobulins, serial dilution curves were obtained for each sample using assay buffer as a diluent. An initial sample dilution of between 1:10 and 1:40 was used with serial 1:2 or 1:3 dilutions thereafter. After incubating for 2 hours at room temperature plates washed five times. Detection antibody and Streptavidin-HRP conjugate (Enzyme Concentrate Avidin-HRP, BD Biosciences) were both diluted 1:250 together in assay buffer and 50 µl added to each well. After 1 hour plates were washed three times and 100 µl/well of TMB substrate (KPL) added. Plates were incubated in the dark for up to 30 minutes or until

sufficient blue colour had developed. Optical density was read on an MRX II microplate reader (Dynex Technologies) at 630 nm running Revelation (Dynex Technologies) software.

OVA-specific IgG1 and IgG2a ELISA were carried out with an identical protocol with the following modifications. Initial sample dilution for IgG1 was 1:500 with 1:3 serial dilutions and for IgG2a between 1:20 and 1:50 with 1:2 dilutions. Detection antibody was added alone as a separate step with a one hour incubation, followed by washing and the addition of Streptavidin-HRP conjugate (Sigma) incubated for 30 minutes.

2.4.4 Multiplexed immunoassay

Multiplexed immunoassay is a method by which multiple cytokines or other mediators are measured simultaneously in a small volume of fluid. This is achieved by using latex microbeads that are stably labelled with a characteristic mix of two fluorochromes and have an antibody to one specific analyte covalently attached to the surface. When incubated in the test solution, the antibody will bind its target cytokine in proportion to its concentration, and bound cytokine after washing can be detected using a biotin-labelled specific antibody in a sandwich-style immunoassay. Each bead can then be identified by its intrinsic twin fluorescent characteristics by flow cytometry. A streptavidin-labelled third fluorochrome is used to quantify the antibody bound to on each bead which will be propotional to the concentration of the analyte in the test solution. This system can be made to quantify multiple ligands by using microbeads with different intrinsic fluorescent characteristics that are bound to mediator specific antibodies.

Biosource Multiplex kits LHC0009 (Cytokine Human 25-Plex), LMC0006 (Cytokine Mouse 20-Plex) and LMC1031 (RANTES Mouse Singleplex) were used according to the manufacturer's instructions. All reagents and buffers were sourced from the kits. Briefly, the supplied 96 well filter plate was pre-wetted with Wash solution before aspiration of well contents using vacuum apparatus. If more than one kit was being used, beads were premixed. Otherwise beads were resuspended and 50 µl bead mixture added per well and washed twice by the addition and aspiration of Wash solution from the wells. 50 µl/well Incubation Buffer was

added followed by 100 µl/well standard or 50µl Assay Diluent plus 50µl sample. Plates were protected from the light by aluminium foil and incubated on shaking apparatus at room temperature for 2 hours. The wells were then washed twice and 100 µl/well biotinylated detection antibody added and incubated for 1 hour as above. Following two more washes, 100 µl/well Steptavidin-RPE was added and incubated for 30 minutes as above. The wells were washed three more times before resuspending the beads in 100 µl/well Wash solution and acquiring data on a Luminex 100TM analyser(Luminex Corp., Texas, USA). Data was analysed using Luminex software (Luminex Corp.).

2.4.5 Mast cell degranulation assay

Degranulation of BMMCs in response to the calcium ionophore ionomycin (Sigma) was assessed by the measurement of ß-hexosaminidase release. Cells were added to 96 well plates at a concentration of $1x10^6$ cells/ml. PBS or ionomycin at a final concentration of 1 µg/ml was added and the plates incubated at 37°C for 30 minutes. The plates were centrifuged at 1400 rpm for 5 minutes at 4°C (Jouan CR3i centrifuge) and the supernatants aspirated. The cell pellets were lysed by the addition of 1% Triton-X (Sigma) in PBS. 25 µl of supernatant or lysate was then placed in each well of an Immulon 4 HBX plate (Thermo Labsystems) and 50 µl of *p*-nitrophenyl-*N*-acetyl-ß-d-glucosamine (NAG,Sigma) substrate solution 1mM in 0.05M citrate buffer was added. After incubation in the dark at 37°C for 1 hour the reaction was quenched by the addition of sodium carbonate buffer 150 µl/well. Optical density was read on an MRX II microplate reader (Dynex Technologies) at 405 nm running Revelation (Dynex Technologies) software.

2.4.6 Lung lavage analysis

Differential cell counts were performed on cytospin preparations from BAL samples stained and mounted as in Section 2.3.5. Slides were randomised and blinded before counting. Using a light microscope at least 400 cells per slide were identified by staining and morphological characteristics at x1000 magnification (Figure 2.1).

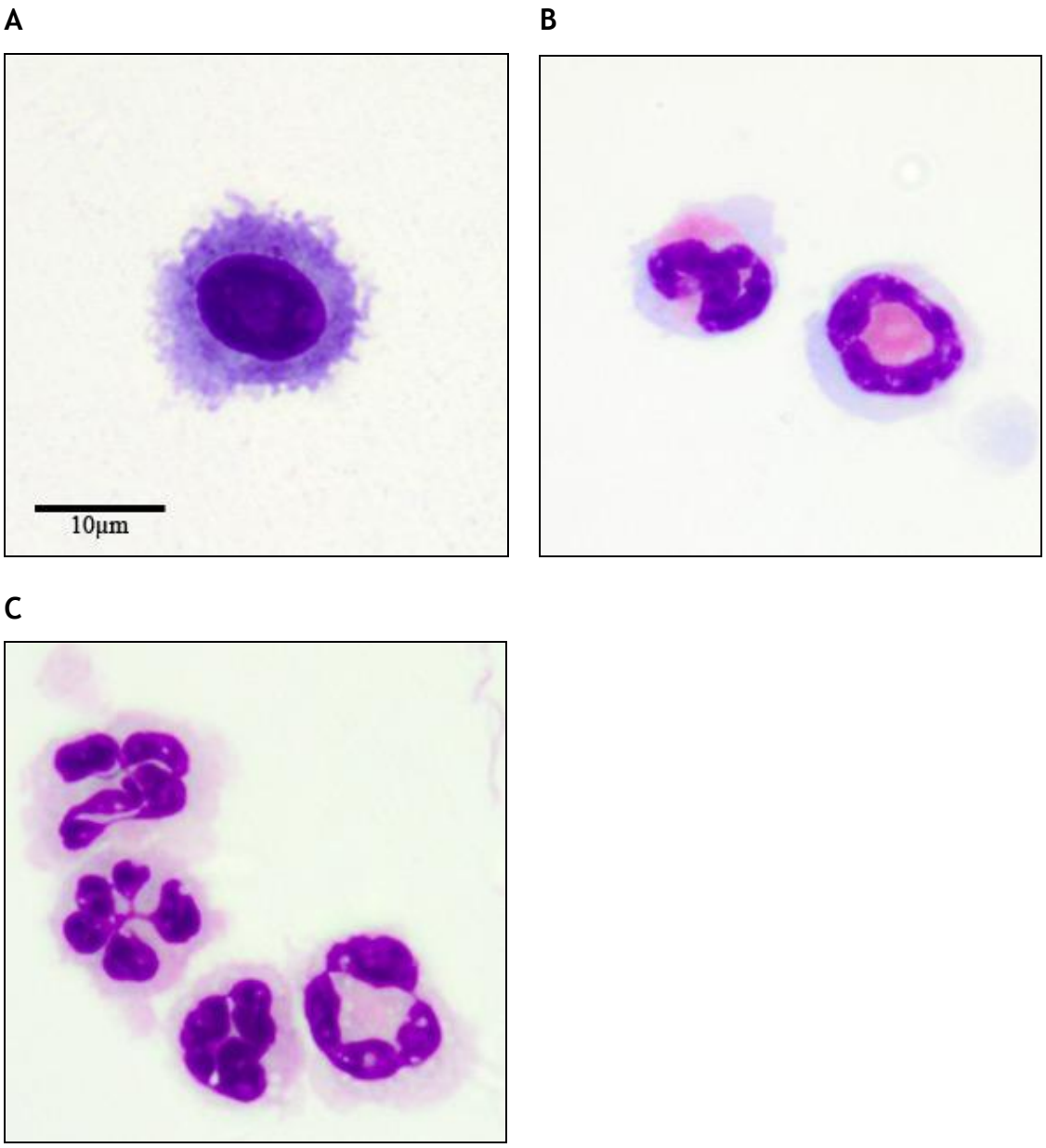


Figure 2.1 – Cytospin preparation of murine bronchoalveolar lavage

Allergic airways inflammation was induced in BALB/c mice as described in 2.3.2. BAL was collected and cytospins prepared and stained with Rapi-Diff II Romanowsky stain as described above. Shown are representative images of (**A**) a macrophage, (**B**) eosinophils and (**C**) neutrophils.

2.4.7 Lung histology

The lungs and heart were removed *en bloc* as detailed in Section 2.3.5 and placed in 10% NBF for at least 48 hours. They were then mounted in paraffin blocks, and 6 µm sections stained with haematoxylin and eosin (H&E; kindly performed by Mr. Roderick Ferrier, Department of Pathology, Western Infirmary, Glasgow). Sections were examined by light microscopy and peribronchial and perivascular inflammation assessed.

2.5 Statistics

Data were obtained from *in vitro* experiments and samples from *in vivo* experiments. Cell numbers, relative cell percentages, mediator concentrations and Penh (a dimensionless measure of AHR) were subject to statistical analysis. These are all quantitative variables which are normally distributed with equal variance. Therefore mean values were calculated and expressed with standard errors of the mean (SEM), and tests of significance performed using with 95% confidence intervals and unpaired Student's two-sample T-test, one-way ANOVA or two-way ANOVA depending on the data. A p value of less than 0.05 was considered significant. Statistical analysis was performed using Excel (Microsoft, Redmond, USA) and Minitab (Minitab Inc, State College, USA) software packages.

Prior to undertaking *in vivo* work power calculations were performed where possible to inform sample size. For example, in the model of allergic airways inflammation, experiments were designed to detect a difference of 20% in mean BAL eosinophil percentage with a significance of 0.05 and a power of 0.95. From preliminary experiments the standard deviation of the BAL eosinophil percentage is 10%. As shown in Figure 2.2 a sample size of 8 mice per group is required.

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 = 10


            Sample  Target
Difference    Size   Power  Actual Power
        20       8    0.95      0.960221

The sample size is for each group.
```

Figure 2.2 - Power calculation for the sample size required in experimental asthma

Chapter 3

The role of mast cell ST2 and the effect of activation by IL-33

In this chapter I will investigate the effect of IL-33 on mast cell lines before examining the role of ST2 in mast cell development and the effect of IL-33 on wild type and ST2 gene knock-out bone marrow mast cells.

3.1 Introduction

Initial studies of ST2 focused on fibroblasts, where the transcripts were first identified(321). Following the discovery that ST2 is selectively expressed on Th2 cells but not Th1 cells(359,360,365), T cells and Th2 inflammation became a major focus of ST2 research. Mast cells, however, received relatively little attention in studies of ST2.

Not only do mast cells express ST2 from a very early stage of cell development(380) but expression on mature mast cells appears to be ubiquitous and at high levels(348,381). Despite this, it has been reported that $ST2^{-/-}$ mice have normal mast cell numbers and that histamine and IL-4 production is not impaired in mast cells generated from such mice(381). It seems likely that ST2 is a functional receptor on mast cells as treatment with IL-33 induces NFκB, Erk1/2, p38, and JNK phosphorylation(323).

In an attempt to understand the role of ST2 on mast cells I set out to generate bone marrow derived mast cells (BMMCs) from wild type and $ST2^{-/-}$ mice and to study the effect of IL-33 on these cells. My hypothesis is 'IL-33 stimulates BMMCs to release cytokines and chemokines'.

3.2 The role of ST2 on human and murine mast cell lines

Before attempting to establish a bone marrow culture system I sought to obtain proof of concept utilising mast cell lines. The HMC-1 human mast cell line is immortalised due to a constitutively active form of c-kit(433), the stem cell factor (SCF) receptor. Although it does not express receptors for IgE(434) it has been widely used in studies of mast cells. The P815 murine mast cell line was described over 50 years ago(435) and like the HMC-1 cell line has a constitutively active form of c-kit(436) and also lacks the high affinity IgE receptor, FcεRI(437).

3.2.1 IL-33 induces cytokine production by human HMC-1 cells

First I attempted to establish if mast cells could be induced to release cytokines when stimulated with IL-33. HMC-1 cells were harvested when they reached confluence and stimulated with increasing concentrations of recombinant human IL-33 (hIL-33). In order to establish a time course, supernatants were harvested after 3, 24 and 48 hours of culture. Since activated mast cells are capable of releasing both pre-formed and newly synthesised TNFα(438) and mast cell derived TNFα appears to be important in asthma(33,163), I chose to measure TNFα release by ELISA. IL-33 induced a dose dependent rise in TNFα concentration which was significant at 24 hours but not 48 hours (Figure 3.1). TNFα was detected at higher concentrations the longer the incubation period, although this effect was also evident in the absence of IL-33, suggesting that there is a basal level of TNFα production which is enhanced by IL-33. In summary the optimum activation of mast cells with IL-33 was achieved after 24 hours.

To examine the possibility that the IL-33 induced increase in TNFα concentration was due to a pro-proliferative effect, cell counts were performed at each time point for each concentration. IL-33 had no effect on cell numbers following 48 hours of culture (Figure 3.1D) and consequently the effect of IL-33 on TNFα release was preserved even when calculated as pg/10^5 cells (Figure 3.1E). In addition there was no effect on cell viability (data not shown).

Given that the administration of IL-33 *in vivo* results in the accumulation of eosinophils(323) I looked for IL-33 induced HMC-1 production of IL-5 and eotaxin, which have been shown to play a key role in eosinophil maturation, mobilization and chemotaxis(74). IL-33 did not induce the release of eotaxin or IL-5 at detectable levels after 48 hours of culture (Figure 3.2). IL-5 and eotaxin were also undetectable after 3 or 24 hours of culture with IL-33 (data not shown).

To determine the optimum dose of IL-33 and duration of treatment for further study a more complete dose response was characterised using between 0.01 and 100 ng/ml of hIL-33. As TNFα had been almost undetectable following 3 hours of culture samples were only collected at 24 and 48 hours. As shown in Figure 3.3 a dose response curve was obtained which was most robust at 24 hours, with evidence of a significant response from 10 ng/ml of IL-33.

Although there was evidence of a dose responsive release of TNFα by IL-33 stimulated mast cells, levels are low, rising to just above 100 pg/ml following 48 hours of stimulation with 100 ng/ml of IL-33. This may reflect the fact that HMC-1 mast cells appear to be relatively poor producers of TNFα(439). Consequently I used multiplexed immunoassay as a screening tool to look for the production of other cytokines or chemokines by HMC-1 cells stimulated with IL-33. IL-33 induced the production of the chemokines MIP-1α, MIP-1ß and MCP-1 at ng/ml levels and TNFα, IL-6 and IL-8 at lower levels (Figure 3.4). IL-1ß, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13, IL-15, IL-17, IFNα IFNγ, eotaxin, RANTES, IP-10, MIG and GM-CSF were not induced at appreciable levels (data not shown).

Although MCP-1 was produced at the highest levels baseline production in the absence of IL-33 stimulation was also high, meaning that IL-33 induced just over a twofold rise in MCP-1 production. MIP-1α was also produced at high levels, but as baseline production was lower the rise represent a 9 fold increase in MIP-1α production. In order to characterise the IL-33 induced production of MIP-1α a full dose response curve was obtained (Figure 3.5). Although levels of IL-33 induced MIP-1α were highest following 48 hours of culture, baseline MIP-1α production was also higher and a significant response to IL-33 was only observed at concentrations of 10 ng/ml and above. Following 24 hours of culture a better dose response curve was obtained with significant MIP-1α induction over baseline observed with concentrations of IL-33 as low as 1 ng/ml.

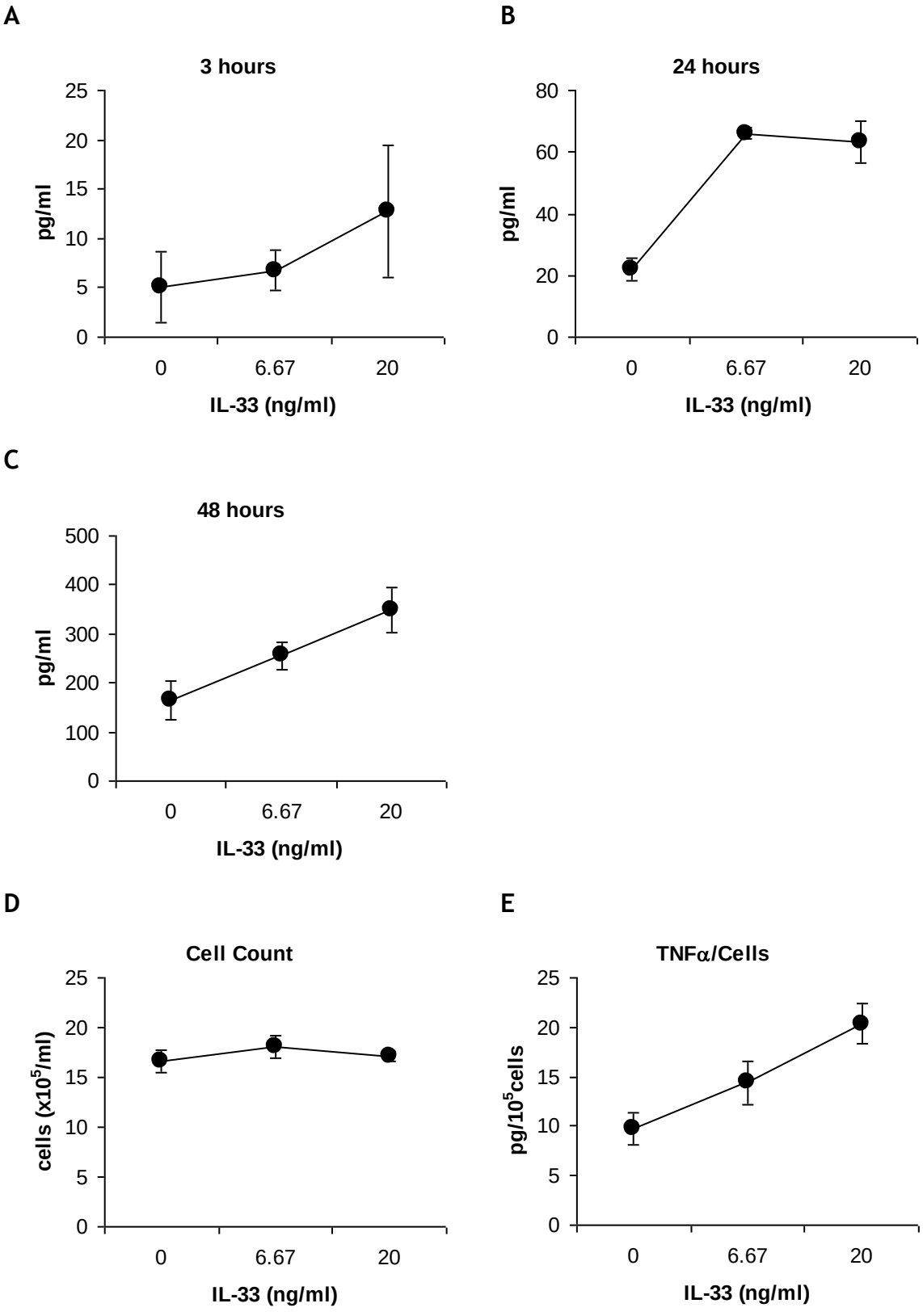


Figure 3.1 – IL-33 induces TNFα production by HMC-1 cells

HMC-1 cells were harvested and washed when they reached confluence before being plated into 24 well plates in duplicate. hIL-33 was added at 0, 6.7 or 20 ng/ml and aliquots of the supernatant were harvested at (**A**) 3, (**B**) 24 and (**C**) 48 hours. TNFα was measured by ELISA. At 48 hours cell numbers were counted and cell concentration (**D**) and TNFα production per cell (**E**) calculated. Results are shown as the mean of duplicate culture wells +/- SEM. One-way ANOVA $p<0.05$ for the effect of IL-33 on TNFα at 24 hours, not significant at 3 and 48 hours.

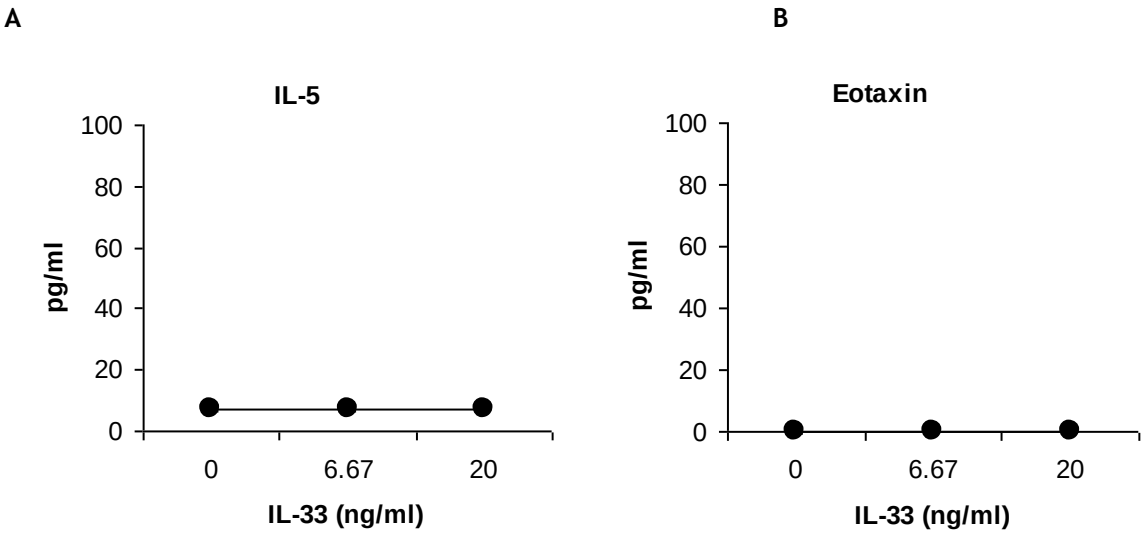


Figure 3.2 – IL-33 does not induce IL-5 or eotaxin production by HMC-1 cells

HMC-1 cells were cultured with hIL-33 as in Figure 3.1 and supernatants were collected after 48 hours. IL-5 (**A**) and eotaxin (**B**) were measured by ELISA. Results are shown as the mean of duplicate culture wells +/- SEM.

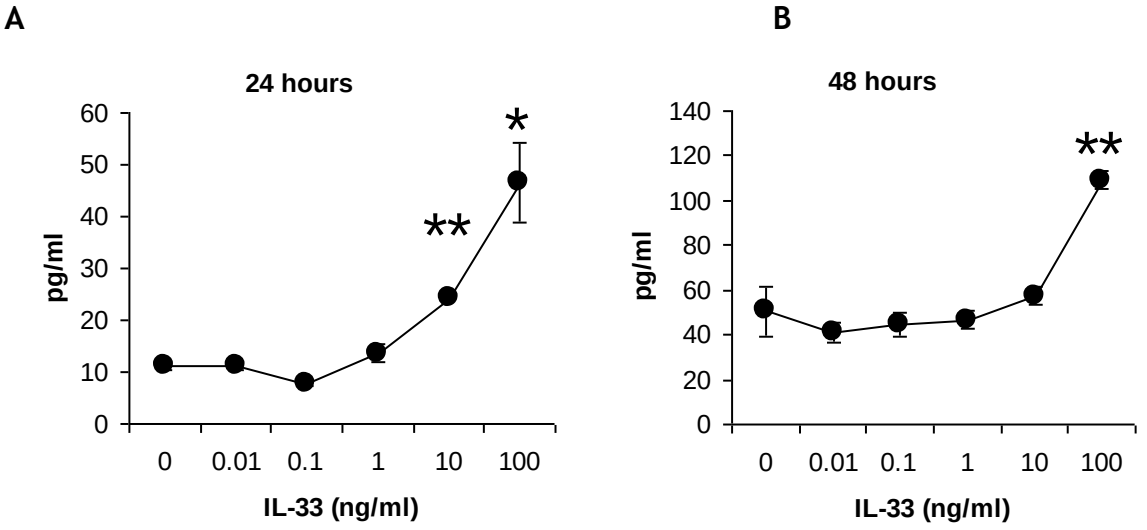


Figure 3.3 – IL-33 induced TNFα production by HMC-1 cells is dose dependent

HMC-1 cells were harvested when they reached confluence and were washed before being plated into a 24 well plate in triplicate. hIL-33 was added at between 0.01 and 100 ng/ml and aliquots of the supernatant were harvested at 24 (**A**) and 48 (**B**) hours. TNFα was measured by ELISA. Results are shown as the mean of triplicate culture wells +/- SEM. * $p<0.05$, ** $p<0.01$ compared with PBS.

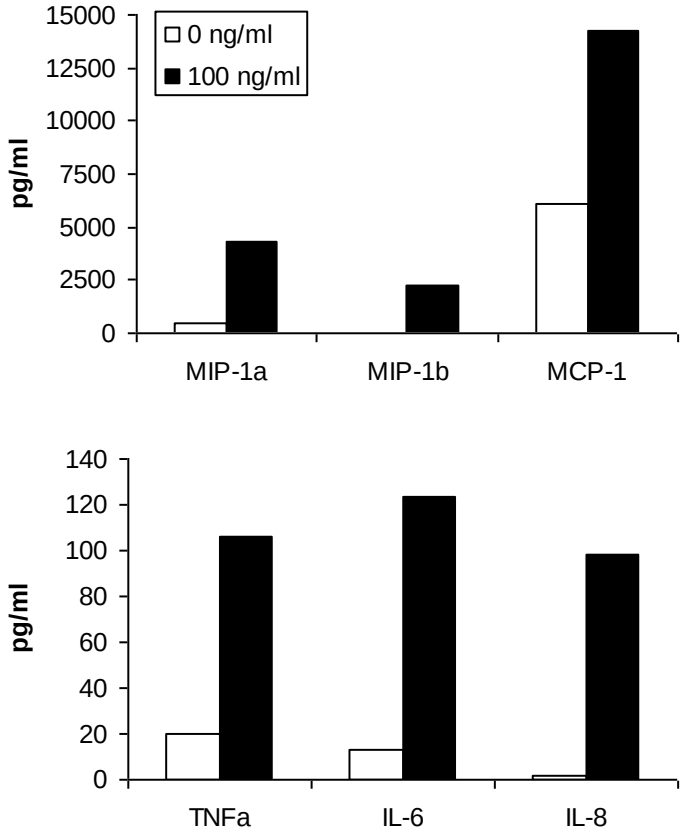


Figure 3.4 - IL-33 induces the production of cytokines and chemokines by HMC-1 cells

HMC-1 cells were cultured with 0 or 100 ng/ml of hIL-33 as in Figure 3.3 and supernatants were collected after 48 hours. MIP-1α, MIP-1ß, MCP-1, TNFα, IL-6 and IL-8 were measured by multiplexed immunoassay. Results are from single samples.

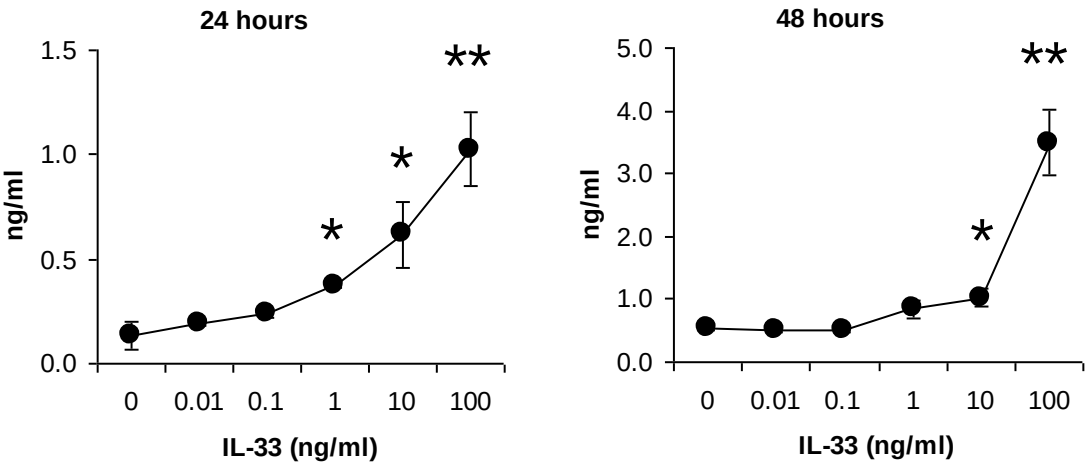


Figure 3.5 - IL-33 induced MIP-1α production by HMC-1 cells is dose dependent

HMC-1 cells were cultured with between 0.01 and 100 ng/ml of hIL-33 as in Figure 3.3 and aliquots of the supernatant were harvested at 24 and 48 hours. MIP-1α was measured by ELISA. Results are shown as the mean of triplicate culture wells +/- SEM. * $p<0.05$, ** $p<0.01$ compared with PBS.

3.2.2 IL-33 induces cytokine production by murine P815 cells

The data above demonstrate that IL-33 is able to induce dose dependent chemokine and cytokine production from a human mast cell line. I hypothesised that this effect was not species specific and that a similar effect would be observed in a murine cell line. I set out to test this hypothesis using cells of the P815 murine mast cell line, which has been shown to express ST2 on the surface(348). Before carrying out further *in vitro* studies I sought not only to confirm that P815 cells expressed ST2 on their surface in my hands, but also to validate my FACS reagents and technique for the measuring the expression of ST2. P815 cells were freshly isolated from culture and stained with ST2-FITC antibodies before being analysed by FACS. These unstimulated P815 cells grown under standard culture conditions were uniformly ST2 positive (Figure 3.6).

Given that the response to IL-33 was most evident after 24 hours of stimulation in HMC-1 cells this time point was also chosen in the study of P815 cells. Although there was no evidence of cytokine production at 3 hours in HMC-1 cells, this time point was also studied to examine the possibility of early cytokine release from P815 cells. Our group have previously found that human IL-33 is bioactive on murine T cells *in vitro* and so in these preliminary experiments hIL-33 was used.

P815 cells cultured in the presence of hIL-33 showed a similar pattern of TNFα production to HMC-1 cells with evidence of a dose response but with only low levels detected (Figure 3.7A). In contrast to HMC-1 cells there was no detectable MIP-1α at 24 hours at even the highest concentration of IL-33 (Figure 3.7B). IL-6 on the other hand was produced at high levels with a significant response to concentrations of IL-33 as low as 0.1 ng/ml (Figure 3.7C). Once again I looked for the production of cytokines associated with eosinophilia but found no evidence of IL-5, eotaxin-1 or eotaxin-2 production (Figure 3.7D,E,F). At 3 hours all tested cytokines and chemokines were undetectable (data not shown).

These data demonstrate that a murine mast cell line responds to IL-33 in a dose dependent manner with the release of pro-inflammatory cytokines and chemokines. While there are differences between the responses in human and murine mast cells, most notably the failure of the murine mast cell line to

produce MIP-1α, it is unclear whether these differences represent a true species difference or are simply a reflection of the differences between the specific transformed cell lines.

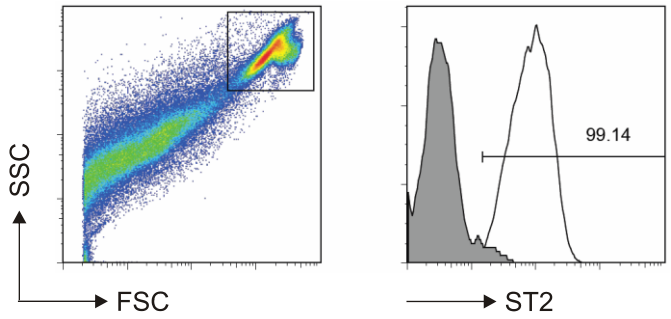


Figure 3.6 – P815 cells are ST2 positive

P815 cells were isolated from ongoing cell culture and stained with ST2-FITC (white) or isotype-FITC antibodies (grey).

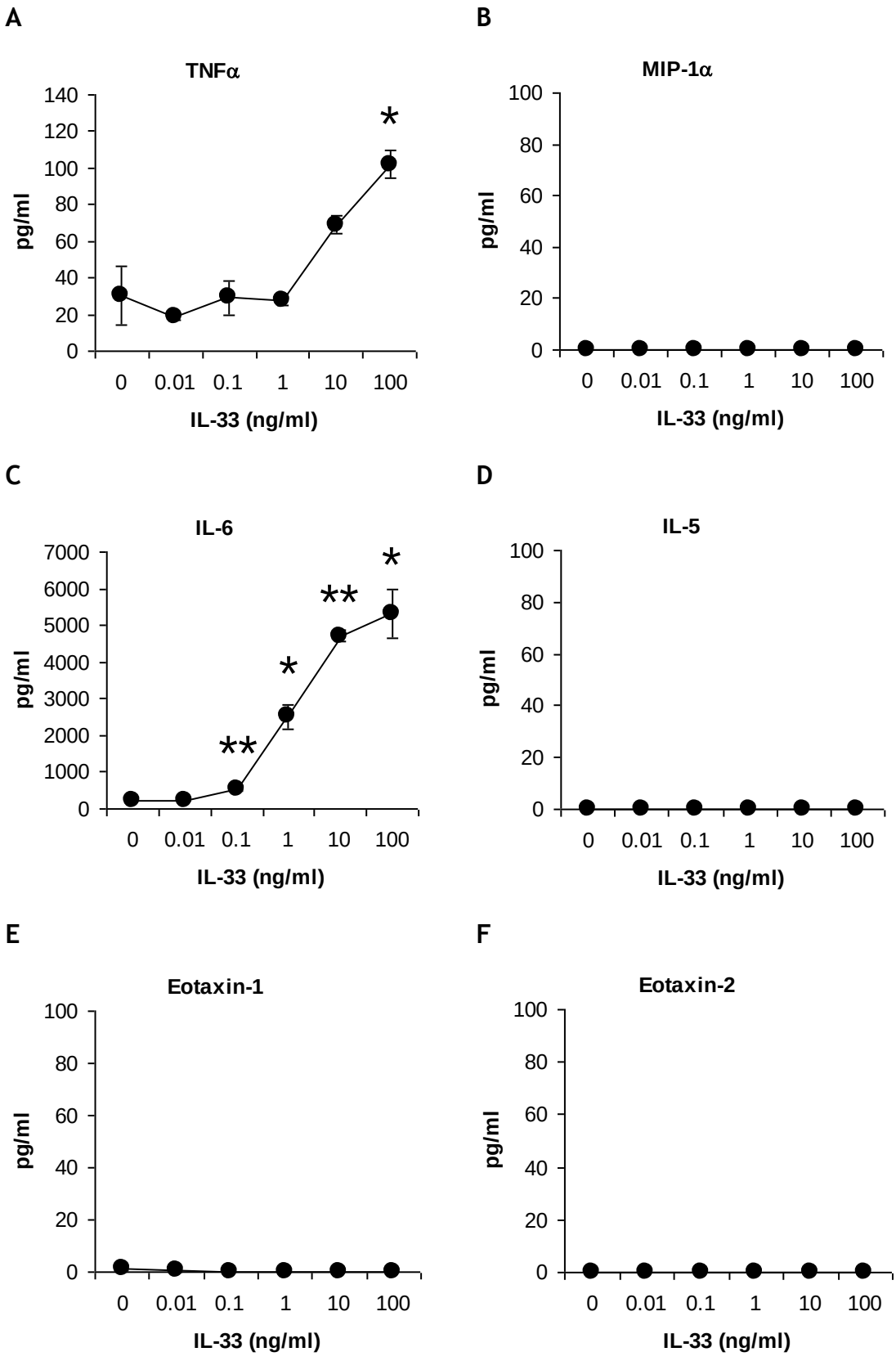


Figure 3.7 – IL-33 induces the release of TNFα and IL-6 from P815 cells

P815 cells were harvested and washed before being plated into a 24 well plate in triplicate at a concentration of $1x10^6$/ml. hIL-33 was added at between 0.01 and 100 ng/ml and aliquots of the supernatant harvested at 24. Cytokines and chemokines were measured by ELISA. Results are shown as the mean of triplicate culture wells +/- SEM. * $p<0.05$, ** $p<0.01$ compared with PBS.

3.3 Bone marrow derived mast cell culture

There were a number of reasons to study the effect of IL-33 on murine BMMCs. Most importantly, I hoped to generate BMMCs from mice lacking the ST2 receptor and thereby demonstrate a specific ligand-receptor dependence of the observed effects. Secondly, the differences seen in the responses of P815 and HMC-1 cells raised questions over how representative each cell line is of the wider population of mast cells. In the case of HMC-1 and P815 cell lines not only are they immortalised due to a constitutively activated SCF receptor but the absence of the high affinity IgE receptor, FcεRI makes them unlike most naturally occurring mast cells.

3.3.1 Bone marrow mast cell maturation

While it is possible to directly obtain mature mast cells from mice, from the peritoneum for example, the relatively small numbers obtained limit the studies possible and most investigators culture mast cells from bone marrow progenitors. Mast cells can be obtained from unrefined adult bone marrow cells by prolonged culture with mast cell growth factors, most commonly IL-3(162,348,381) with the addition of SCF in some cases (440-442). On the basis of the experience within the laboratory(A.J. Melendez, personal communication) I opted to culture murine bone marrow cells with SCF (50 ng/ml) for the first three weeks and IL-3 (1 ng/ml) throughout, a protocol which others have also used(443). Mast cell maturity was determined by expression of the α chain of the high affinity IgE receptor (FcεRIα) and the SCF receptor (c-kit). Figure 3.8 shows an example of the typical gating strategy employed.

By utilising serial FACS analysis it was possible to monitor the development of mature mast cells from bone marrow progenitors and so determine the time at which the population was fully mature. As shown in Figure 3.9 after 3 weeks of culture the majority of cultured cells (81%) express neither FcεRIα nor c-kit, although 12% are already FcεRIα$^+$c-kit$^+$. With increasing culture time the expression of FcεRIα and c-kit rises. By 6 weeks 85% of cells are c-kit positive and 74% FcεRIα$^+$c-kit$^+$. Further culture results in 99% of cells c-kit$^+$ and 89% FcεRIα$^+$c-kit$^+$ by 8 weeks. Consequently BMMCs were considered to be mature

after 8 weeks of culture, although maturation was always confirmed by the presence of at least 95% c-kit$^+$ cells by FACS.

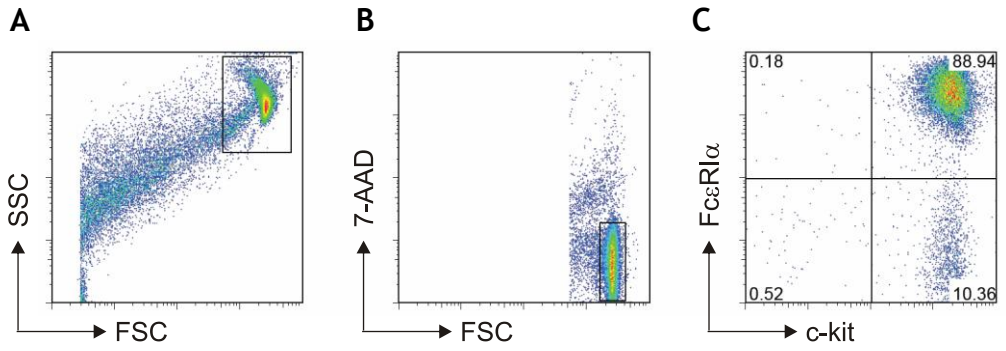


Figure 3.8 - Mature mast cells express FcεRIα and c-kit

BMMCs were harvested after 8 weeks of culture and stained with anti-FcεRIα and anti-c-kit antibodies. Cells were identified as a population with high FSC and SSC (**A**). Live cells were then gated by excluding 7-AAD positive cells (**B**). The expression of c-kit and FcεRIα on these cells was then analysed (**C**).

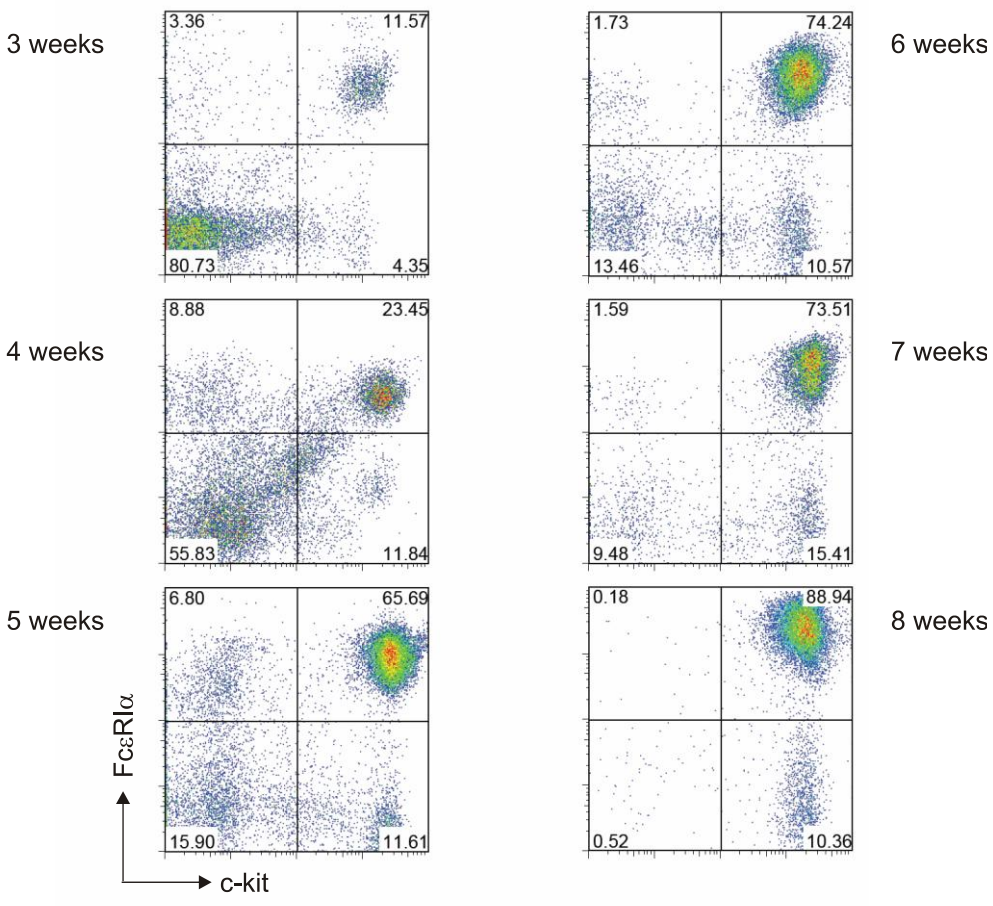


Figure 3.9 - The expression of FcεRIα and c-kit on mast cells increase with culture time

BMMCs were harvested after between 3 and 8 weeks of culture and analysed as described in Figure 3.8.

3.3.2 Expression of ST2 on bone marrow mast cells

Although other investigators have reported the presence of ST2 on the surface of BMMCs(348,380,381) I sought to confirm that ST2 was expressed on the BMMCs I had obtained using the culture protocol described. Mature, 8 week old, BMMCs are 99% ST2 positive with a high fluorescence intensity, suggesting that not only do the majority of mature BMMCs express ST2, but that they express it at a relatively high surface density (Figure 3.10). This is particularly striking when compared to other ST2 expressing cells such as T cells(359,360). It has been reported that mast cells express ST2 from a very early stage(380) and Figure 3.10 also shows that a small proportion of cells within the BMMC culture express ST2 after as little as 3 weeks of culture. As the culture progresses the proportion of $ST2^+$ cells rises.

Given the dynamic changes in the expression of FcεRIα, c-kit and ST2 during the course of BMMC culture, and the expression of ST2 on a subset of cells from an early stage, I sought to study the relationship between the expression of ST2 and the established mast cell markers FcεRIα and c-kit. When cells were gated according to their expression of ST2 (Figure 3.11) there was a very strong correlation with c-kit. After 3 weeks of culture greater than 95% of ST2- cells were $c\text{-}kit^-$ and over 90% of these were $Fc\varepsilon RI\alpha^- c\text{-}kit^-$. Greater than 95% of $ST2^+$ cells were $c\text{-}kit^+$ and over 85% of these were $Fc\varepsilon RI\alpha^+ c\text{-}kit^+$.

The corollary of this was to study the expression of ST2 by different populations of BMMCs depending on their surface expression of FcεRIα and c-kit. Figure 3.12 shows the expression of ST2 by $Fc\varepsilon RI\alpha^- c\text{-}kit^-$, $Fc\varepsilon RI\alpha^+ c\text{-}kit^-$, $Fc\varepsilon RI\alpha^- c\text{-}kit^+$ and $Fc\varepsilon RI\alpha^+ c\text{-}kit^+$ populations at different stages of BMMC culture. At no point is ST2 convincingly expressed by $Fc\varepsilon RI\alpha^- c\text{-}kit^-$ or $Fc\varepsilon RI\alpha^+ c\text{-}kit^-$ cells. $Fc\varepsilon RI\alpha^- c\text{-}kit^+$ cells have a bimodal ST2 expression with $ST2^-$ and $ST2^+$ subpopulations present from very early in the culture. $Fc\varepsilon RI\alpha^+ c\text{-}kit^+$ cells on the other hand are 100% ST2 positive at all times.

These data suggest a relationship between the expression of ST2 and of c-kit. By plotting the two surface markers against each other it possible to see the relationship more clearly (Figure 3.13).

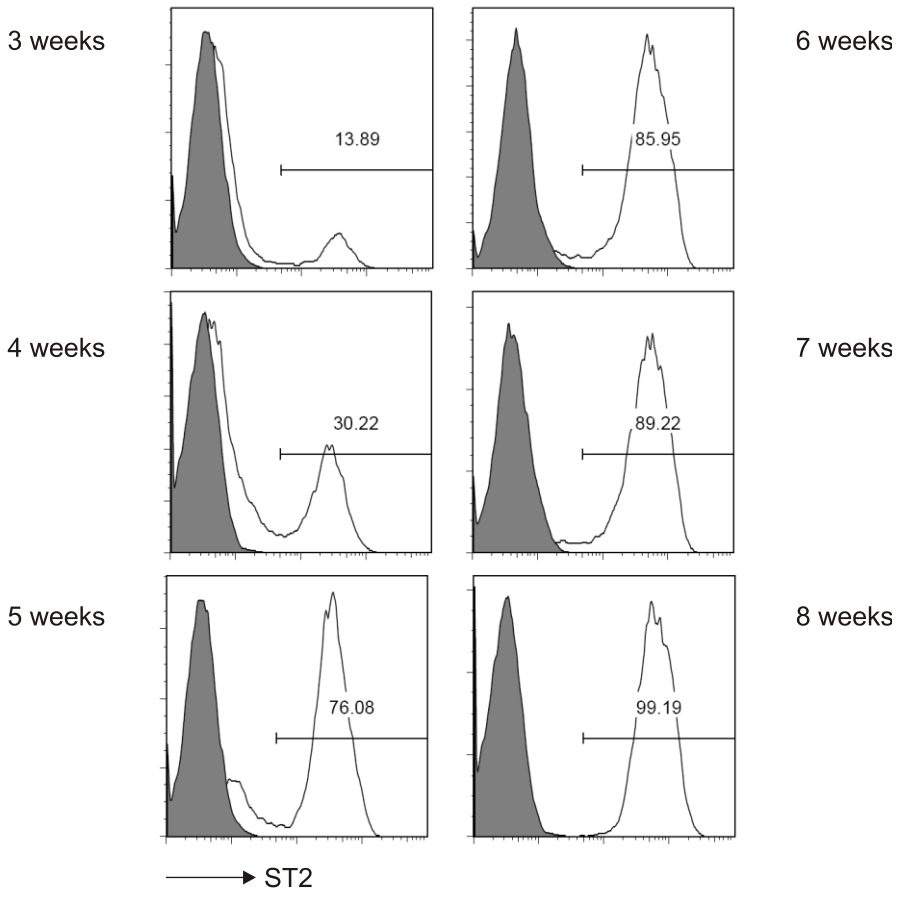


Figure 3.10 – The expression of ST2 on mast cells increases with culture time

BMMCs were harvested after between 3 and 8 weeks of culture and analysed by FACS as detailed in Figure 3.8. Cells were also labelled with anti-ST2 (unshaded) or isotype matched antibody as a control (shaded).

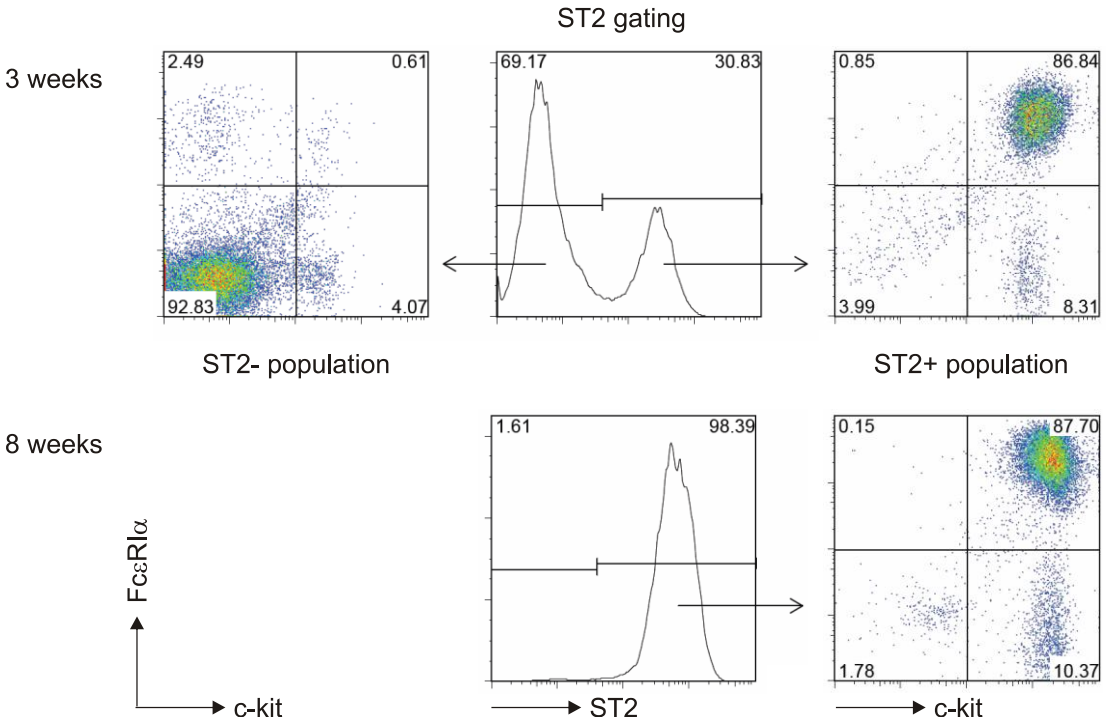


Figure 3.11 – The expression of c-kit and FcεRIα differs according to the expression of ST2

BMMCs were harvested after 3 or 8 weeks of culture and analysed by FACS as detailed in Figure 3.10. Cells were then gated according to the expression of ST2 and the expression of c-kit and FcεRIα by the ST2- and ST2^{+} populations analysed.

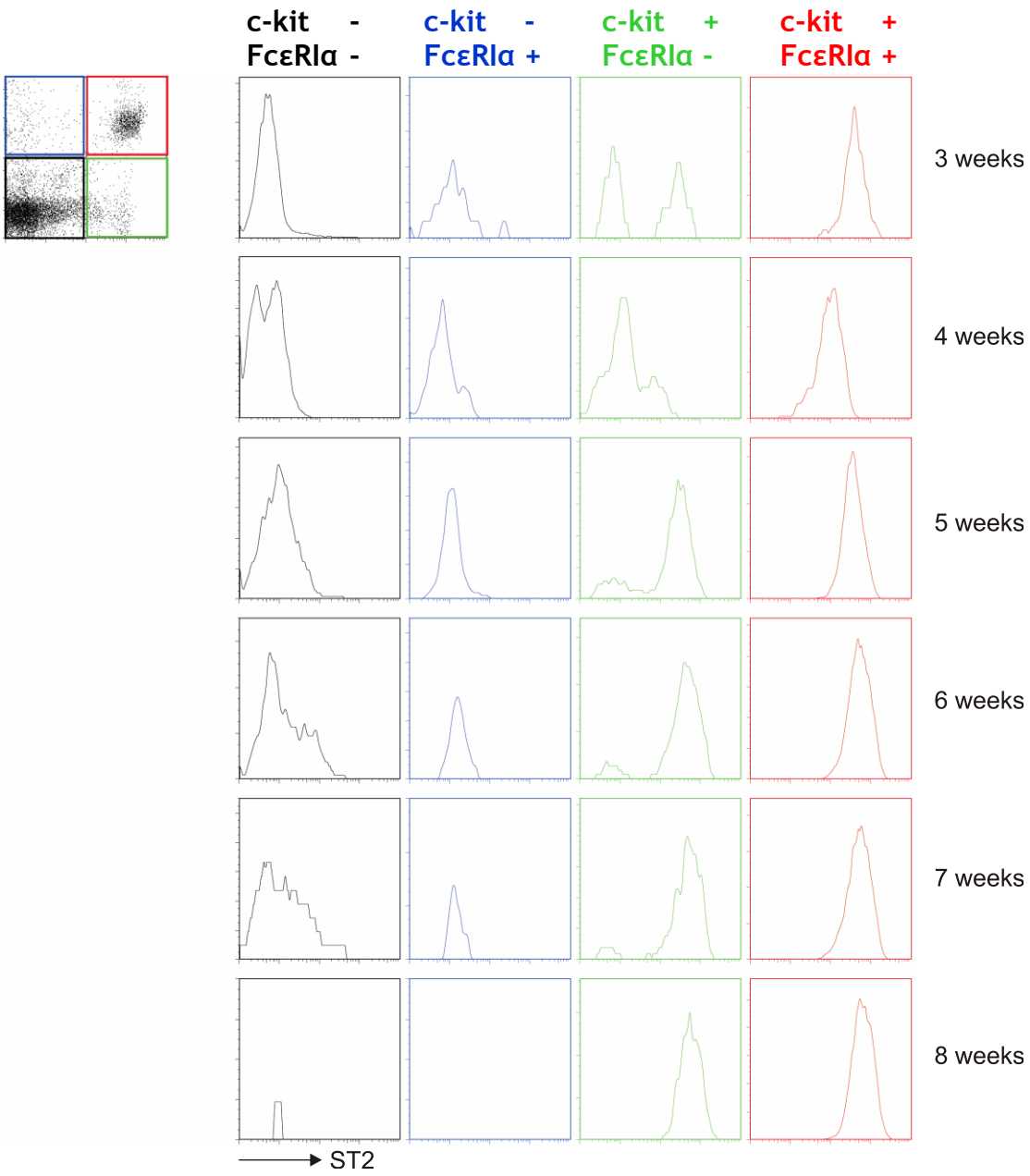


Figure 3.12 - The expression of ST2 differs according to the expression of c-kit and FcεRIα

BMMCs were harvested after between 3 and 8 weeks of culture and analysed by FACS as detailed in Figure 3.10. Cells were then gated according to the expression of c-kit and FcεRIα and the expression of ST2 by these populations analysed.

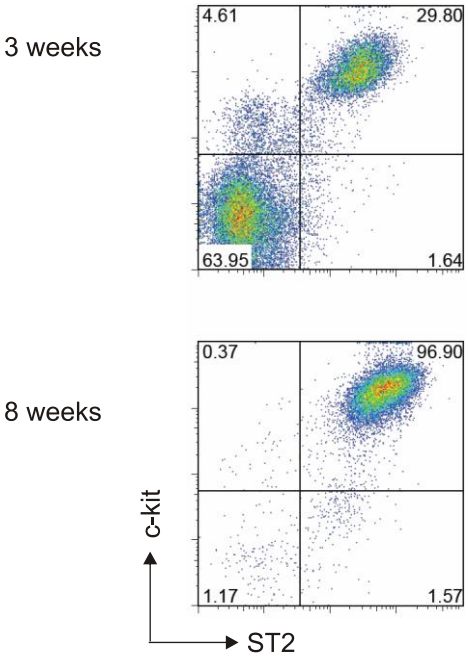


Figure 3.13 – The expression of ST2 correlates with the expression of c-kit

BMMCs were harvested after 3 or 8 weeks of culture and analysed by FACS as detailed in Figure 3.10.

3.4 ST2 deficient bone marrow mast cells

The expression of ST2 at high levels on all mast cells from an early stage in development raises the possibility that ST2 may play an important role in the development or function of mast cells. It is known that BMMCs can be generated from mice in the absence of ST2(381,382) and that such $ST2^{-/-}$ mast cells produce normal levels of IL-4 upon stimulation with PMA and ionomycin, and of histamine upon IgE crosslinking(381). However there are no data regarding the development and surface phenotype of such mast cells from $ST2^{-/-}$ mice. To investigate the possibility that the absence of ST2 would alter BMMC development, mast cells were cultured from wild type (WT) and $ST2^{-/-}$ BALB/c mice and their expansion and surface expression of FcεRIα and c-kit measured over time.

3.4.1 Growth and maturation of mast cells lacking ST2

FACS analysis of mature WT and $ST2^{-/-}$ BMMCs demonstrates that the surface expression of FcεRIα and c-kit is unaffected by the absence of ST2. WT BMMCs are 98% $c\text{-}kit^{+}$ and 85% $Fc\varepsilon RI\alpha^{+}c\text{-}kit^{+}$ and $ST2^{-/-}$ BMMCs are 99% $c\text{-}kit^{+}$ and 88% $Fc\varepsilon RI\alpha^{+}c\text{-}kit^{+}$ (Figure 3.14). Similarly, there was no appreciable difference in the expression of c-kit and FcεRIα on less mature BMMC populations (data not shown). These data also confirm that anti-ST2 antibody binding is specific as there is no staining of BMMCs from $ST2^{-/-}$ mice.

Figure 3.15A shows the total number of cells grown from bone marrow cells cultured with IL-3 and SCF culture over successive weeks. There is no difference between the growth curves for WT or $ST2^{-/-}$ BMMCs, demonstrating that the absence of ST2 has no impact on the number of BMMCs obtained at any of the time points studied. Interestingly total cell numbers rise steadily to a peak at 3 weeks of culture before falling rapidly at week 4. This decline correlates with the withdrawal of SCF suggesting that SCF is required to maintain the population of BMMCs. This was confirmed when BMMCs were cultured with SCF and IL-3 throughout (Figure 3.15B). Although a peak in cell numbers was still seen, in this case at 2 weeks, the fall in numbers was more gradual when SCF was continued. Surface expression of FcεRIα, c-kit and ST2 were not affected by the sustained

use of SCF during culture (data not shown). For subsequent experiments both IL-3 and SCF were added for the full duration of BMMC culture.

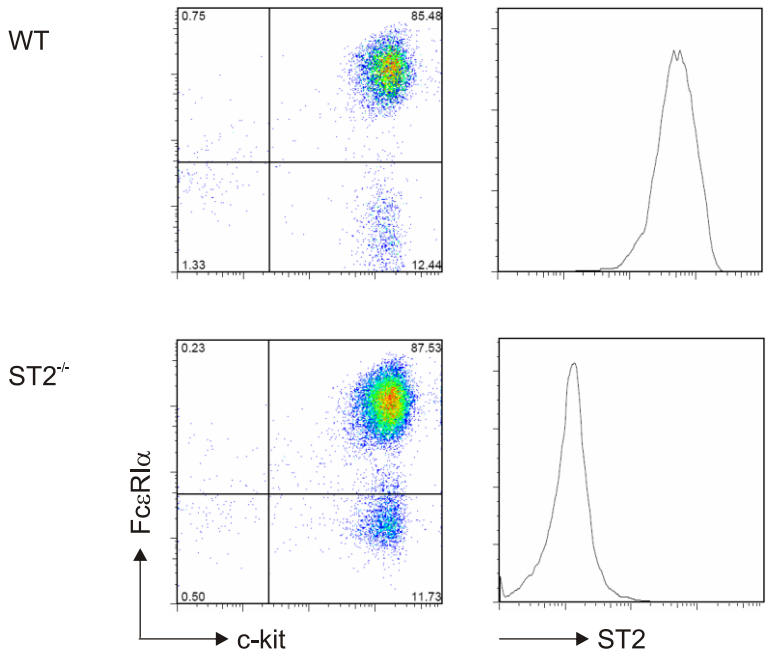
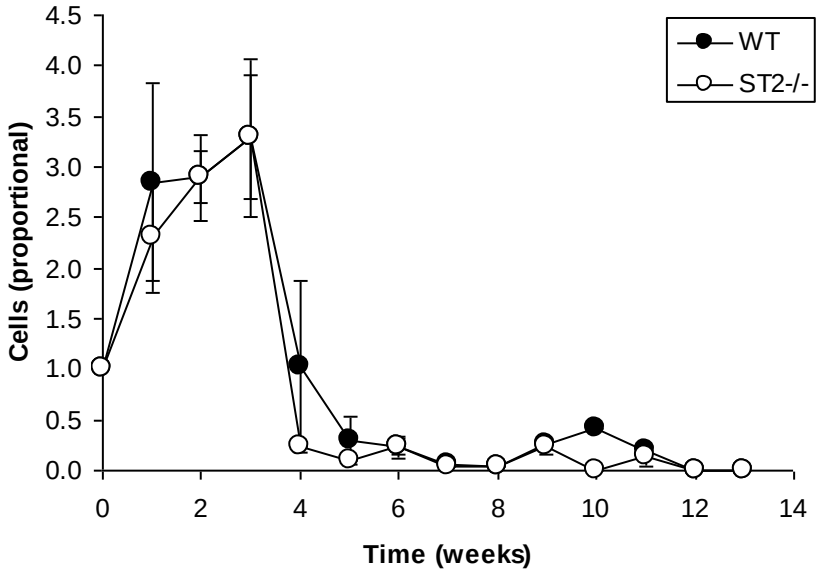


Figure 3.14 - FcεRIα and c-kit expression is unaffected by the absence of ST2

The surface expression of c-kit, FcεRIα and ST2 on mature 8 week old WT and ST2$^{-/-}$ BMMCs was analysed by FACS. Results are representative of three separate experiments.

A



B

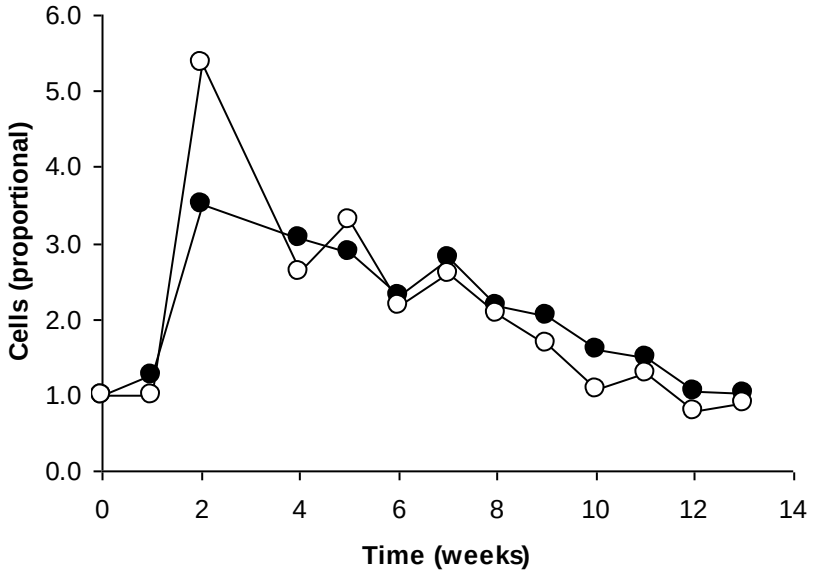


Figure 3.15 - ST2 gene knockout does not affect BMMC numbers

Wild type and $ST2^{-/-}$ bone marrow cells were cultured with SCF for 3 weeks and IL-3 throughout(**A**) or with both IL-3 and SCF throughout(**B**). Total cell counts were performed each week and this was calculated as a proportion of the number of cells originally seeded. Data in A are from 4 separate cultures per group and are shown as the mean +/- SEM. Data from all groups is not available at every time point. Data in B are from a single culture per group.

3.4.2 Function of mast cells lacking ST2

Having generated WT and $ST2^{-/-}$ cells with the surface phenotype of mast cells I sought to confirm the finding that mast cell function is unaffected by the absence of ST2. One of the hallmarks of mast cell activation is degranulation. Mast cells can be stimulated to degranulate by the calcium ionophore ionomycin and the granule product ß-hexosaminidase measured. There was no significant difference between the proportional ß-hexosaminidase release by activated WT and $ST2^{-/-}$ mast cells (Figure 3.16). There was also no difference in the total ß-hexosaminidase activity within WT and ST2-/- mast cells (data not shown).

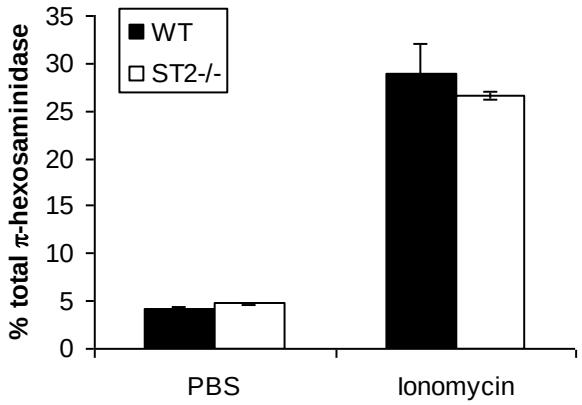


Figure 3.16 - ST2 gene knockout does not affect mast cell degranulation

Mature BMMCs were plated into a 96 well plate at a concentration of $1x10^6$ cells/ml before the addition of PBS or ionomycin at a final concentration of 1 µg/ml. After a 30 minute incubation the ß-hexosaminidase activity within the supernatant and cell lysate was assayed and the proportion of total activity contained in the lysate calculated. Results are shown as the mean of duplicate culture wells +/- SEM and are representative of three separate experiments.

3.5 The effect of IL-33 on bone marrow mast cells

Having established that human and murine mast cell lines produce pro-inflammatory cytokines and chemokines in response to stimulation with IL-33, and having cultured BMMCs from WT and $ST2^{-/-}$ mice, which appear to be identical in terms of growth, phenotype and function, I set out to examine the effect of IL-33 on these cultured mast cells.

3.5.1 The effect of IL-33 on wild type mast cells

Preliminary experiments were carried out to establish an effect of IL-33 on WT BMMCs and to optimise the sampling times and IL-33 concentration. MIP-1α, TNFα and IL-6 were chosen as readouts based on the data from mast cell lines. At 10 ng/ml IL-33 induced the release of MIP-1α, TNFα and IL-6 as measured at 24 hours (Figure 3.17).

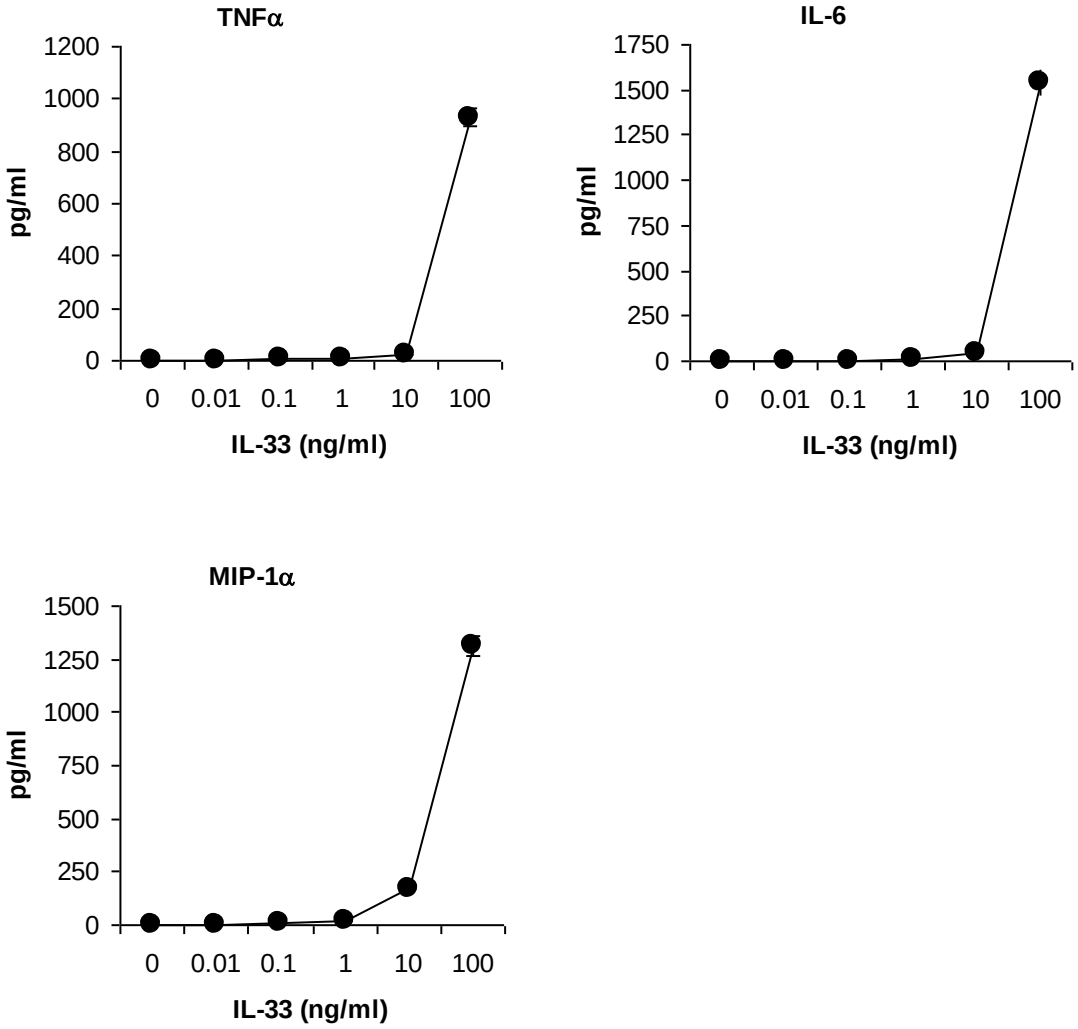


Figure 3.17 - IL-33 induces cytokine and chemokine release from BMMCs

Mature WT BMMCs at a concentration of $1x10^6$/ml were cultured in duplicate with mIL-33 at between 0.01 and 100 ng/ml and aliquots of the supernatant were harvested at 24 hours. MIP-1α, TNFα and IL-6 were measured by ELISA. Results are shown as the mean of duplicate culture wells +/- SEM. * $p<0.05$, ** $p<0.01$ compared with PBS.

3.5.2 The need for ST2 in the mast cell response to IL-33

While the data above show that IL-33 can induce cytokine and chemokine release from a freshly derived population of murine mast cells they do not confirm the specificity of action of IL-33. One possible confounding factor would be the contamination of the protein preparation with other bioactive molecules, for example TLR ligands such as LPS. In addition IL-33 could potentially act via another receptor in addition to ST2. In order to address these question mast cells from mice lacking ST2 were used as controls. Based on the preliminary data WT and $ST2^{-/-}$ BMMCs were stimulated with IL-33 at concentrations of 10 or 100 ng/ml for 24 hours. The supernatants were analysed by a combination of multiplexed immunoassay and ELISA. There was a significant and dose responsive release of the MIP-1α, MCP-1, GM-CSF, VEGF, IL-6 and IL-13(Figure 3.18) from WT but not $ST2^{-/-}$ BMMCs. The following were not detected; IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, TNFα, IFNγ, eotaxin-1, eotaxin-2, FGF, MIG, IP-10, KC (data not shown).

MIP-1α was produced at high levels, often in the ng/ml range, a finding which was highly repeatable across multiple batches of bone marrow mast cells, with peak production ranging from 500 to 2000 pg/ml. While the levels of IL-6 produced were much lower this finding was equally robust. Similar results were obtained with MCP-1, with peak concentrations varying between 66 and 310 pg/ml. IL-13 was not detected in experiments where MIP-1α was produced at levels of less than 1000 ng/ml. VEGF and GM-CSF were also not detected in all experiments.

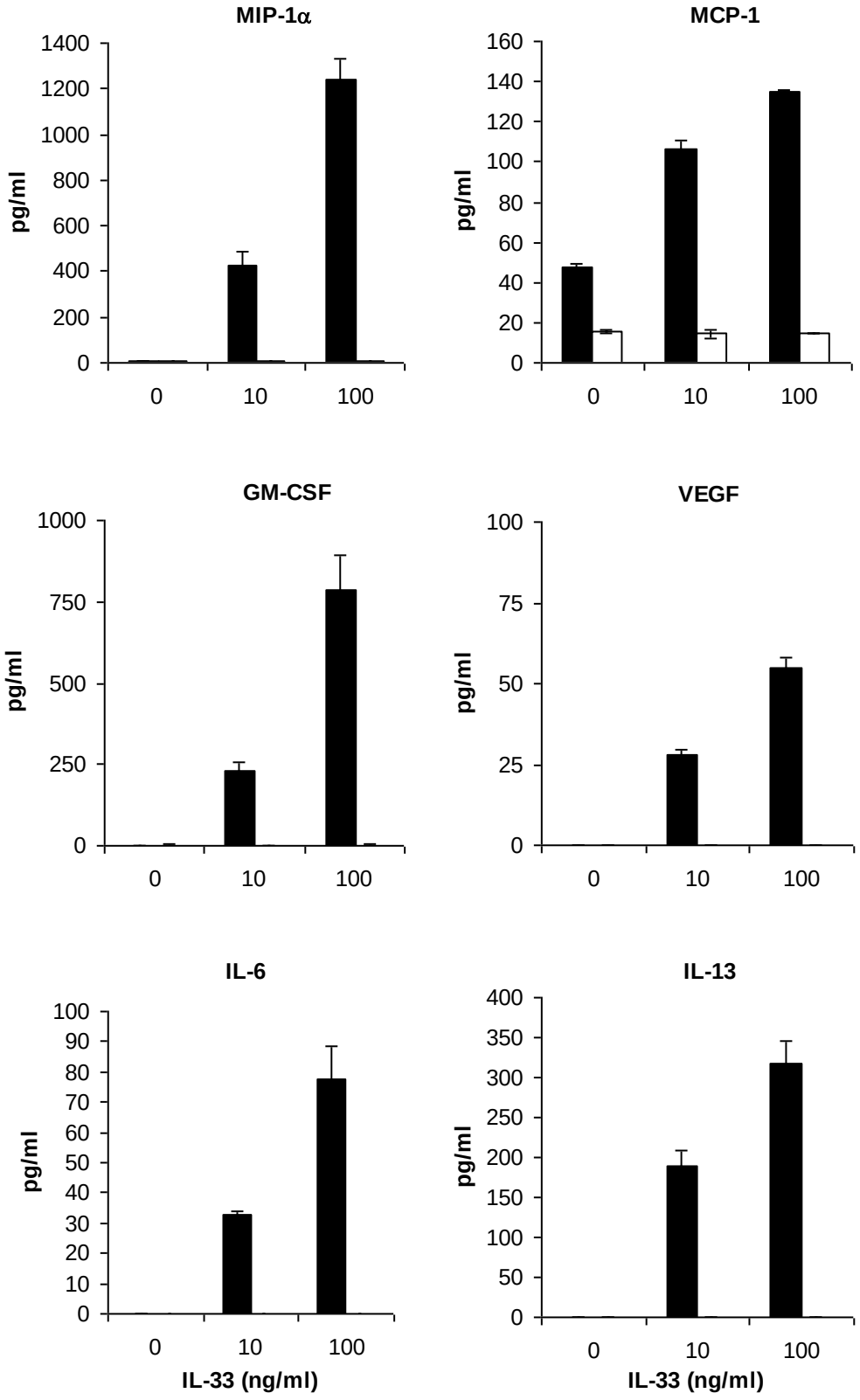


Figure 3.18 - IL-33 induced cytokine and chemokine production by BMMCs is ST2 dependent

Mature WT (■) and ST2$^{-/-}$ (□) BMMCs at a concentration of 1x10^6/ml were cultured in duplicate with mIL-33 at between 0.01 and 100 ng/ml and aliquots of the supernatant were harvested at 24 hours. Cytokines and chemokines were measured by multiplexed immunoassay or ELISA. Results are shown as the mean of duplicate culture wells +/- SEM. One-way ANOVA on WT BMMC for the effect of IL-33 on MIP-1α, MCP-1, VEGF and IL-13 $p<0.005$, GM-CSF and IL-6 $p<0.01$.

3.6 Conclusions

The work presented in this chapter attempts to address the role of the expression of ST2 by mast cells. These experiments demonstrate that normal development from bone marrow precursors to fully mature cells as well as mast cell function measured by mediator release does not appear to be affected by the absence of ST2. In addition, exogenous IL-33, acting via ST2, stimulates mast cells to produce pro-inflammatory cytokines and chemokines.

The possibility that ST2 may be involved in mast cell development is raised as a consequence of the detection of cell surface ST2 early in mast cell development, which appears to be unique among leucocytes. An early study reported that almost 8% of unsorted bone marrow cells expressed ST2, with this proportion falling to 2% after six days of IL-3 culture before climbing steadily to 99.5% after 4 weeks(348). With the exception of unsorted bone marrow cells ST2 was only expressed on c-kit$^+$ cells.

I have described a similar phenomenon during mast cell culture. When ST2 is plotted against c-kit there is a strong correlation, with over 90% of cells expressing either both or neither surface markers. After 3 weeks of culture there was a small (<5%) population of cells which where ST2$^-$ but expressed c-kit, although at a lower level than on ST2$^+$ cells. At all time points studied c-kit$^-$ cells are ST2$^-$ and c-kit$^+$FcεRIα$^+$ cells are ST2$^+$, whereas the intermediate c-kit$^+$FcεRIα$^-$ cells contain ST2$^-$ and ST2$^+$ populations, with the proportion of the latter increasing with time. This suggests that during mast cell development ST2 is expressed on c-kit$^+$ single positive cells before they express FcεRIα. The data are compatible with the possibility that mast cells develop from c-kit$^+$ST2$^-$ progenitors or that the c-kit$^+$ population contains subpopulations of c-kit$^+$ST2$^+$ mast cell progenitors and c-kit$^+$ST2$^-$ cells which lack the potential to become mast cells. Although the previously published report did identify a proportion of c-kit$^-$ cells expressing ST2 at the beginning of culture, inspection of the data raises the possibility that this may be a gating artefact, and the authors themselves refer to these cells as ST2^{low}(348).

ST2 has also been used as a marker of mast cell precursors. The Lin$^-$c-kit$^+$Sca-1$^-$ population of bone marrow cells is known to contain mast cell progenitors(444).

When these cells were sorted by ß7 integrin, the majority of the mast cell generating activity was within the $ß7^+$ population. Further sorting of this population by ST2 revealed that while the $ß7^+ST2^-$ population was multipotent and could generate mast cells as well as other haematopoietic cells, the $ß7^+ST2^+$ population generated mast cells exclusively, suggesting that ST2 is a marker of commitment to the mast cell lineage (380).

While these data raise the possibility that ST2 is involved in mast cell development, data from $ST2^{-/-}$ mice has thus far suggested otherwise. Two independent groups have generated $ST2^{-/-}$ mice, from which it was possible to culture normal mast cells, as assessed by FACS(381,382). These mast cells were also functionally normal with regards to cytokine release following non-specific activation, and IgE mediated histamine release. There was, however, no comment on how the growth and development of these cells compared with those from wild type mice. Mast cells were also detected in the gut and skin of $ST2^{-/-}$ mice, although at reduced numbers in the latter(381). While these data demonstrate that ST2 is not essential for mast cell development the possibility that mast cell development is somehow impaired in the absence of ST2 has not been addressed. I have found no evidence of any difference in the number of mast cells generated, the time taken to reach maturity or the expression of c-kit and FcεRIα in mast cells generated from ST2 deficient mice. Consequently there is still no explanation for the expression of ST2 at high levels at an early stage of mast cell development, prior to the expression of the IgE receptor.

It is important to note that these culture systems are likely to be devoid of IL-33 and also contain an excess of other mast cell growth factors. It is possible that under the influence of IL-3 and SCF the role of ST2 is unimportant, but that IL-33 could play a role in mast cell development and maturation in their absence. In keeping with this there is now evidence that IL-33 can enhance the survival of both murine BMMCs and human cord blood derived mast cells (CBMCs) when incubated in the absence of other mast cell growth factors(445,446).

At the outset of these studies it seemed likely that IL-33 would have an effect on mast cells, given their high level of ST2 expression and the finding that IL-33 induced NFκB and MAP kinase activation in mast cells(323). However the nature of this effect was unknown. I have shown that IL-33 induces the release of

inflammatory mediators from mast cells. Specifically the pro-inflammatory cytokine IL-6, the Th2 cytokine IL-13 and the chemokines MIP-1α and MCP-1, and at times the classical growth factors GM-CSF and VEGF.

Since completing this work these findings have been confirmed by four other studies. All found that IL-33 is a potent inducer of cytokine release from murine bone marrow mast cells, although the exact cytokine profile varied (Table 3.1). This variation may well be due to differences in protocol, including BMMC culture, sampling time and, perhaps most significantly, mouse strain. The only other data from BALB/c mast cells published by Kondo *et al*(447) correlated closely with the results I obtained. Human CBMCs have been shown to release IL-1ß, IL-3, IL-5, IL-6, IL-8, IL-13, TNFα, eotaxin-2, MCP-1, MIP-1α, TCA3, RANTES and TARC in response to stimulation with IL-33(446,448,449).

	IL-1ß	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12	IL-13	TNFα	IFNγ	MCP-1	MIP-1α
Pitman	-	-	-	-	+	-	-	+	-	-	+	+
Kondo (447)			-	-	+			+			+	+
Ali (450)	+				+			-	+			
Ho (445)			-		+			+				
Moulin (443)	+	+	-	-	+	-	-		+	-	+	

Table 3.1 – Mediators released by IL-33 treated mast cells: comparison with published data

A comparison of the mediators produced by murine BMMCs stimulated with IL-33 organised by investigator. Assay of the supernatant of IL-33 stimulated BMMCs revealed the presence (+) or absence (-) of the corresponding protein. In some cases the particular protein was not measured (grey boxes).

Investigators have also looked for the possibility that IL-33 could induce mast cells degranulation. Several papers have reported that human CBMCs(446,448) or murine BMMCs (443,445) show no evidence of degranulation in response to IL-33 treatment, even if pre-incubated with IgE. However building on the work presented here others within the laboratory have found that murine peritoneal mast cells and human CBMCs do degraulate in response to IL-33 provided that they are preincubated with IgE for a sufficiently long period(449).

Other studies attempted to confirm that the effect was IL-33 specific by using blocking antibodies against ST2(443) or IL-1RAcP, the other subunit of the IL-33 receptor(450). However these antibodies were required at µg concentrations, up to 10,000 times the concentration of IL-33 used, and inhibition was not always

complete. I have presented data here from $ST2^{-/-}$ BMMCs demonstrating that in the absence of ST2 there is a complete abrogation of IL-33 induced cytokine and chemokine production, confirming that IL-33 acts on mast cells in a completely ST2 dependent manner.

The mast cell product of IL-33 stimulation most consistently detected is IL-6 (Table 3.1). Having originally been identified as a B-cell differentiation factor IL-6 is traditionally considered to be primarily involved in the proliferation and differentiation of both B- and T-lymphocytes(451). However IL-6 has also been shown to promote mast cell survival(452), drive helper T cells towards a Th2 phenotype(453) and to be an important co-factor in IL-4 induced IgE synthesis(454). In addition IL-6 has been implicated in the transition from acute to chronic inflammation and the associated resolution of neutrophilic inflammation and initiation of macrophage recruitment(455), partly acting via the induction of MCP-1 release(456). As a consequence IL-6 is thought to play an important role in auto-immune diseases such as rheumatoid arthritis and inflammatory bowel disease(457).

While less frequently measured, the chemokines MIP-1α and MCP-1 are both consistently produced by IL-33 stimulated mast cells (Table 3.1). MCP-1 is a potent monocyte chemoattractant(458,459) and MIP-1α was originally identified as a neutrophil chemoattractant released by LPS treated macrophages(460), although both can be produced by a number of cell types and are capable of recruiting other inflammatory cells(461-464). In addition they play a role in the Th1-Th2 balance. MCP-1 drives T cells towards a Th2 phenotype(465-467) and $MCP\text{-}1^{-/-}$ mice have a marked defect in Th2 responses(468), while MIP-1α induces Th1 differentiation(466,467).

With this in mind it is not difficult to see how IL-33 induced release of IL-6, MIP-1α and MCP-1 by mast cells could play an important role in the initiation of inflammation. Given the data suggesting a role of ST2 in allergic inflammation and the strong eosinophilic response IL-33 promotes *in vivo* it is seems reasonable to consider the role that IL-33 induced release of IL-6, MIP-1α and MCP-1 by mast cells might play in asthma.

Not only have elevated levels of IL-6 and the IL-6 receptor been detected in asthma(469-472), but IL-6 can induce subepithelial fibrosis(473) and lymphocyte

infiltration(474) in the airway. Mast cells can also be an important source of IL-6 in allergic inflammation with mast cells accounting for over 90% of IL-6 positive cells in biopsies of allergic rhinitis(475). MCP-1 and MIP-1α are elevated in clinical asthma(258,476,477), and also play a role in experimental asthma. MCP-1 is associated with lymphocyte and macrophage accumulation, mast cell degranulation and AHR(179,478,479) and MIP-1α with eosinophil recruitment(41,478,480). The other mediator commonly released by mast cells upon IL-33 stimulation is IL-13 (Table 3.1) which has a well established role in asthma as previously discussed.

Having demonstrated a role for ST2 on mast cells, in the next two chapters I sought to examine the role of ST2 in airways inflammation by studying the impact of ST2 gene knock-out in a model of asthma (Chapter 4), and the effect of ST2 activation by IL-33 in the airways (Chapter 5).

Chapter 4

The role of ST2 in murine models of asthma

In this chapter I will establish, modify and validate a murine model of asthma before using this to study the requirement for ST2 in allergic airways inflammation.

4.1 Introduction

There is much to suggest that ST2 may play an important role in asthma. ST2 expression is most widely recognised on Th2 cells and mast cells, and in the previous chapter mast cells were shown to release pro-inflammatory mediators upon stimulation via ST2. Both Th2 cells and mast cells are though of as important elements of human and murine asthma. Activation of ST2 *in vivo*, by the systemic administration of IL-33, results in local eosinophilia and elevated levels of IgE, IL-5 and IL-13(323), phenomena which are characteristic of asthma. There is also evidence that the absence of ST2 can result in dramatically impaired Th2 responses *in vivo*(382). ST2 is detected at increased levels in both experimental(360,372,395) and clinical asthma(398) giving further support to the notion that ST2 may be important in allergic airways inflammation.

However, at the outset of this work the literature regarding the role of ST2 in murine models of asthma was far from clear. Studies utilising neutralising antibodies against ST2 that modified disease in a number of different models all supported a role for ST2 in experimental asthma(298,372,396). Conversely ST2 gene-deleted mice demonstrated no difference in airway inflammation(381). This last study used an OVA and alum driven 35 day model of asthma with mice on a C57Bl/6 background. The main experimental endpoints including airways eosinophilia and raised total IgE titres occurred equally in wild type and $ST2^{-/-}$ mice, although AHR was not measured. BALB/c mice are generally considered more prone to Th2 inflammation than C57Bl/6 mice. As discussed in Chapter 1 there can be significant differences in experimental outcome depending on the strain of mouse used, for example IL-5 gene deletion generally results in the loss of AHR in C57Bl/6 but not BALB/c mice(94,100). In addition the study of $ST2^{-/-}$ mice by Hoshino *et al*(381) utilised an intensive challenge protocol with mice receiving 15 OVA challenges in total, which resulted in high BAL eosinophil counts of greater than 50 $x10^5$ cells/ml.

In these examples there is incomplete evidence to conclude that ST2 has a role in airway inflammation. The effects of ST2 gene-deletion might be obscured by redundant mechanisms and it is conceivable that any contribution that ST2 might make to airways inflammation was masked by strong stimuli involving multiple challenges and powerful adjuvants.

In an attempt further examine the role of ST2 in experimental asthma I sought to study the effect of ST2 gene knock-out on AHR as well as eosinophilic inflammation in an antigen based model using mice from a BALB/c background.

4.2 Developing a murine model of asthma

The most well studied murine models of asthma employ systemic sensitisation with OVA, combined with alum as a Th2 adjuvant, followed by airways challenge with OVA alone. There are many published variations of this model, with changes in the dose, timing and number of administrations of OVA during sensitisation and challenge, and differing methods of delivery to the airways. There was considerable previous experience within our laboratory using a 28 day model of asthma(429-431), adapted from a previously published model(432). Therefore I set out to reproduce this model before making any modifications to the protocol.

4.2.1 Induction of allergic airways inflammation in a 28 day model

Mice immunised and challenged with OVA using the 28 day protocol developed significant airways eosinophilia (Figure 4.1). While there was a corresponding decrease in the proportion of macrophages there was nonetheless a significant rise in the absolute number of BAL macrophages. Neutrophils and lymphocytes were found at very low frequency. OVA-specific immunoglobulins, including high titres of IgE, were significantly elevated in the serum (Figure 4.2). In order to confirm the induction of a robust Th2 response the draining lymph nodes were studied. Single cell suspensions from the lymph nodes were restimulated with OVA *in vitro* (Figure 4.3). Cell proliferation was induced which was almost four-fold greater from mice treated with OVA than those treated with PBS. OVA

treated mice also produced high concentrations of the Th2 cytokines IL-4 and IL-5, in addition to the Th1 cytokine IFNγ. PBS treated mice produced higher levels of IFNγ with no IL-4 or IL-5. Cells from either group treated with medium alone did not proliferate or produce cytokines (data not shown). These results are all in keeping with the expected findings in this model of asthma.

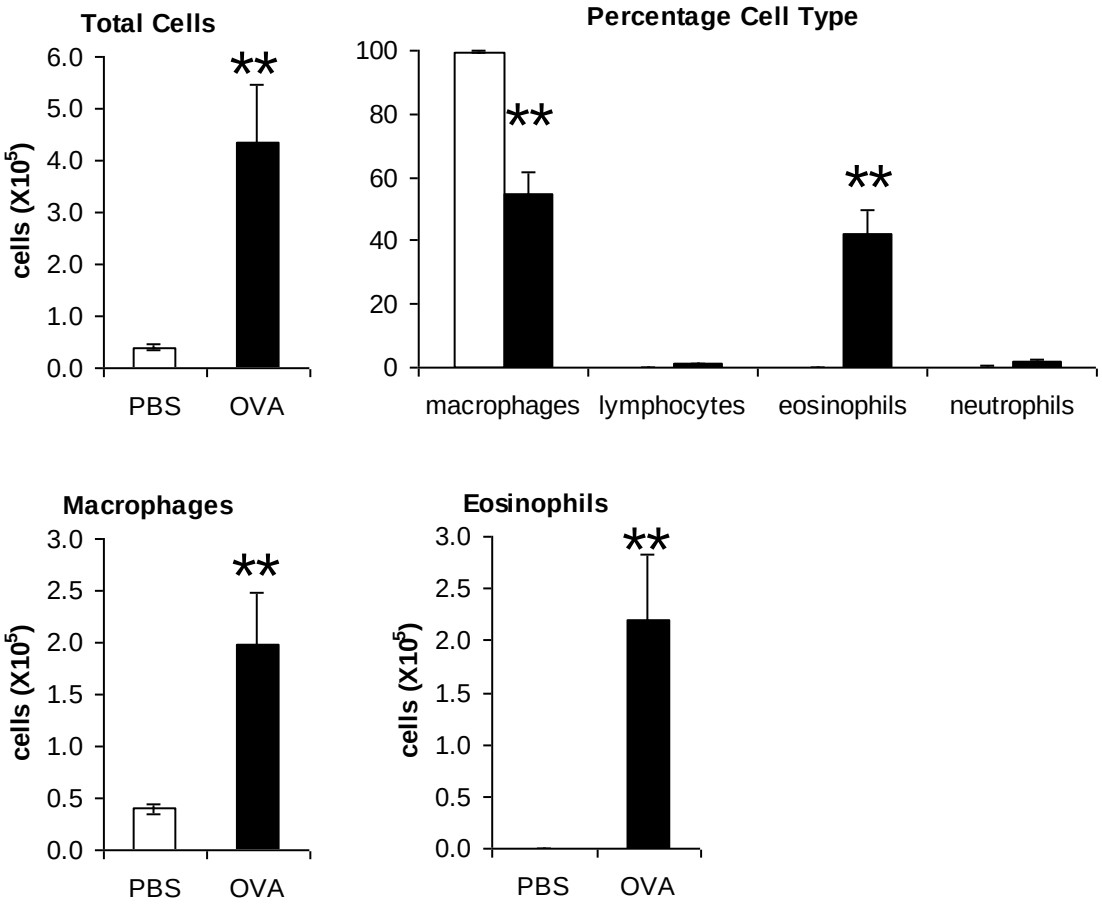


Figure 4.1 – A 28 day model of asthma induces pulmonary eosinophilia

BALB/c mice were immunised with OVA (100 µg) in 1% alum on days 0 and 14 and were challenged with intranasal OVA (30 µg) on days 14, 25, 26 and 27 (■). Control mice received PBS alone (□). BAL was collected on day 28. Total and differential cell counts were performed. Data are shown as the mean +/- SEM. n=9. * $p<0.05$, ** $p<0.01$ compared with PBS.

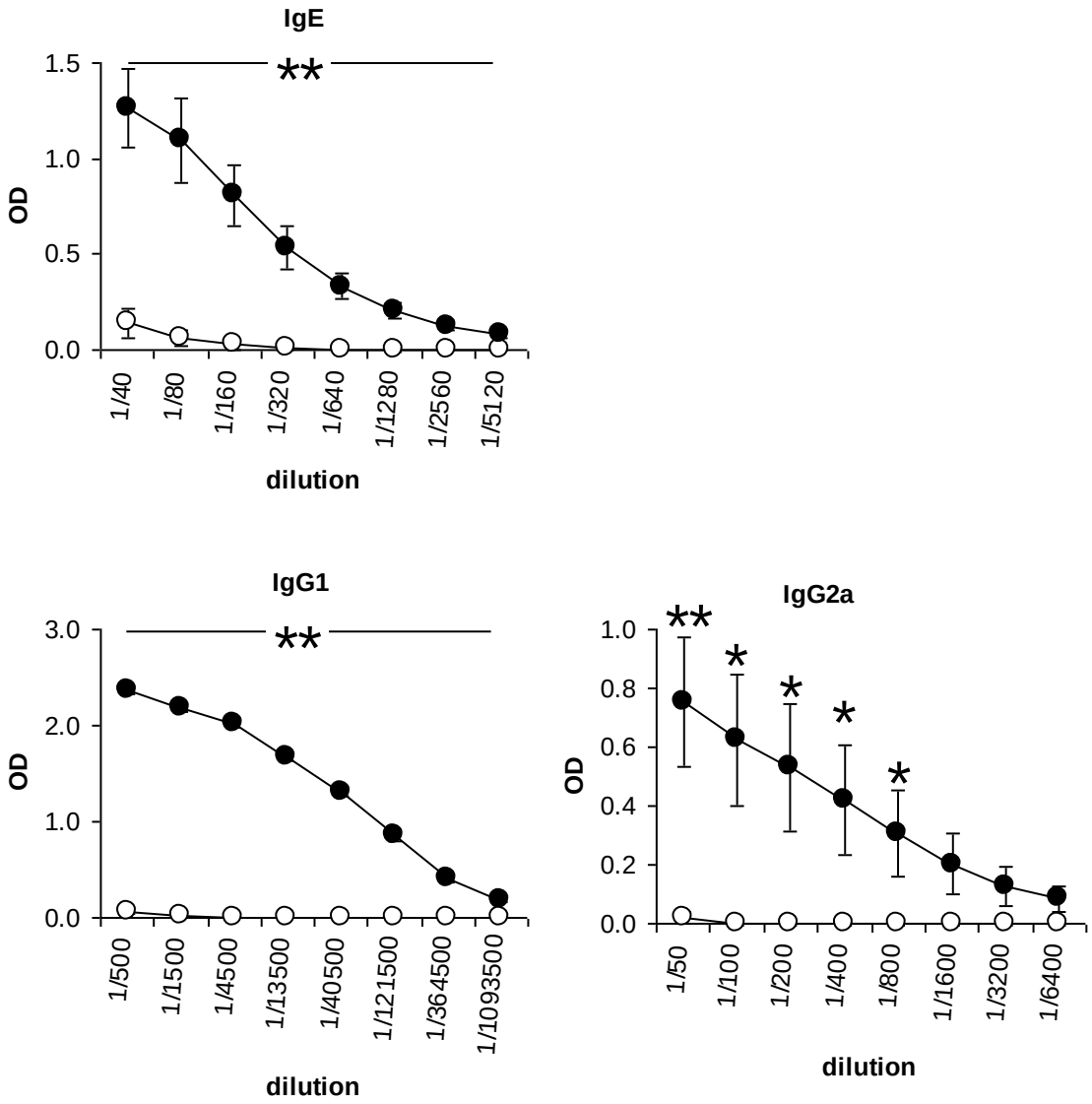


Figure 4.2 – A 28 day model of asthma induces antigen specific immunoglobulins

Mice were immunised and challenged with OVA (●) or PBS (○) as is Figure 4.1. Serum was collected the day after the final challenge. OVA specific IgE, IgG1 and IgG2a were measured in the serum at the indicated dilutions. Results are shown as the mean of each group of mice +/- SEM. n=9 mice. * $p<0.05$, ** $p<0.01$ compared with PBS.

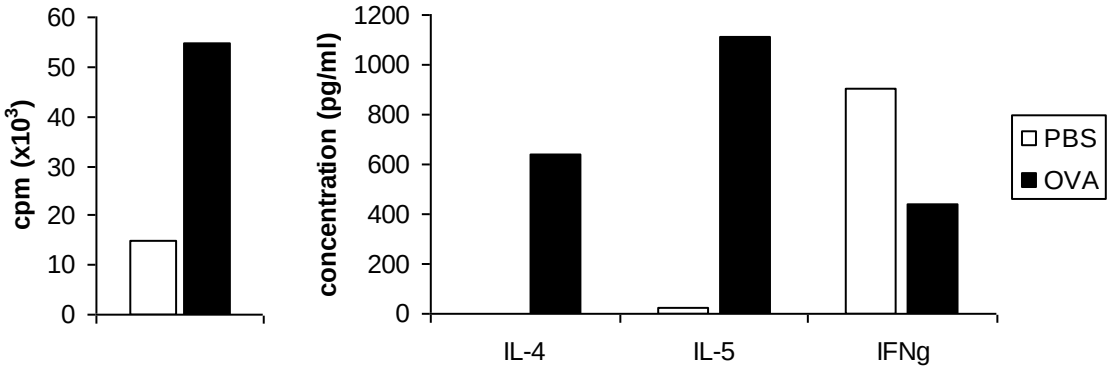


Figure 4.3 - A 28 day model of asthma induces antigen specific Th2 lymphocytes

Mice were immunised and challenged with OVA (■) or PBS (□) as is Figure 4.1 and lymph nodes harvested the day after the final challenge. Single cell suspensions were stimulated with OVA (1 mg/ml). (**A**) After 72 hours proliferation was measured by ^{3}H thymidine uptake. (**B**) After 96 hours supernatants were collected and IL-4, IL-5 and IFNγ measured by ELISA. Results are shown as pooled samples from groups of 9 mice.

4.2.2 Intranasal versus intratracheal dosing

The most common methods of delivering antigen to the murine airway are either by placing conscious animals in a chamber containing nebulised antigen or by directly instilling the antigen into the airways of anaesthetised mice. There is no evidence that either method is superior to the other, therefore I chose direct instillation due to our established local experience with intranasal delivery.

One concern with intranasal delivery is that not all of the instilled solution is guaranteed to reach the lungs, with the possibility that some is retained in the nasopharynx, or aspirated into the stomach. Although less common than intranasal administration, some investigators have reported using intratracheal delivery (282). By direct cannulation of the trachea it should be possible to deliver the entire solution into the lungs.

Both delivery methods were compared using the 28 day model of asthma. Although total BAL eosinophil numbers appeared lower in the intratracheal group, there was no significant difference in BAL eosinophil percentage or absolute number (Figure 4.4), with no associated reduction in intra- or inter-experiment variability. Given that intratracheal administration was more technichally demanding, more time consuming and more likely to lead to trauma, the decision was made to use intranasal administration in all subsequent experiments.

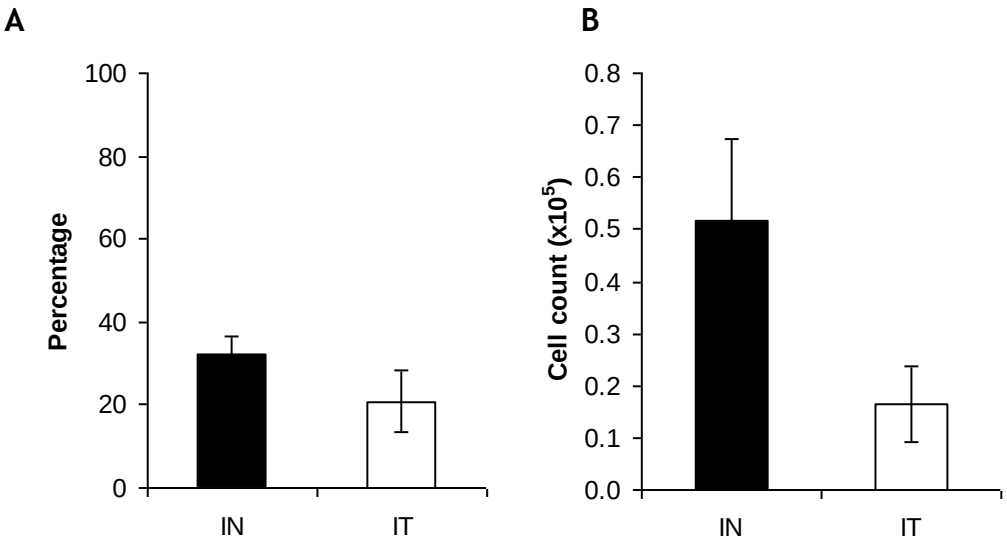


Figure 4.4 – Intratracheal OVA administration is not superior to intranasal administration

BALB/c mice were immunised with OVA and alum on days 0 and 14 and were challenged with OVA on days 14, 25, 26 and 27 via the intranasal (■) or intratracheal (□) route. BAL was collected the day after the final challenge and eosinophil percentage (**A**) and absolute numbers (**B**) counted. Data are shown as the mean +/- SEM. n=6.

4.2.3 Validation of BAL differential cell counts

One of the key endpoints in asthma models is the measurement of BAL eosinophilia. While FACS analysis can be used to analyse the cellular composition of BAL the gold standard remains microscope counting of stained cytospin preparations. Although internal bias is prevented by rigorous blinding any cytological system may be at risk of subjective error. In order to confirm inter-observer consistency I undertook a blind comparison of results with another experienced investigator in the laboratory. Asthma was induced and BAL cytospins prepared. Each individual then performed differential counts on all the samples from the experimental group. Samples were unblinded independently and results compared.

As shown in Figure 4.5 there was good inter-observer correlation.

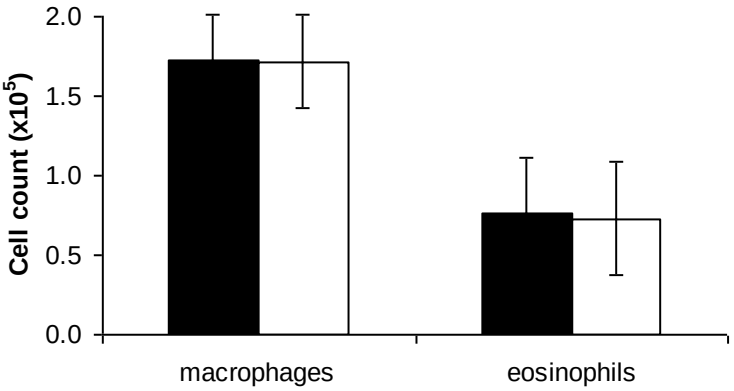


Figure 4.5 – BAL differential cell counts are reproducible

Mice were immunised and challenged with OVA and BAL cells collected as is Figure 4.1. Differential cell counts were performed by Investigator 1 (■) and investigator 2 (□). Data are shown as the mean +/- SEM. n=4

4.2.4 Pooled versus individual LN culture

While the primary endpoints in the murine asthma models are generally lung eosinophil infiltration, IgE antibody and AHR; additional important information can come from the measurement of other variables. When antigen is delivered to the lung it can act directly on local cells, but is also taken up by dendritic cells which then migrate to the draining lymph nodes to become professional antigen presenting cells where they activate antigen specific lymphocytes(292). By antigen stimulation of the cells within the lymph nodes that drain the lung it is possible obtain information on the profile of the cytokines these cells are producing.

Historically this had been carried out by pooling all the draining lymph nodes from the mice in each group and restimulating a fixed number with OVA. There are frequently variations in the extent of BAL eosinophilia within any treatment group, which can include quite extreme outliers. I was concerned that the cells from such over- or under-responsive mice could skew the data from pooled lymph node samples. In addition when lymph nodes are pooled there is no information on the variance within any given group making statistical analysis difficult. Consequently I set out to determine whether it would be feasible to culture the draining lymph nodes from individual mice separately, and to study the data obtained in this way.

Although the total numbers of cells retrieved from the draining lymph nodes of a single animal is small, I established that with optimisation of culture conditions it was almost always possible to carry out individual lymph node cultures. Interestingly, despite similar cell counts in both groups (Figure 4.6A), the mean levels of proliferation (Figure 4.6B) and cytokine production (Figure 4.6C) were lower in the individual lymph node cultures than in pooled lymph nodes. This raises the possibility that when lymph nodes are pooled the response can be dominated by a small proportion of highly reactive cells from one or two mice.

Individual lymph node culture provides addition data, allows for statistical analysis and may prevent skewing of the data by a small subset of mice. Consequently all subsequent lymph node culture was performed by isolating individual lymph nodes from each animal.

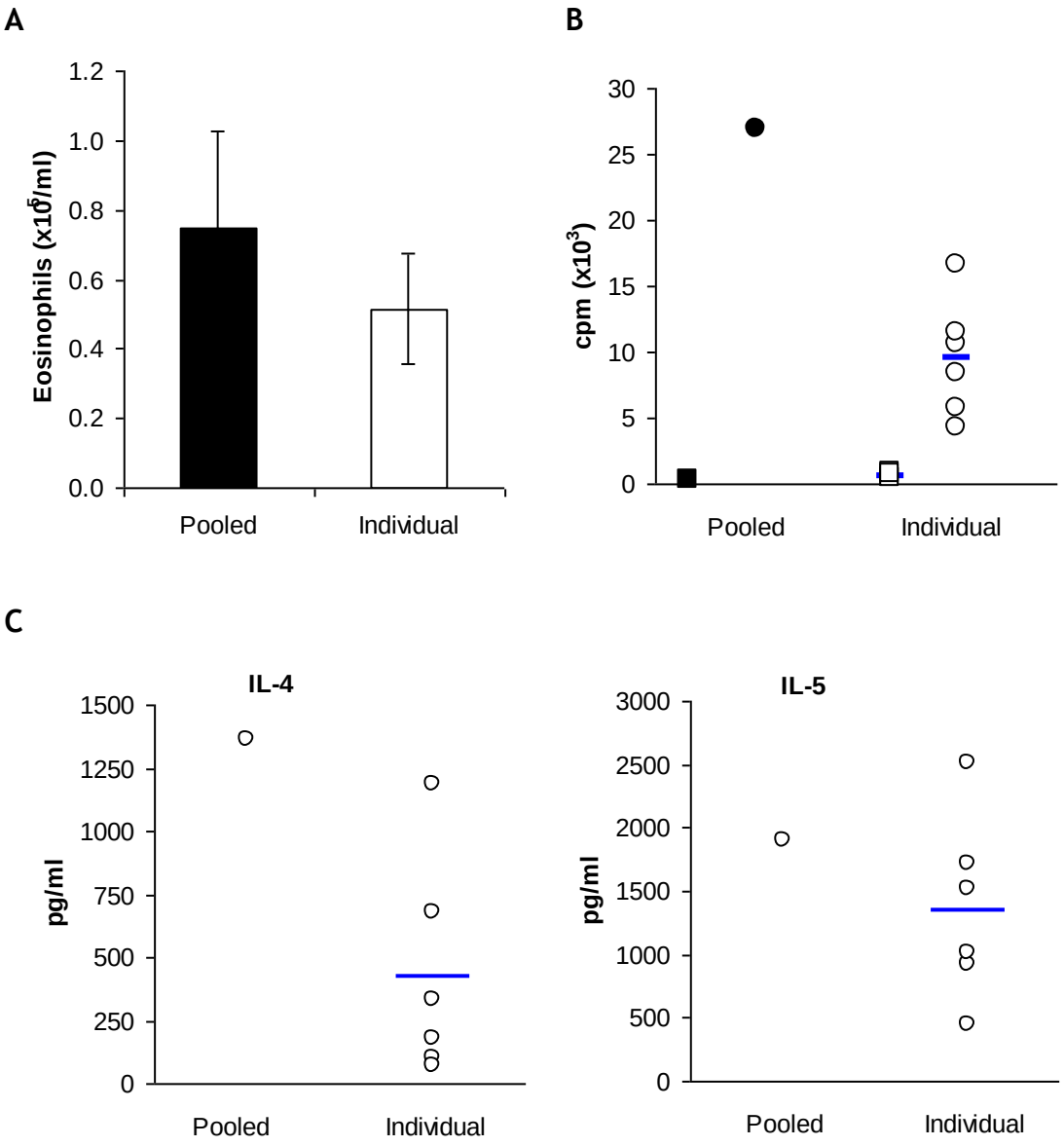


Figure 4.6 – Individual lymph node culture provides additional information

BALB/c mice were immunised with OVA and alum before being challenged with intranasal OVA as in Figure 4.4. (**A**) Total eosinophils within the BAL were counted. Data are shown as the mean +/- SEM. Draining lymph nodes were either pooled from all mice (■,●) or separated by individual mice (□,○). A total of $2x10^6$ lymph node cells were cultured with complete medium alone (■ and □) or with OVA at 1 mg/ml (● and ○). (**B**) Proliferation was measured by thymidine uptake. (**C**) Supernatant IL-4 and IL-5 were assayed by ELISA. The result from each culture well is plotted, in the case of individual node cultures the mean is represented by a bar. n=6.

4.2.5 Establishing a method to measure airways hyperresponsiveness

One of the hallmarks of clinical asthma is AHR and this is generally reproduced in murine models of asthma. In both the clinical setting and animal models this is measured by establishing the degree of airflow resistance in subjects before and after inhalation of a bronchoconstricting agent such as histamine or methacholine. In humans, airflow resistance is measured by quantifying air flow during forced expiratory manoeuvres. Such techniques are effort-dependent and require co-operation and are therefore not possible in animals. The gold standard method of measuring AHR in animals requires anaesthesia and intubation. As discussed in Chapter 2, it is also possible to measure AHR in conscious, unrestrained animals using whole body plethysmography, where mice are placed in a chamber within which the pressure differences occurring are recorded and from these changes the parameter of enhanced Pause (Penh) is derived(29).

Before utilising this method to answer experimental questions it was important that I validated the technique. As a positive control I used BALB/c mice in which allergic airways inflammation was induced using a 28 model of asthma which is well recognised to induce AHR(21). As a negative control naïve mice were challenged with intranasal PBS alone. As expected there is a significant rise in Penh, the measure of AHR, with increasing broncho-provocation with methacholine in both the control and the treated group (Figure 4.7).

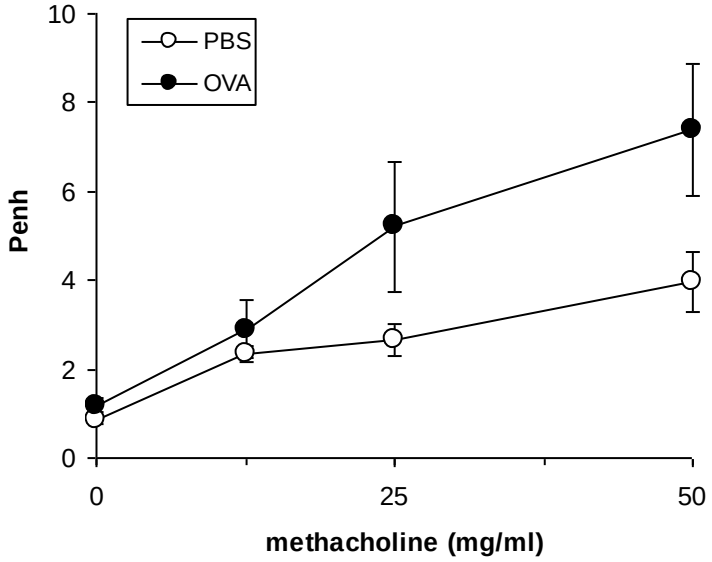


Figure 4.7 - Airways hyperresponsiveness can be measured by Penh

BALB/c mice were immunised with OVA and alum before being challenged with intranasal OVA as in Figure 4.4. Unimmunised control mice were challenged with PBS alone. The day after the final airways challenge Penh was measured with increasing doses of nebulised methacholine. Results are shown as the mean of each group of mice +/- SEM. n=4. Two-way ANOVA $p<0.01$ PBS vs OVA.

4.3 Establishing a twelve day model of asthma

When *S. mansoni* eggs are used to drive pulmonary eosinophilic inflammation ST2 is only essential in mice that have not already been immunised(382), highlighting the fact that differences in sensitisation can affect the contribution of ST2. Speculating that the use of multiple systemic immunisations and an extended period of repeated challenges may obscure any requirement for ST2, a 12 day model which had been described was attractive(49). This model utilised a single immunisation, followed by three airways challenges. Furthermore the move from a 28 day to a 12 day model not only allows for increased productivity in a shorter time frame, but also has economic implications. While the 12 day protocol had been shown to induce AHR, airways inflammation and immunoglobulin generation were not assessed. Therefore I set out to validate and optimise a novel 12 day model of asthma, base on that published by Stock *et al*(49).

4.3.1 Features of asthma can be induced using a twelve day model

The published protocol was for an immunisation using OVA (100 µg) with alum adjuvant followed by daily intranasal challenges of OVA (50 µg) on days 9 to 11 in BALB/c mice (Figure 4.8). Initially I set out to repeat the protocol exactly as described and to look for the presence of eosinophilic inflammation in the airways.

This protocol induced a robust inflammatory response in the airways as represented by total eosinophilia (Figure 4.9). The level of eosinophilia is towards the upper limit of what we would expect to see for the 28 day model and this was even more marked in subsequent repeats (data not shown).

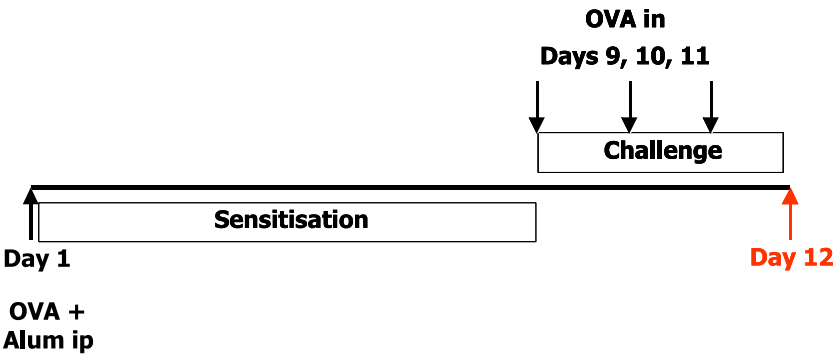


Figure 4.8 - The twelve day model of asthma

Mice are immunised with OVA and alum by the intraperitoneal route on day 1 before being challenged with intranasal OVA once on each of days 9, 10 and 11. On day 12 AHR is measured and/or mice are culled.

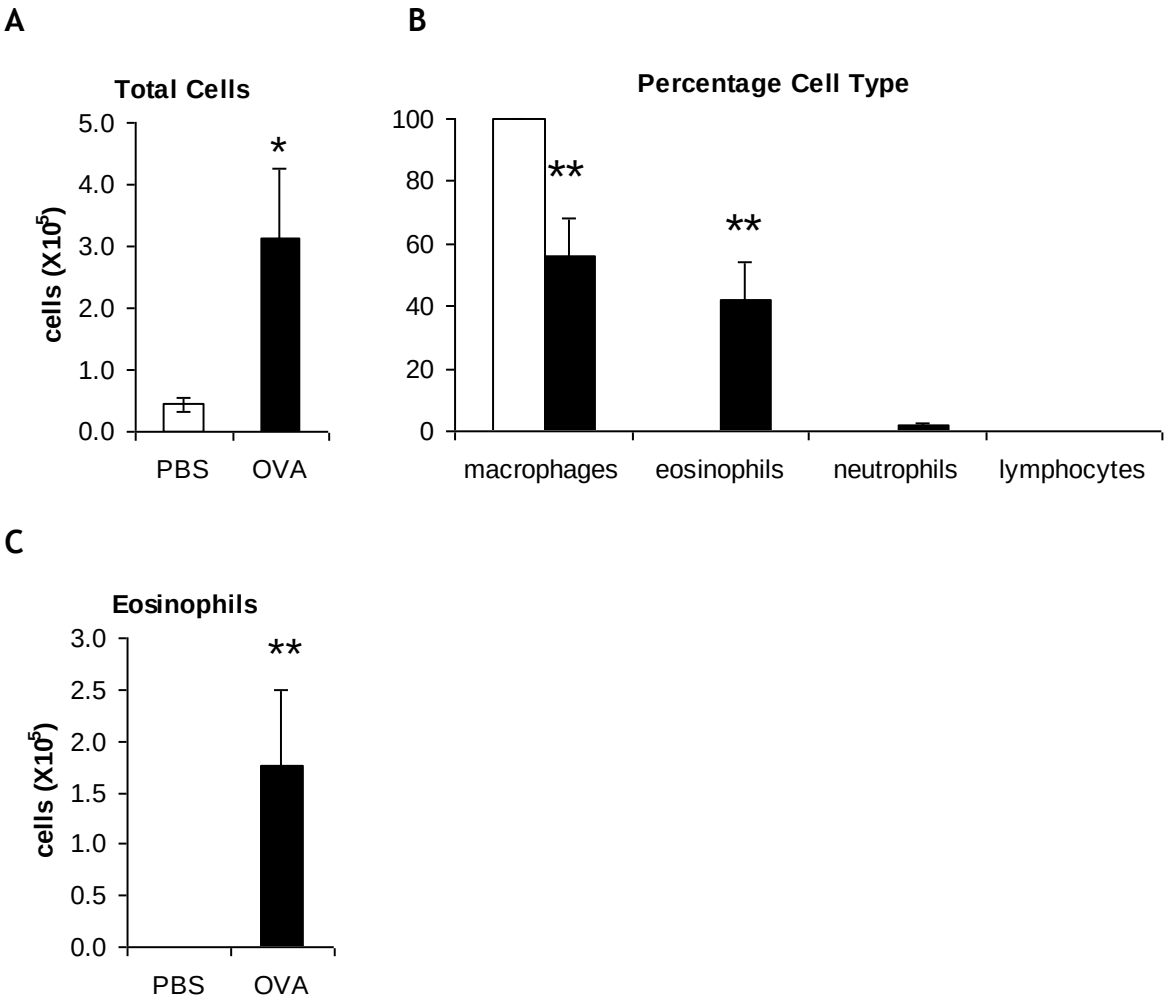


Figure 4.9 – BAL eosinophilia is induced by a twelve day asthma protocol

BALB/c mice were immunised with OVA (100 µg) and alum on day 1 and were challenged with OVA (50 µg) on days 9, 10 and 11 (■). Control mice received PBS alone (□).BAL was collected the day after the final challenge and (**A**) total cell count, (**B**) differential percentages and (**C**) absolute cell numbers counted. Data are shown as the mean +/- SEM. n=5. * $p<0.05$, ** $p<0.01$ compared with PBS.

4.3.2 Dose response to ovalbumin challenge in the twelve day model

Having established that a significant BAL eosinophilia can be generated using a 12 day model of asthma it was important to optimise this model. Following the observation that this model generated a more extensive eosinophilia than normally seen with the 28 day model, I anticipated that the effect of therapeutic intervention might be less readily detected with an inflammatory response of this magnitude. Therefore I adjusted the dose of OVA used at challenge in the 12 day model and performed a dose titration in order to establish a protocol which might be more susceptible to reasonable intervention. Given that a dose of 50 µg per mouse generated a marked eosinophilia I chose this as a starting point and studied this dose and below.

Reducing the dose of OVA given at challenge resulted in a dose related decrease in BAL eosinophil numbers (Figure 4.10). While there was also a reduction in the percentage of eosinophils with lower doses of OVA challenge, even at 2.5 µg eosinophils still comprised 39% of BAL cells. Given that a dose of 10 µg appears to represent the mid-point of the dose response curve it likely that this protocol will be susceptible to interventions which either exacerbate or attenuate eosinophilic inflammation. Consequently a dose of 10 µg was used in subsequent experiments.

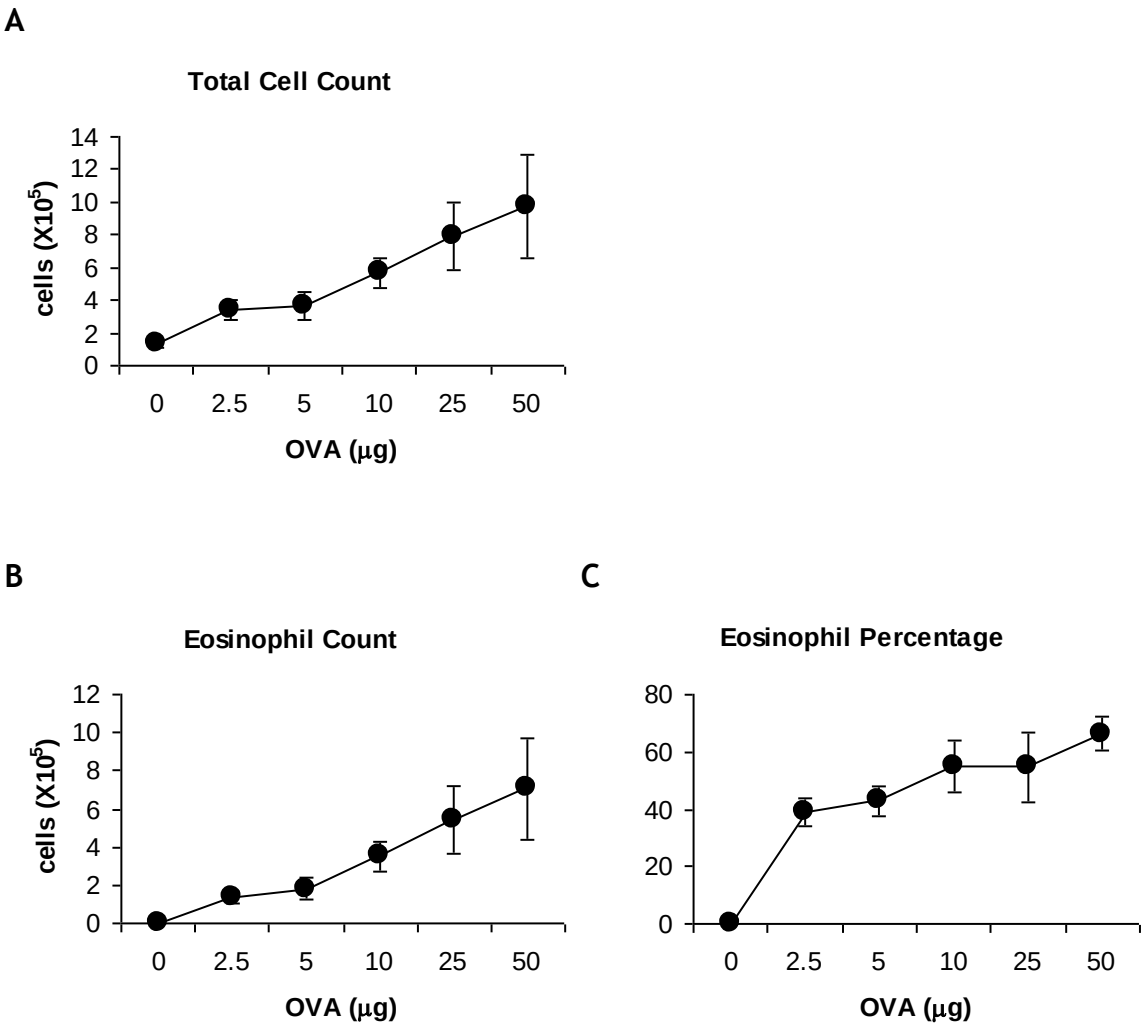


Figure 4.10 - BAL cell content related to OVA challenge dose

BALB/c mice were immunised with OVA (100 µg) and alum on day 1 and were challenged with OVA (0 to 50 µg) on days 9, 10 and 11. BAL was collected on day 12 and (**A**) total and (**B,C**) differential cell counts performed. Data are shown as the mean +/- SEM. n=6 mice.

4.3.3 Validation of a twelve day model

Thus far I have focused on BAL eosinophils as the primary end point when assessing the 12 day model of asthma, but there are other features one would expect to find in such a model. Before using this protocol I set out to confirm that these features of clinical asthma were reproduced. Most importantly BAL eosinophilia should be accompanied by eosinophilic inflammation in the lung tissue, OVA specific IgE in the serum and the generation of AHR.

There is good evidence to suggest that eosinophilic inflammation and AHR can be dissociated, for example some models generate eosinophilia but not AHR in C57Bl/6 mice(50) whereas IL-5 gene deletion results in loss of eosinophilia with preserved AHR in BALB/c mice(100,103). Therefore one cannot assume that because the 12 day protocol generates a robust BAL eosinophilia that AHR will also be generated. Therefore I confirmed that the 12 day protocol selected did indeed induce significant AHR as measured by Penh (Figure 4.11).

It is unresolved whether BAL eosinophilia is always associated with lung tissue eosinophil infiltration, therefore it is important to confirm if these features are present in the 12 day model. When the 28 day and 12 day models were compared directly there was no significant difference in BAL eosinophilia (Figure 4.12) with a similar degree of inflammation also seen on histology (Figure 4.13).

In models of atopic asthma antigen-specific IgE should also be generated. In both the 28 day and 12 day models OVA specific IgE was detected in the serum at comparable levels at a titre of 1/20 (Figure 4.12C), although at a titre of 1/180 IgE was significantly lower in the 12 day model. OVA specific IgG1 and IgG2a were also generated and serum concentrations were once again significantly lower in the 12 day model than the 28 day model (Figure 4.14).

The generation of antigen-specific antibodies provided evidence of effective immunisation. I choose to further examine T-lymphocyte immunisation by observing cells in culture from the draining lymph nodes in this model, to see whether they served as a readout of the antigen recall response.

Cells from the draining lymph nodes of mice immunised and challenged with the 12 day protocol were restimulated with OVA. These cells showed evidence of

activation as measured by proliferation (Figure 4.15A). It was also possible to measure the cytokines produced by these cells upon antigen restimulation (Figure 4.15B). As expected the recall response demonstrated Th2 polarisation with production of high concentrations of the Th2 cytokines IL-4 and IL-5 in much greater relative concentrations than that of the Th1 cytokine IFNγ.

These data demonstrate that the selected 12 day protocol produces all the acute features of clinical asthma expected of a murine model, namely AHR, eosinophilic inflammation, IgE production and a Th2 cytokine response.

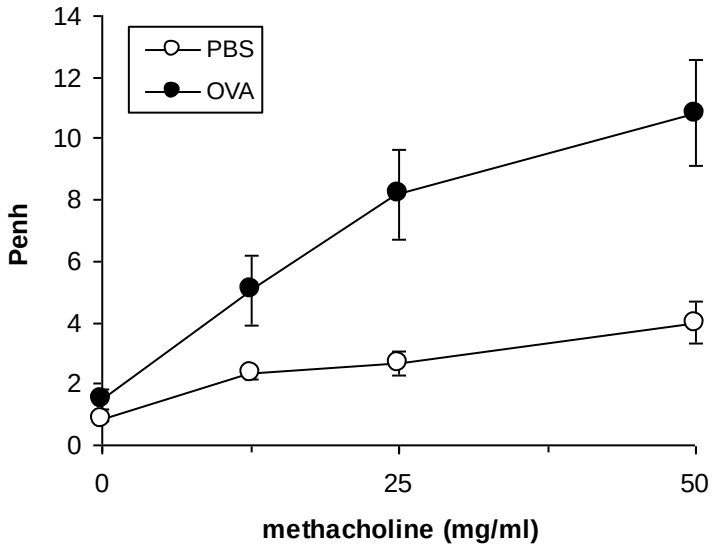


Figure 4.11 – The twelve day model of asthma induces airways hyperresponsiveness

BALB/c mice were immunised with OVA (100 µg) and alum on day 1 and were challenged with OVA (10 µg) on days 9, 10 and 11. Unimmunised control mice were challenged with PBS alone. The day after the final airways challenge Penh was measured. Results are shown as the mean of each group of mice +/- SEM. n=4 mice. * $p<0.05$, ** $p<0.01$ compared with PBS. Two-way ANOVA $p<0.001$ PBS vs OVA.

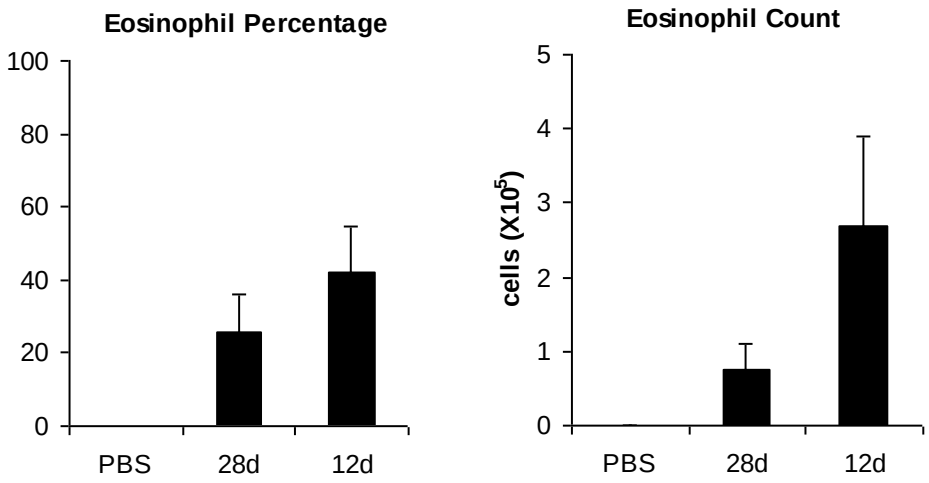
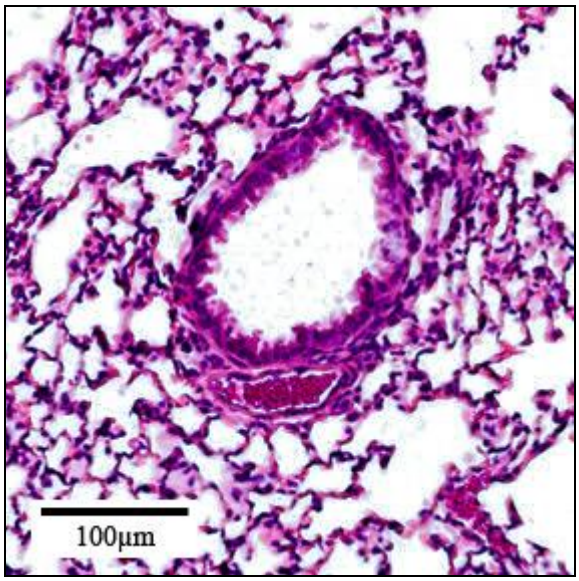


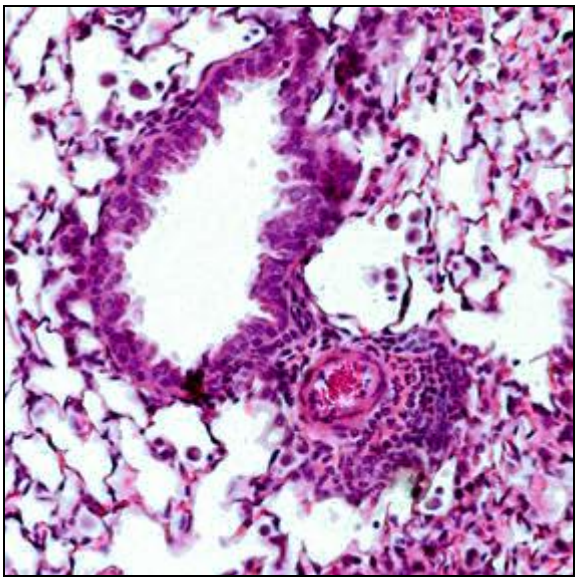
Figure 4.12 - The twelve day model of asthma induces lung eosinophilia

BALB/c mice were immunised and challenged using the 28 day protocol (OVA 100 µg IP days 1 and 14, OVA 10 µg IN days 25, 26 and 27) or the 12 day protocol (OVA 100 µg IP on day 1, 10 µg days 9, 10 and 11). Control mice received PBS challenge alone. The day after the final challenge BAL was collected. Differential counts were performed on BAL samples. Results are shown as the mean of each group of mice +/- SEM. n=4 mice.

PBS



28 Day Model



12 Day Model

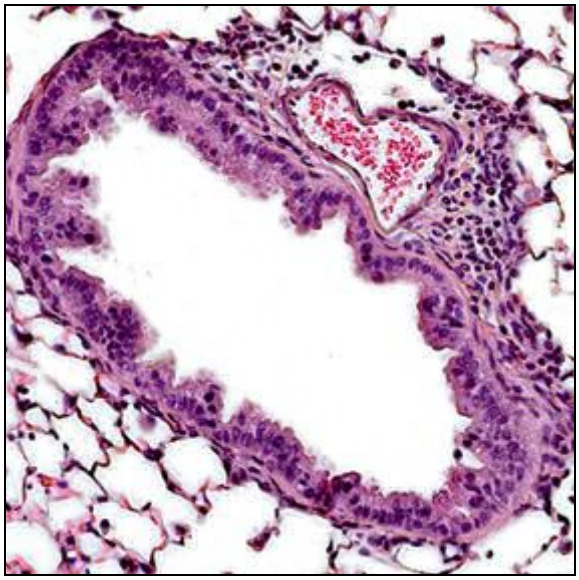


Figure 4.13 - The twelve day model of asthma induces lung eosinophilia

BALB/c mice were immunised and challenged as in Figure 4.12. The day after the final challenge lungs were collected and fixed in formalin. Sections were prepared and stained with H&E. Panels shown are representative sections.

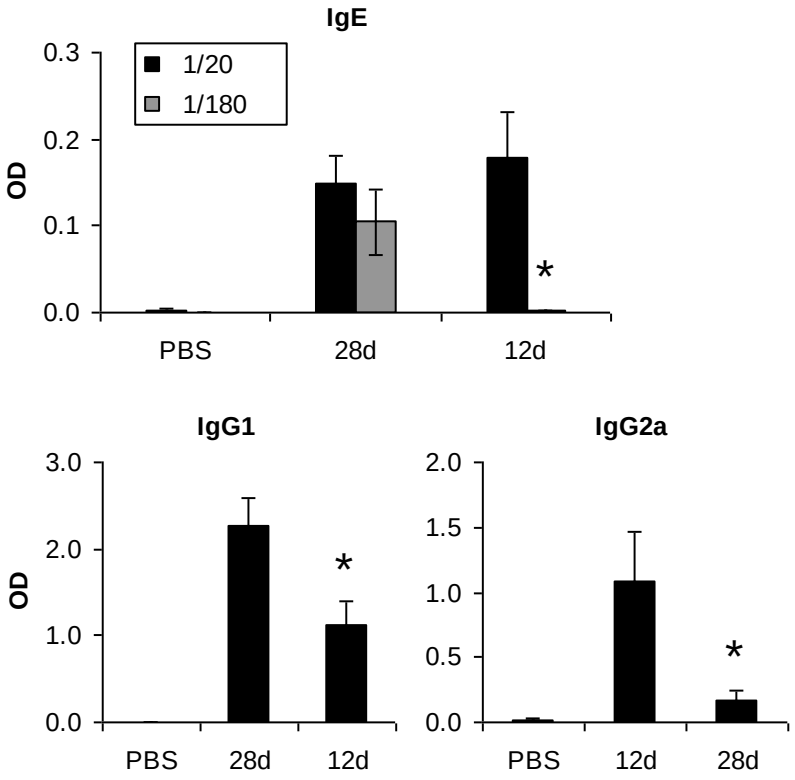


Figure 4.14 - The twelve day model of asthma induces antigen specific immunoglobulins

Mice were immunised and challenged as is Figure 4.12. Serum was collected the day after the final challenge. OVA specific IgE was measured in the serum at dilutions of 1/20 and 1/80. OVA specific IgG1 and IgG2a were measured at dilutions of 1/500 and 1/20 respectively. Results are shown as the mean of each group of mice +/- SEM. n=4 mice. * $p<0.05$ compared with 12d protocol.

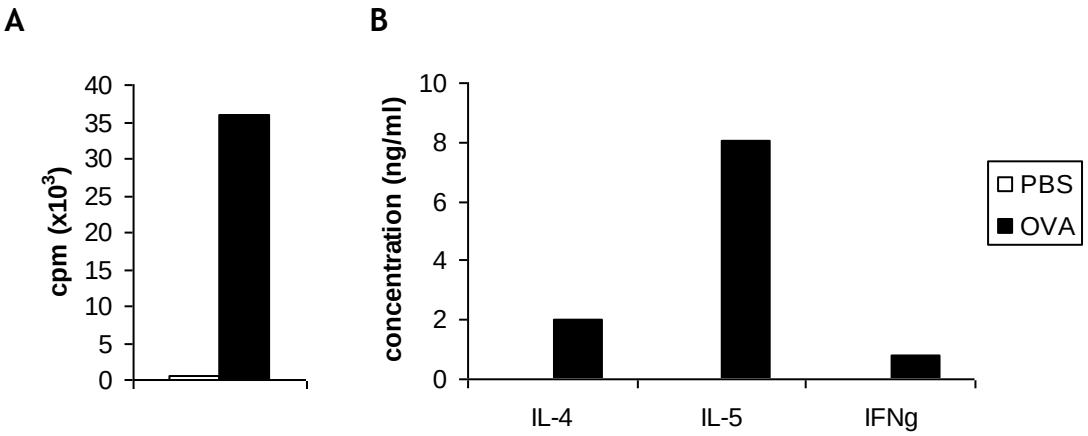


Figure 4.15 - The twelve day model of asthma induces antigen specific Th2 lymphocytes

Mice were immunised and challenged as is in Figure 4.8 and lymph nodes harvested the day after the final challenge. Single cell suspensions were stimulated with PBS or OVA (1 mg/ml). (**A**) After 72 hours proliferation was measured by ^{3}H thymidine uptake. (**B**) After 96 hours supernatants were collected and IL-4, IL-5 and IFNγ measured by ELISA. Results are shown as pooled samples from groups of 4 mice.

4.4 Cytokines and chemokines in the twelve day model

Before studying any potential role for ST2 in the 12 day model of asthma I sought to characterise the model further. Having shown that IL-33, acting via ST2, could be a potent inducer of chemokine production I was interested in the relationship between cytokines, chemokines and the inflammatory response in the airways. There have been a number of studies examining the expression of and requirement for chemokines in asthma. As discussed in Chapter 1 there is evidence that chemokines, such as the eotaxins, as well as cytokines, particularly IL-5, play an important role in eosinophil recruitment to the airways. However there is less information on the temporal expression of chemokines following antigen challenge in allergic airways inflammation. While some data are available in many cases they are at the RNA level only(179,481) or use unconventional models or limited time points(41,179). Therefore I set out to measure cytokine and chemokine levels in the BAL and serum at multiple time points throughout airways challenge.

4.4.1 Kinetics of airways inflammation following multiple challenges

Having chosen to investigate the challenge phase of the 12 day model I designed an experiment where groups of mice were sacrificed at different intervals throughout the challenge period. This would make it possible to study the relationship between cytokines, chemokines and eosinophil accumulation. My hypothesis was that each intranasal challenge would stimulate cytokine and chemokine release and that eosinophil numbers would rise with each challenge, perhaps falling again between challenges. As shown in Figure 4.16 all mice were immunised according to the standard 12 day protocol. The first group were then culled immediately prior to the first intranasal challenge (0h), the next group 3 hours after the first challenge (3h), the next immediately prior to the second challenge (24h), the next 3 hours after the second challenge (27h) and so on. A final group was culled at what would be the standard cull day in the 12 day model (72h).

Throughout the challenge period the total number of cells recovered from the airways increased and was highest at 72 hours after the first challenge (Figure

4.17A); a time point analogous to the standard final day cull in the 12 day model. This rise in total cell numbers was mirrored by an increase in the percentage of eosinophils within the BAL to a peak of 72% at 72 hours (Figure 4.17B). While the absolute number of macrophages increased slowly throughout the challenge period this is eclipsed by the rise in eosinophils by 72 hours (Figure 4.17C).

When the local cytokine and chemokine levels within the BAL were assayed, the Th2 cytokine levels all peaked at 24h after the initial challenge and decreased to almost undetectable levels at 72 hours (Figure 4.18). IL-4, IL-5 and IL-13 increased to concentrations of 1810 pg/ml, 101 pg/ml and 459 pg/ml at 24 hours respectively before falling to 59 pg/ml, 7 pg/ml and 19 pg/ml. The pro-inflammatory cytokine IL-6 followed a similar pattern with a peak concentration of 74 pg/ml at 24 hours (data not shown). Concentrations of IL-1, IL-2, IL-10, IL-12, IL-17, IFNγ and TNFα were below 50 pg/ml.

Given that eosinophilic inflammation within the airways progresses despite a fall in local Th2 cytokine levels I hypothesised that local chemokine production could be responsible for ongoing eosinophil recruitment and activation. Although MIP-1α reached a peak of 217 pg/ml at the later time point of 48h, levels had fallen to only 76 pg/ml by 72h (Figure 4.18). Eotaxin-2 and TARC however did rise progressively throughout the challenge period (Figure 4.18). The changes in eotaxin-2 concentrations correlated particularly well with eosinophil numbers as shown in Figure 4.19. Concentrations of GM-CSF, KC, IP-10, MCP-1, MIG, eotaxin-1 and TCA3 in the BAL were below 50 pg/ml.

Although local cytokine and chemokine concentrations are likely to play an important role in the recruitment of cells to the site of inflammation, cytokines and chemokines released systemically can drive selective haematopoiesis and the mobilisation of cells from the bone marrow. Serum cytokine levels followed a similar pattern to that seen in the BAL with IL-5 and IL-13 peaking at 227 pg/ml and 256 pg/ml at 24 to 27 hours before falling to 0 pg/ml and 40 pg/ml at 72 hours (Figure 4.20A). IL-10 followed a similar pattern, peaking at 116 pg/ml at 24h (data not shown). Concentrations of IL-1, IL-2, IL-4, IL-6, IL-12, IL-17, IFNγ and TNFα in the serum were below 50 pg/ml.

The chemokines eotaxin-1 and KC did show a trend towards rising 3h after each challenge before falling again at 24h, whereas the converse was true of MIG (Figure 4.20B). Concentrations of GM-CSF, IP-10, MCP-1, MIP-1α, eotaxin-2, TCA3 and TARC in the serum were below 50 pg/ml.

Antigen specific IgE is generated in response to immunisation with OVA and there was no evidence that titres were affected by intranasal antigen challenge at dilutions of between 1/10 (Figure 4.21) and 1/1280.

To obtain information on the local specific immune response cells from the lymph nodes draining the lungs were cultured with OVA and the cytokines and chemokines produced assayed (Figure 4.22). The cytokines produced by lymph node cells at the highest concentrations were IL-5 and IL-13, which increased 24 hours after the first challenge and remained elevated thereafter. The other Th2 cytokine IL-4 was produced in increasing amounts from lymph nodes as the challenge period progressed. The pro-inflammatory cytokines IL-17 and IFNγ were also detected at high concentrations, but there was no obvious relationship with the timing of intranasal challenges. A similar pattern, although at lower concentrations, was seen for IL-6 and MCP-1. The chemokines eotaxin-2 and TARC were released at relatively low concentrations, but lymph node cells from later in the challenge period produced these chemokines at higher concentrations than those from the beginning of the challenge period. IL-10, TNFα, GM-CSF, KC, MIP-1α, TCA3 and eotaxin-1 were produced at concentrations of between 100 to 300 pg/ml prior to antigen challenge and did not change significantly throughout the time course (data not shown). IL-1, IL-2, IL-12, IP-10 and MIG were not detected in draining lymph node supernatants.

These data demonstrate that in the 12 day model eosinophil numbers in the airways rise steadily during the challenge period, despite the fact that Th2 cytokine concentrations, both locally in the BAL and systemically in the serum, peak prior to the second challenge before falling to very low concentrations by day 12. Chemokines may be responsible for the accumulation of eosinophils within the airways given that BAL eotaxin-2 concentrations correlate with eosinophil numbers and intranasal antigen challenges appear to result in transient increases in eotaxin-1 and KC within the serum.

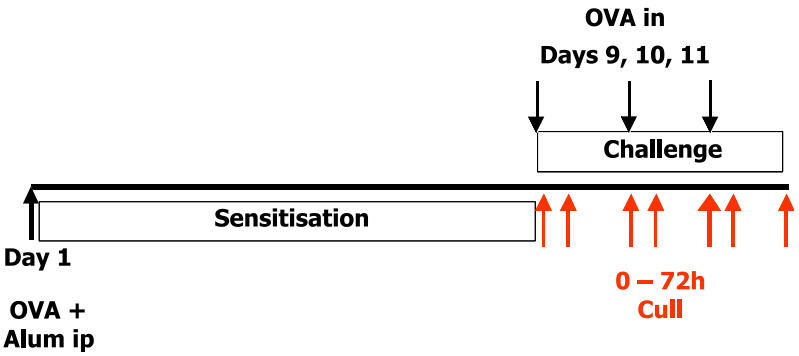


Figure 4.16 - A time course study of the twelve day asthma model

BALB/c mice were immunised with OVA (100 µg) and alum on day 1 and were challenged with OVA (10 µg) on days 9, 10 and 11. BAL, serum and draining lymph nodes were collected from groups of mice immediately prior to each challenge (0, 24 and 48 hours) or at 3 hours after each challenge (3, 27, 51 hours). Samples were collected from a final group at the standard day 12 end point for the model (72 hours).

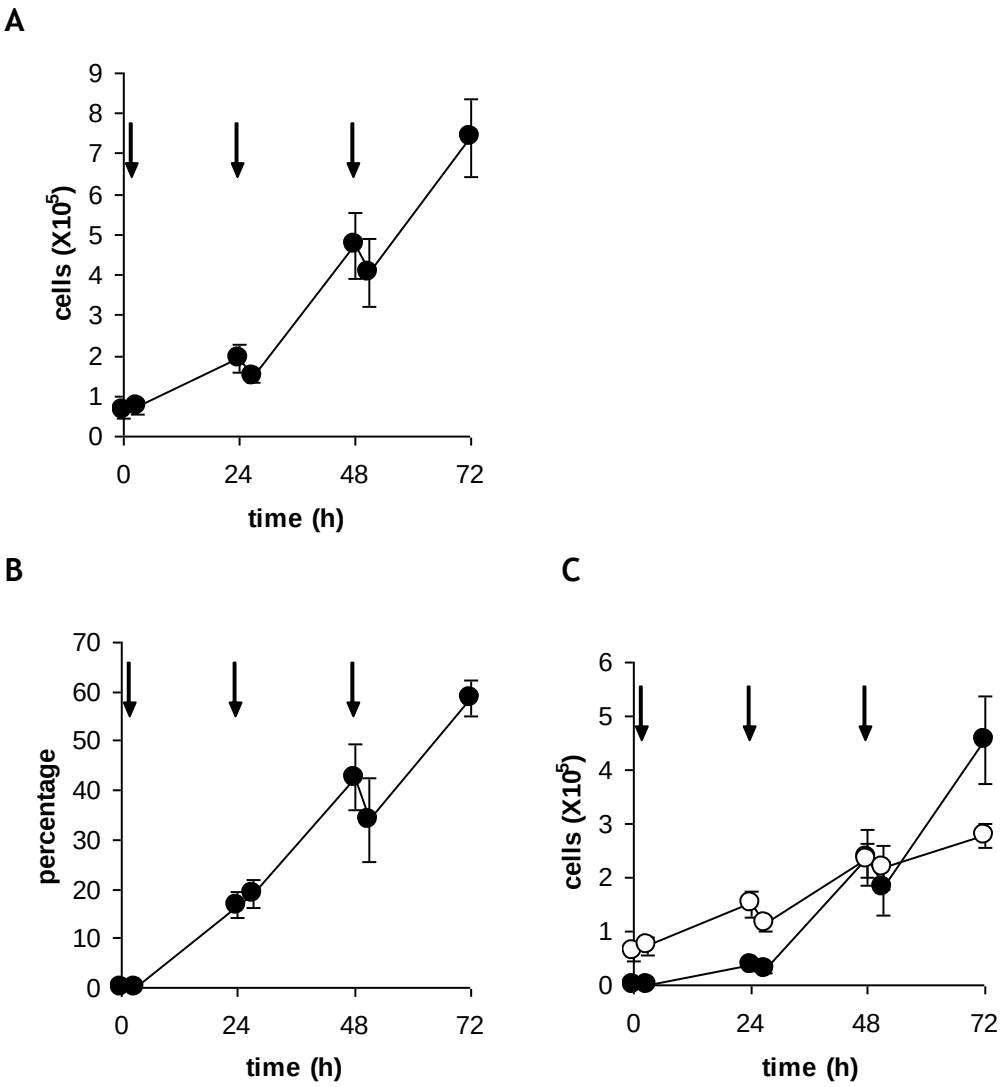


Figure 4.17 - Eosinophil numbers increase with time during challenge

Mice were immunised and challenged as in Figure 4.16. (**A**) Total BAL counts were performed. (**B**,**C**) Differential counts were performed and macrophage (○) and eosinophil (●) proportions calculated. Arrows indicated intranasal challenges (0h, 24h and 48h). Results are shown as the mean of each group of mice +/- SEM. n=8 mice.

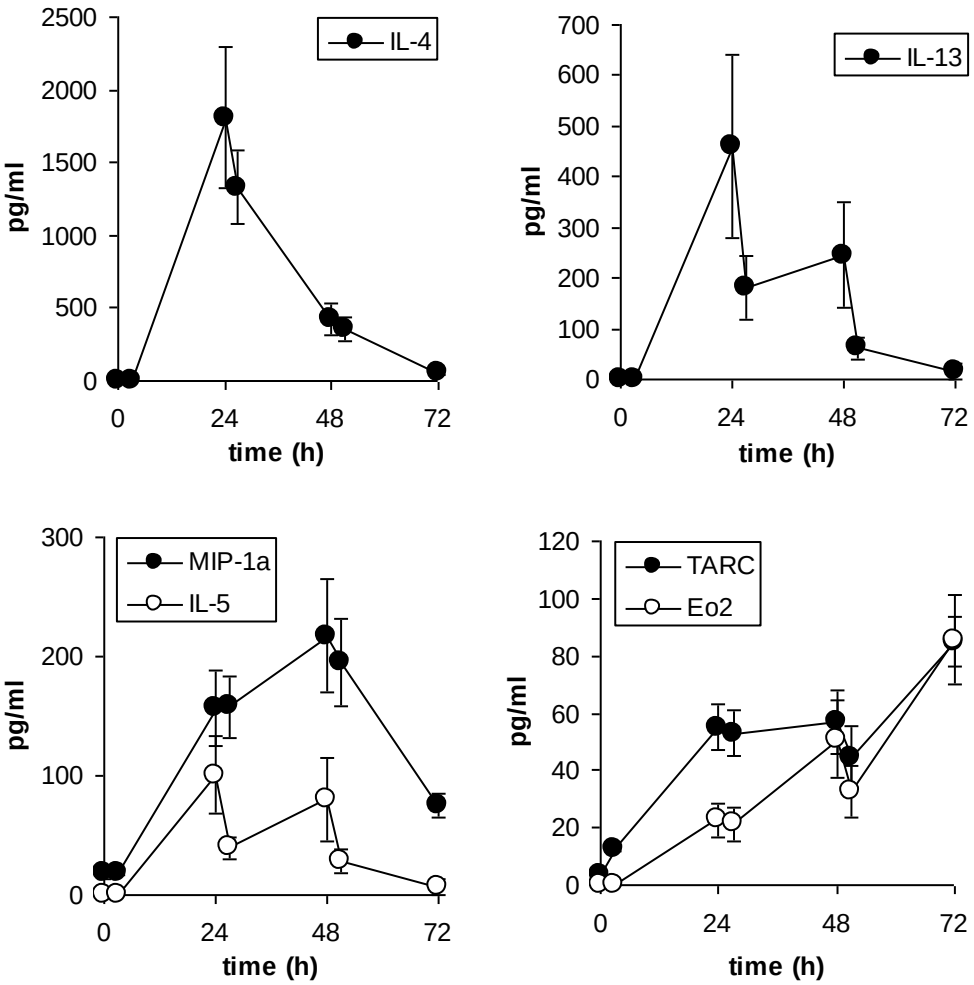


Figure 4.18 – BAL cytokine and chemokine concentrations change throughout the challenge period

Mice were immunised and challenged as in Figure 4.16. BAL was collected from groups at each time point and cytokines and chemokines measured by ELISA or multiplexed immunoassay. Eo2 = eotaxin-2. Results are shown as the mean of each group of mice +/- SEM. n=8 mice.

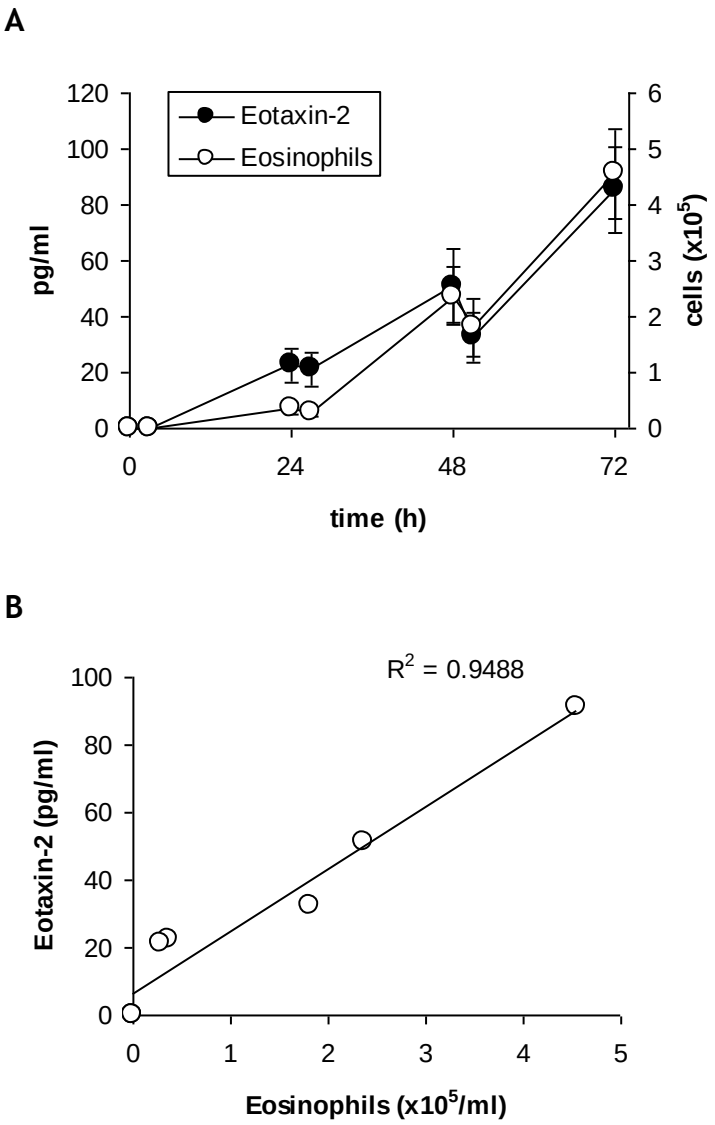
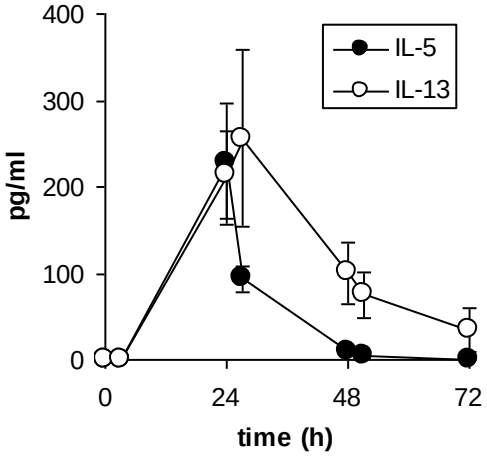


Figure 4.19 - Airways eosinophilia correlates with local eotaxin-2 concentrations

Mean total eosinophil numbers (Figure 4.17C) were plotted against BAL eotaxin-2 concentration (Figure 4.18D) for mice treated as in Figure 4.16.

A



B

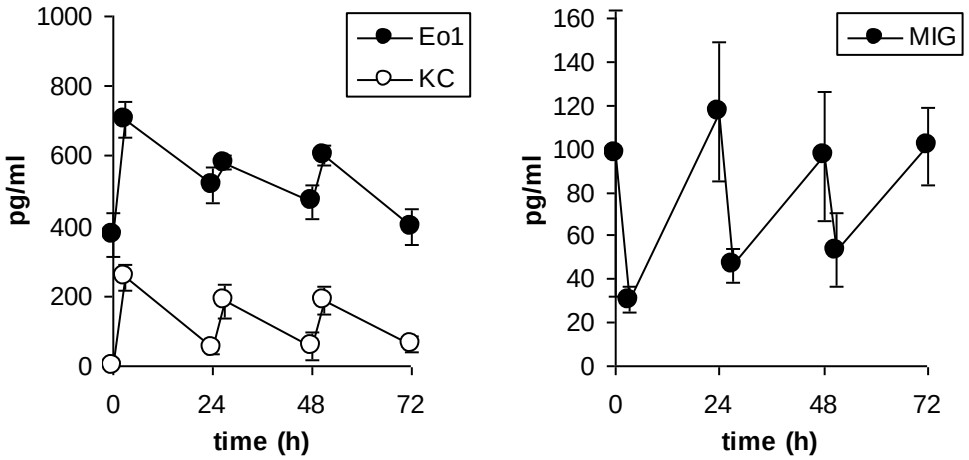


Figure 4.20 – Serum cytokine and chemokine concentrations change throughout the challenge period

Mice were immunised and challenged as in Figure 4.16. Serum was collected from groups at each time point and cytokines and chemokines measured by ELISA or multiplexed immunoassay. Results are shown as the mean of each group of mice +/- SEM. n=8 mice

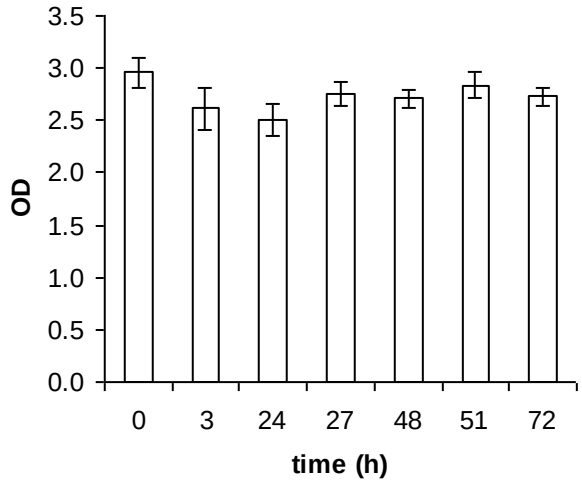


Figure 4.21 - Serum IgE titres are unaffected by intranasal antigen challenge

Mice were immunised and challenged as in Figure 4.16. Serum was collected from groups at each time point and OVA-specific IgE measured by ELISA at a dilution of 1/10. Results are shown as the mean of each group of mice +/- SEM. n=8 mice

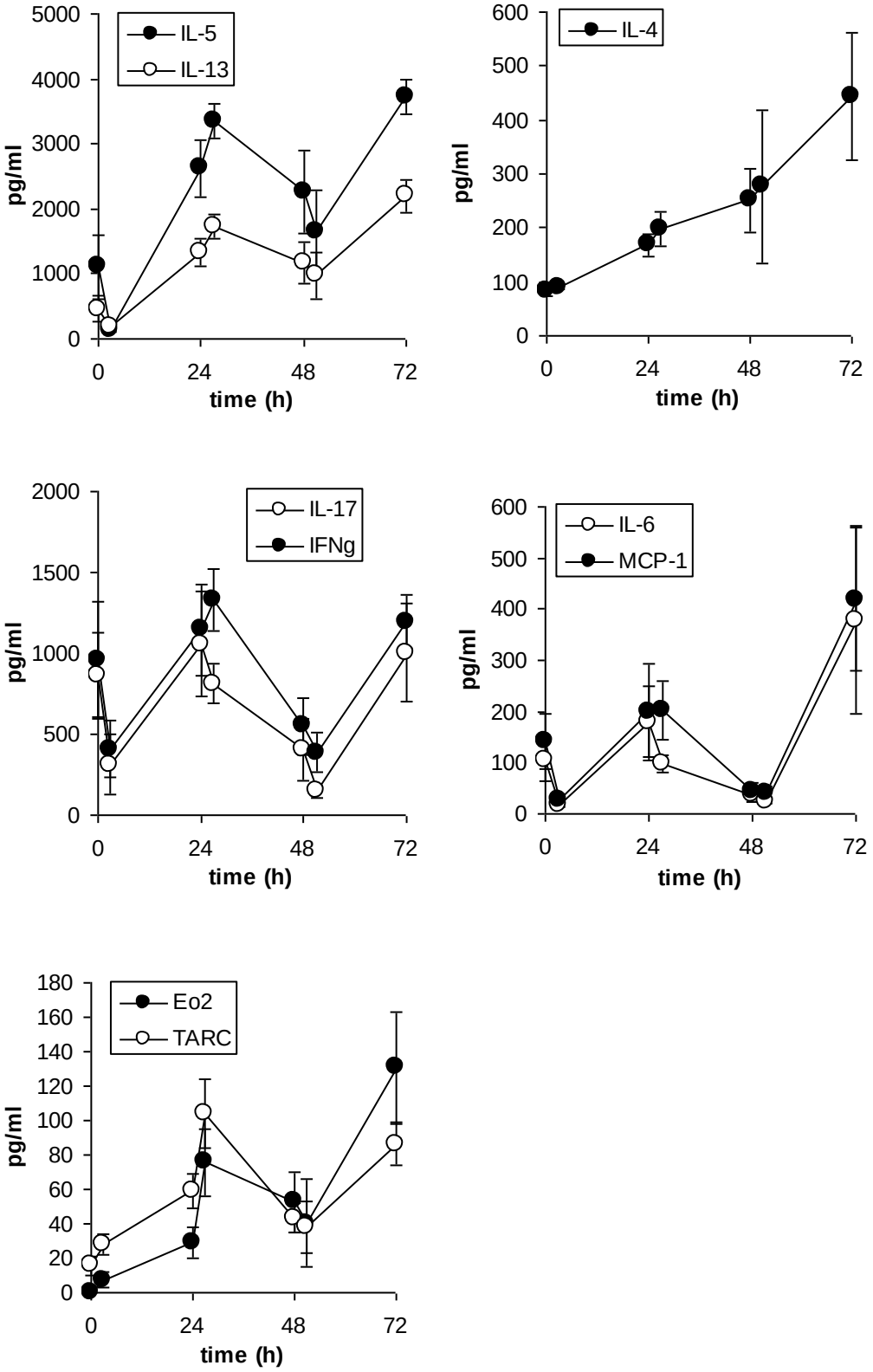


Figure 4.22 – Th2 cytokines and eotaxins are produced by draining lymph node cells

Mice were immunised and challenged as in Figure 4.16. Draining lymph nodes were collected from groups at each time point and single cell suspensions from individual mice restimulated with OVA 1 mg/ml. Supernatants were collected after 96 hours and cytokines and chemokines measured by ELISA or multiplexed immunoassay. Eo1 = eotaxin-1, Eo2 = eotaxin-2. Results are shown as the mean of each group of mice +/- SEM. n=8 mice

4.4.2 Kinetics of airways inflammation following a single challenge

During the three day challenge period of the 12 day model, BAL Th2 cytokine concentrations decreased early while chemokine concentrations continued to rise. My hypothesis was that the stimulus for the increasing chemokine concentrations was the repeated antigen challenges. To test this hypothesis I chose to study the changes occurring after a single antigen challenge. I predicted that, as in the three challenge protocol, Th2 cytokines would increase transiently and then fall rapidly after a single challenge, but that chemokine concentrations would also fall in the absence of further challenges, and that this would result in a fall in eosinophil numbers.

Mice were immunised with OVA and alum as previously. In order to make comparisons I choose to study the same time period as that used with three challenges, from days 9 to 12 (0 to 72 hours). Therefore the challenge given on day 9 would be identical in both experiments, but in the single challenge protocol there would not be the usual second and third challenge on days 10 and 11. Groups of mice were culled at 24 hour intervals following the challenge and BAL and serum collected (Figure 4.23).

Following a single intranasal OVA challenge the percentage of eosinophils in the BAL increased steadily throughout the following 72 hour period and reached a maximum of 27% at 72 hours post challenge (Figure 4.24A). This was associated with a rise in total eosinophil numbers over time to a maximum of 0.5×10^5 cells/ml at 72 hours (Figure 4.24B).

In the BAL IL-4 peaked 24 hours after challenge before falling rapidly (Figure 4.24C). A similar pattern was seen with serum IL-5 (Figure 4.24D). BAL IL-5, serum IL-4 and BAL and serum IL-13 were undetectable. BAL eotaxin-2 concentrations increased more slowly to a peak at between 48 and 72 hours.

These data demonstrated that, as in the three challenge model, BAL and serum Th2 cytokine concentrations peak at 24 hours after challenge before rapidly falling again. Surprisingly BAL eosinophils continued to rise in the absence of

further challenges with the highest number recorded at the final 72 hours time point. This was associated with a continued rise in eotaxin-2 concentration.

On day 12 of the single challenge protocol eosinophils are still in the minority at 27% of total BAL cells, and absolute numbers are low at 0.5 x 10^5 cells/ml. This is in contrast to an eosinophilia of 59% or 4.6 x 10^5 cells/ml on day 12 in the three challenge model. Taking this into account I chose to continue to work with the three challenge 12 day model in which I had demonstrated the generation of AHR and histological changes earlier.

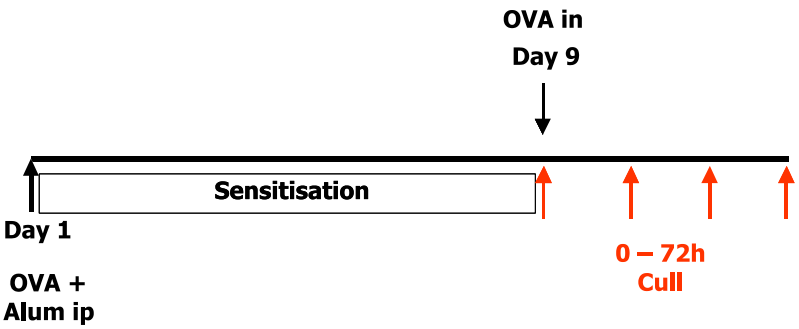


Figure 4.23 - A time course study of the twelve day asthma model

BALB/c mice were immunised with OVA (100 µg) and alum on day 1 and were challenged with OVA (10 µg) on day 9. BAL, serum and draining lymph nodes were collected from groups of mice immediately prior to the challenge (0h) and then every 24 hours thereafter up to 72 hours.

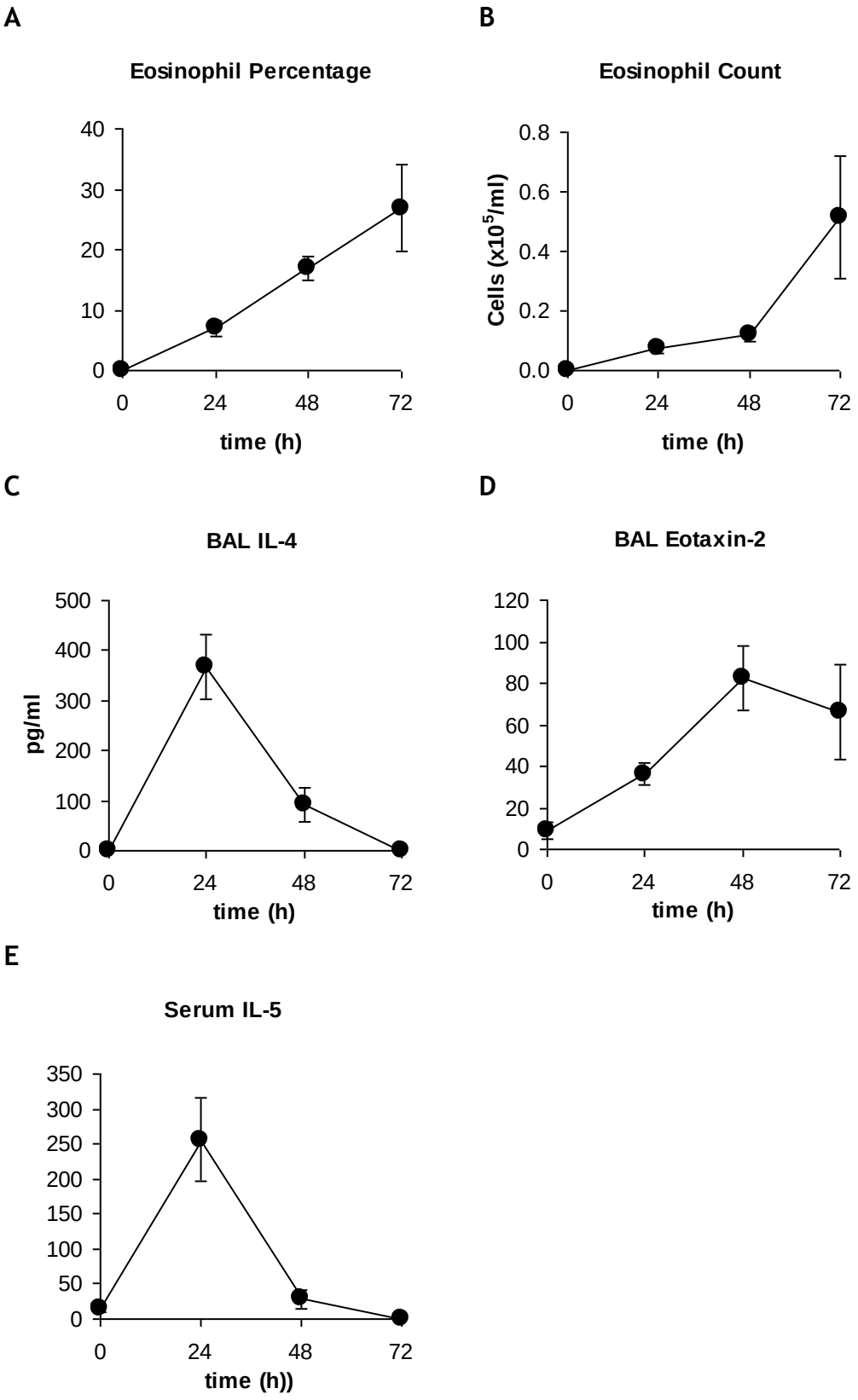


Figure 4.24 – A single airways challenge induces eosinophilia and Th2 cytokines

Mice were immunised and challenged as in Figure 4.23. BAL and serum samples were collected immediately prior to challenge (0h) or at the noted interval after challenge. (**A**,**B**) BAL differential counts were performed. (**C**,**D**,**E**) Cytokine and chemokine concentrations in BAL and serum were determined by ELISA. Results are shown as the mean of each group of mice +/- SEM. n=5 mice.

4.5 The role of ST2 in murine asthma models

As discussed earlier the importance of ST2 in experimental asthma remains unclear. Neutralising antibodies against ST2 have attenuated eosinophilic inflammation(298,372,396). Whereas, at the outset of this work, the only study of asthma in $ST2^{-/-}$ mice found no difference in inflammation when a 35 day OVA based model of asthma utilising 15 airways challenges was used in C57Bl/6 mice. Given the conflicting data utilising antibodies, the intensive challenge protocol used in the $ST2^{-/-}$ study and the possibility that the strain could contribute significantly to the outcome; as in $IL\text{-}5^{-/-}$ studies where AHR is lost in C57Bl/6 but not BALB/c mice(94,100), I chose to study the effect on ST2 gene deletion on a BALB/c background in the 12 day model of asthma.

4.5.1 The effect of ST2 gene knock-out in the twelve day model

Using the 12 day asthma model validated above, we observed that in ST2 deficient mice airways inflammation was significantly reduced compared with similarly challenged wild type (WT) mice. There was a reduction in the total number of inflammatory cells within the airways (Figure 4.25A). This was predominantly due to a reduction in the percentage and absolute number of eosinophils (Figure 4.25B,C), although there was also a reduction in macrophage numbers(Figure 4.25C).

Cytokines and chemokines were generally only detected at low concentrations in the BAL in both groups and although there appeared to be a trend towards a reduction in BAL IL-4, eotaxin-2 and TARC in $ST2^{-/-}$ mice the changes were not statistically significant (Figure 4.26A). Concentrations of IL-1, IL-2, IL-5, IL-10, IL-12, IL-13, IL-17, IFNγ, TNFα, GM-CSF, FGF, IP-10, MCP-1, KC, TCA3 and eotaxin-1 in the BAL were below 50 pg/ml.

With the exception of eotaxin-1, cytokines and chemokines were detected at low concentrations in the serum (Figure 4.26B). There was a significant reduction in serum IL-13 in $ST2^{-/-}$ mice, although the concentration in WT mice was only 40 pg/ml. Concentrations of IL-1, IL-2, IL-4, IL-5, IL-6, IL-12, IL-17, IFNγ, TNFα, GM-CSF, IP-10, MCP-1, MIP-1α, TARC and TCA3 in the serum were below 50 pg/ml.

When draining lymph node cells were restimulated with OVA IL-5 and IL-13 were produced at ng/ml concentrations with other cytokines and chemokines produced at lower concentrations (Figure 4.27). The only statistically significant difference between WT and $ST2^{-/-}$ mice was a reduction in the concentration of TNFα in $ST2^{-/-}$ mice.

These data demonstrate that the absence of ST2 significantly attenuates allergic airways inflammation, but do not offer any insights into the mechanism behind this.

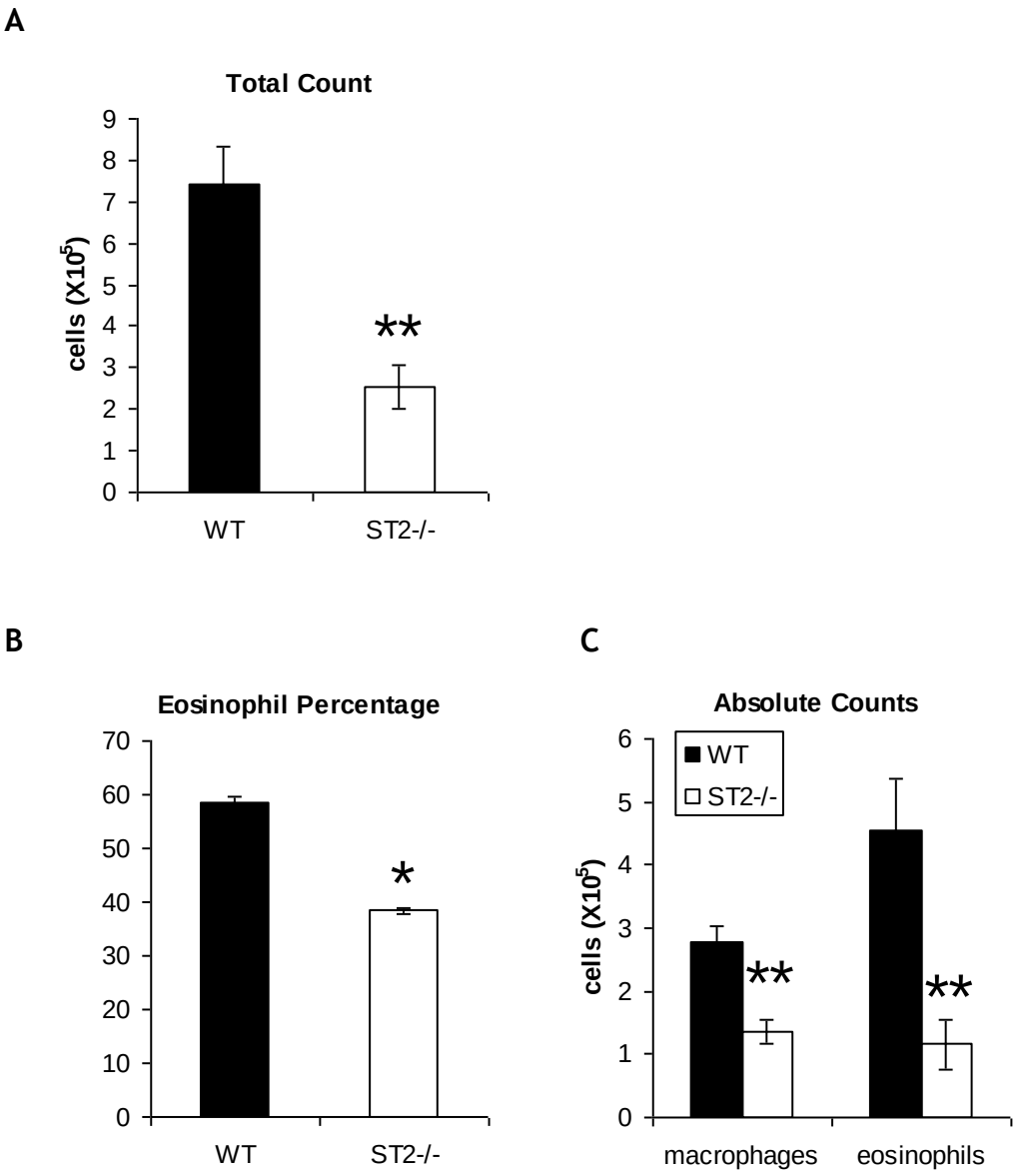


Figure 4.25 - ST2 gene knockout attenuates airways inflammation

BALB/c WT and $ST2^{-/-}$ mice were immunised with OVA and alum prior to being challenged with OVA utilising the 12 day protocol (Figure 4.8). The day after the final challenge BAL was performed. (**A**) Total cell numbers were counted. (**B**,**C**) Differential counts were performed. Results are shown as the mean of each group of mice +/- SEM. n=7 mice. * $p<0.05$, ** $p<0.01$. Results are representative of three separate experiments.

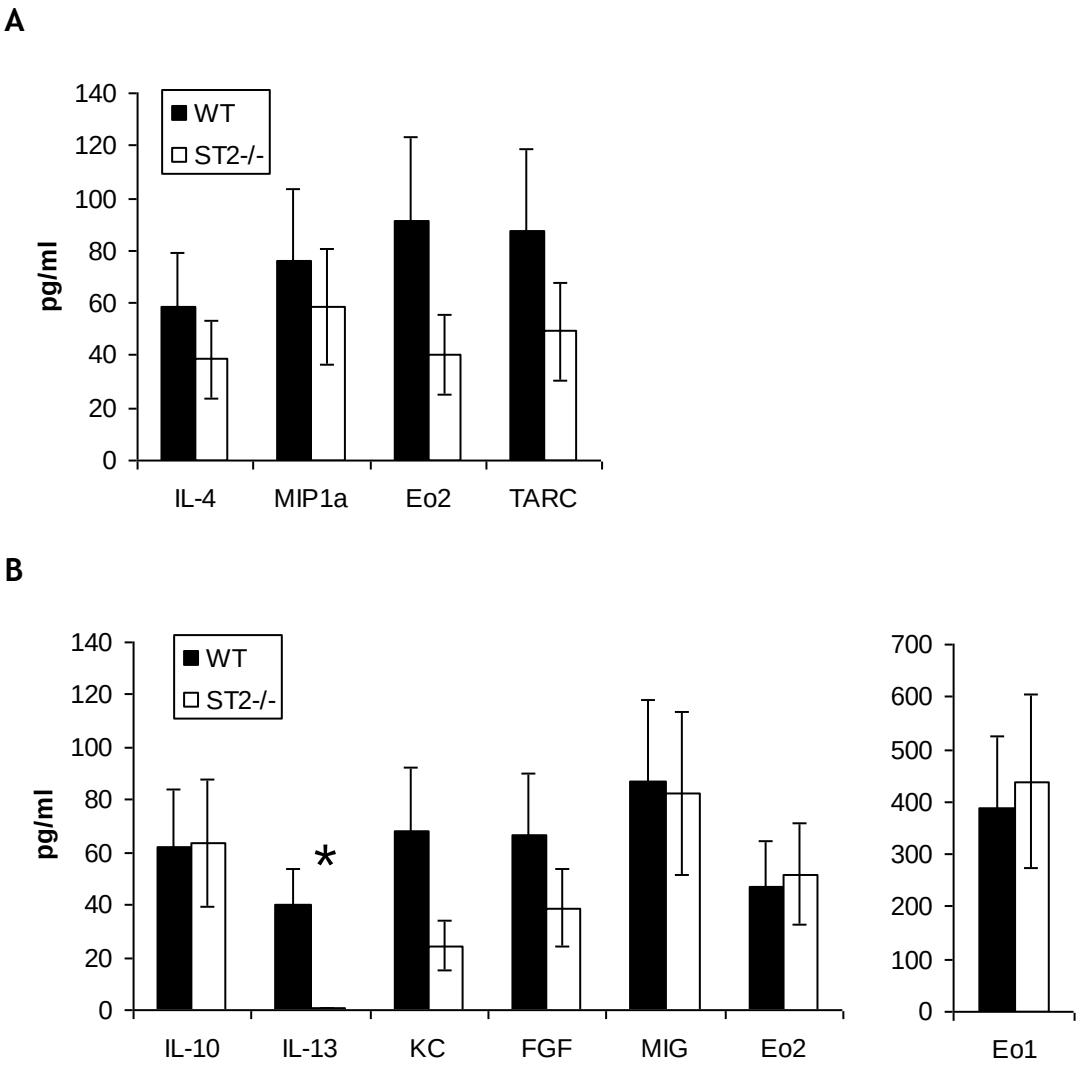


Figure 4.26 –ST2 gene knockout does not affect BAL and serum cytokine and chemokines

BAL was obtained from immunised and challenged WT and $ST2^{-/-}$ mice as in Figure 4.25. Cytokine and chemokine concentrations were measured by ELISA and multiplexed immunoassay in (**A**) BAL and (**B**) serum. Eo1 = eotaxin-1, Eo2 = eotaxin-2. Results are shown as the mean of each group of mice +/- SEM. n=7 mice. * $p<0.05$.

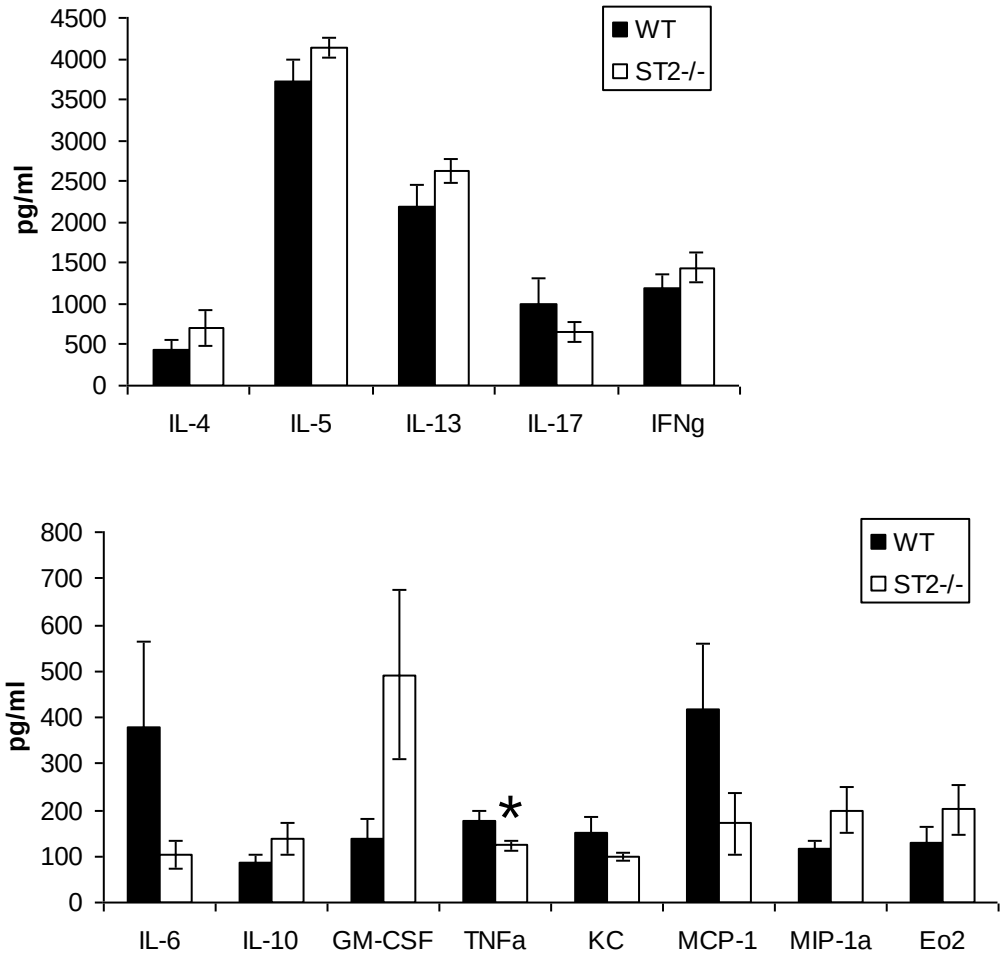


Figure 4.27 - ST2 gene knockout does not affect draining lymph node responses

WT and $ST2^{-/-}$ mice were immunised and challenged as in Figure 4.25. Draining lymph nodes were collected on day 12 and single cell suspensions from individual mice restimulated with OVA 1 mg/ml. Supernatants were collected after 96 hours and cytokines and chemokines measured by ELISA or multiplexed immunoassay. Eo2 = eotaxin-2. Results are shown as the mean of each group of mice +/- SEM. n=7 mice. * $p<0.05$.

4.5.2 Establishing an alum-free model of asthma

Having demonstrated that ST2 is important in a 12 day model of asthma in BALB/c mice I sought to explore the mechanism for this. In addition to the previously cited paper(381), during the course of this work further data came to light published by Mangan *et al*(383). Both these studies are summarised in Table 4.1. Using a 30 day OVA and alum based model in BALB/c mice Mangan *et al* found that, while histological inflammation and AHR were unaffected by ST2 gene deletion, BAL eosinophils were elevated. The fact that I found a reduction in airways eosinophilia and Hoshino *et al* did not could be attributed to differences in mouse strain as well as model. However the fact that Mangan *et al* and I reached opposite conclusions using the same strain of $ST2^{-/-}$ BALB/c mice suggests that the model used may be crucial. I have used a 12 day model in which mice were immunised with OVA 100 µg in alum before being challenged on days 9 to 11 with 10 µg of intranasal OVA. Mangan *at al* immunised with 2 µg of OVA in alum on days 1 and 14 before challenging with nebulised OVA on days 28 to 30.

Investigator	Mice	Sensitisation	Challenges	Length	EØ
Hoshino(381)	C57Bl/6	OVA + Alum d0 + d12	15	32 days	NC
Mangan(383)	BALB/c	OVA + Alum d1 + d14	3	31 days	↑
Pitman	BALB/c	OVA + Alum d1	3	12 days	↓

Table 4.1 – The effect of ST2 gene deletion in experimental asthma

Key – Sensitisation: antigen and adjuvant administered on indicated days. Challenges: number of airways challenges given. Length: Total duration of model. EØ: effect on lung eosinophil numbers.

It is possible that the requirement for ST2 in experimental asthma is upon mast cells. The data I presented in Chapter 3 suggests that ST2 can be an important receptor on mast cells. As discussed previously conventional models of asthma featuring immunisation with OVA and alum are mast cell independent(157,162). The 12 day model used here has not been extensively studied, the difference between it and more conventional models, and the importance of mast cells in this model are unknown. One of the only models utilising alum in which mast cell deficiency resulted in reduced eosinophilia was very similar. Mice were

immunised with OVA and alum on days 1 and 5 before being challenged with OVA twice on day 12(159).

I hypothesised that ST2 was required in models of asthma which were mast cell dependent. The omission of alum has been shown to induce mast cell dependency in models which otherwise do not require mast cells. Therefore I chose to study the role of ST2 in one of these published models.

I tested two alum-free models based on published protocols. The first paper to highlight the role of mast cells in alum-free models used seven sensitisations of OVA (10 µg) alone over 14 days followed by three intranasal challenges of OVA (200 µg) on days 40, 43 and 46(162). A more recent paper demonstrated that the omission of alum from a shorter more conventional model resulted in mast cell dependence. Mice were sensitised with OVA (20 µg) alone on days 0 and 14 before being challenged with nebulised OVA on days 28, 29 and 30(163).

Both protocols were adapted slightly in line with our own laboratory experience, specifically they were both shortened from 47 and 31 days to 28 days, and in the later model intranasal delivery was used in place of nebulised delivery. Figure 4.28 details the two models, Protocol A and Protocol B. I set out to confirm that with these minor changes these models generated airways and tissue inflammation, AHR and antigen specific IgE.

Although BAL total cell counts were increased in both models this was only statistically significant in Protocol A (Figure 4.29A). The proportion of eosinophils within the BAL was significantly elevated in both protocols but was again more marked in Protocol A (Figure 4.29B). This translated to a rise in total BAL eosinophils which was greater and more statistically significant in Protocol A than Protocol B (Figure 4.29C). IL-4, IL-5 and IL-13 were not detected in the BAL.

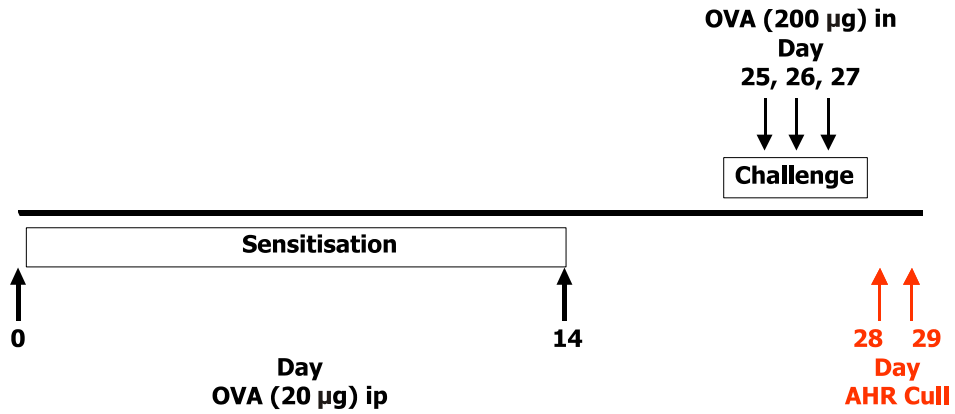
Significant AHR was generated in both models (Figure 4.30). Antigen specific IgE was also generated using both protocols, although levels were only significantly higher than PBS with Protocol B (Figure 4.31).

The reason for the generally more pronounced inflammatory response, but reduced IgE titres in Protocol A is unclear. The only difference between the two

protocols was in the immunisation, with Protocol A using a total of 40 µg of OVA administered at two immunisations and Protocol B using a total 70 µg administered at seven immunisations.

Protocol A was selected for further study. Not only was BAL eosinophilia more marked, but the reduced number of immunisations was preferable in terms of animal welfare. Histological sections confirmed that Protocol A also induced tissue eosinophilia (Figure 4.32).

Protocol A



Protocol B

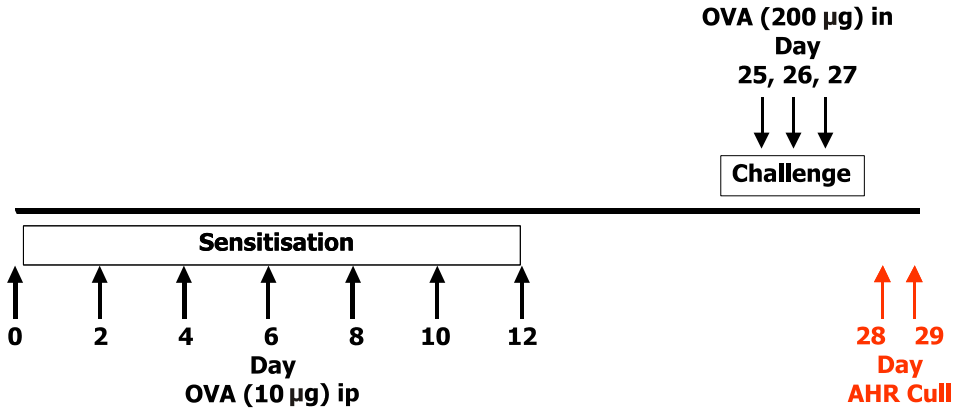


Figure 4.28 - Two alum-free models of asthma

(**Protocol A**) Mice were immunised with OVA (20 µg) alone on days 0 and 14 before being challenged with intranasal OVA (200 µg) on days 25, 26 and 27. AHR was measured on day 28 and mice culled on day 29. (**Protocol B**) Mice were immunised with OVA (10 µg) alone on days 0, 2, 4, 6, 8, 10 and 12 before being challenged with intranasal OVA (200 µg) on days 25, 26 and 27. AHR was measured on day 28 and mice culled on day 29.

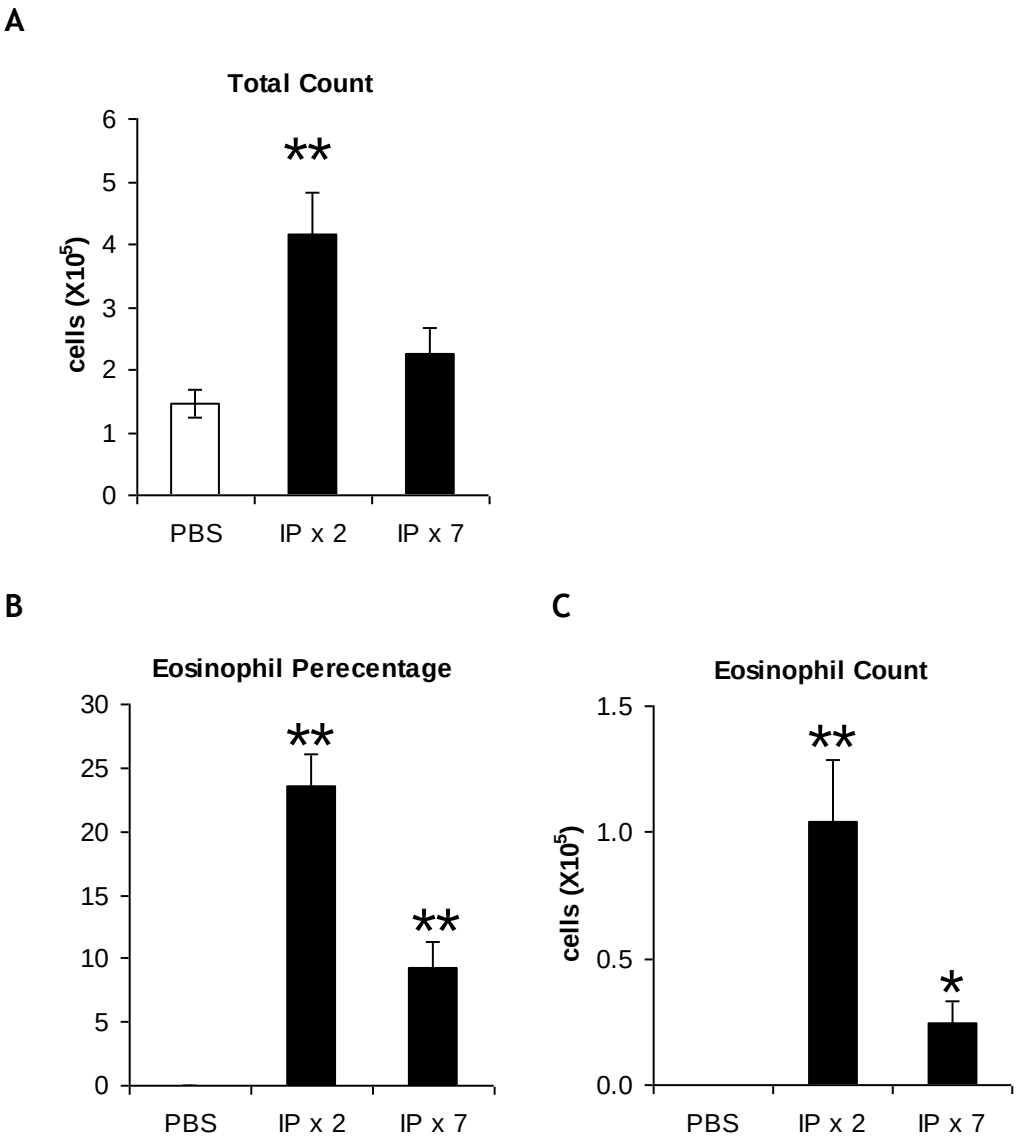


Figure 4.29 - BAL eosinophilia is generated in the absence of alum

Mice were immunised, challenged and culled according to Protocol A (IP x 2) or Protocol B (IP x 7) as detailed in Figure 4.28. Control mice (PBS) were sensitised with OVA (20 µg) on days 0 and 14 before being challenged with PBS alone on days 25, 26 and 27. (**A**) Total cell counts were performed upon BAL. (**B**,**C**) BAL differential counts were performed. Results are shown as the mean of each group of mice +/- SEM. n=6 mice. * $p<0.05$, ** $p<0.01$.

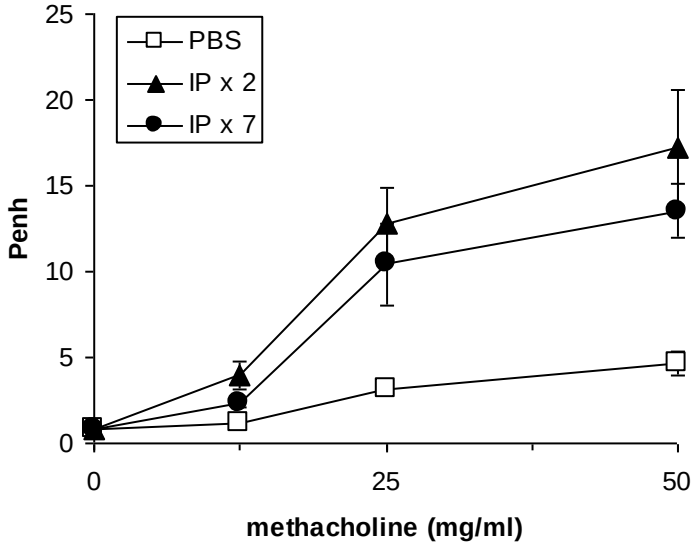


Figure 4.30 – Airways hyperresponsiveness is generated in the absence of alum

Mice were immunised and challenged as in Figure 4.29 with Penh being measured on day 28. Results are shown as the mean of each group of mice +/- SEM. n=6 mice. Two-way ANOVA $p<0.001$ IP x 2 compared with PBS, $p<0.001$ IP x 7 compared with PBS.

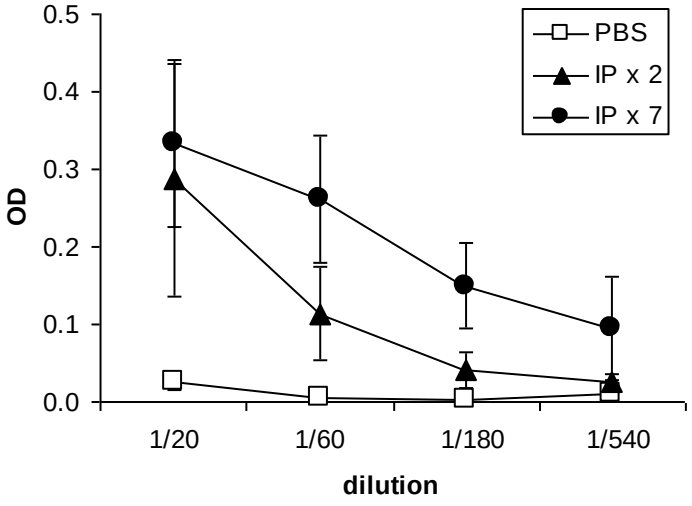


Figure 4.31 – Antigen specific IgE is generated in the absence of alum

Mice were immunised, challenged and culled as in Figure 4.29. OVA specific IgE was measured in serum by ELISA at dilutions from 1/20 to 1/540. Results are shown as the mean of each group of mice +/- SEM. n=6 mice. Two-way ANOVA $p<0.05$ IP x 2 compared with PBS, $p<0.001$ IP x 7 compared with PBS.

PBS **IP x 2**

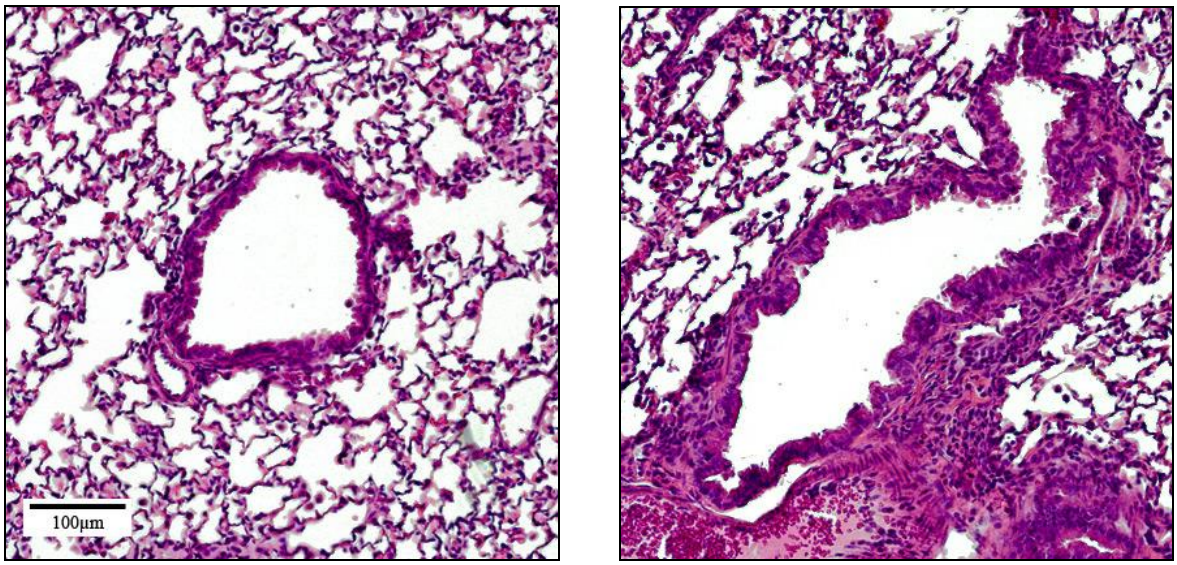


Figure 4.32 - Histological inflammation is induced in the absence of alum

Mice were immunised, challenged and culled as in Figure 4.29. Lungs were fixed in formalin and stained with H&E. Panels shown are representative sections.

4.5.3 The role of ST2 in an alum-free model of asthma

In order to determine whether or not there was an alum dependent requirement for ST2 in murine asthma I induced asthma in WT and $ST2^{-/-}$ mice with and without alum. Mice were immunised and challenged with OVA alone (Figure 4.28A) or using an identical protocol with the addition of 1% alum to both the day 0 and day 14 immunisation.

When immunised and challenged with OVA alone (OVA group) BAL total cell counts, eosinophil percentage and total eosinophil numbers were all significantly reduced in $ST2^{-/-}$ mice as compared with WT mice. Conversely, in the group immunised with OVA and alum (OVA-Alum group) BAL total cell counts and eosinophilia were no different between WT and $ST2^{-/-}$ mice (Figure 4.33). There was also evidence of attenuated eosinophilic inflammation on histology (Figure 4.34).

Despite ST2 gene deletion attenuating airway inflammation, the increased AHR in both asthma models was maintained. As expected AHR was generated in both the OVA alone model and the OVA-Alum model in WT mice. In both models AHR was unaffected by ST2 gene deletion (Figure 4.35).

BAL cytokines and chemokines provided no clear explanation for the significant reduction in eosinophils in $ST2^{-/-}$ mice immunised with OVA alone. IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IFNγ, TNFα, GM-CSF, KC, FGF, IP-10, MIG, MCP-1, MIP-1α and RANTES were undetectable or present at concentrations of less than 50 pg/ml, and did not differ significantly between protocols or strains. Eotaxin 1 and 2 were detected at slightly higher concentrations, and there was a non-significant trend for lower concentrations of eotaxin-2 in $ST2^{-/-}$ mice in both the OVA and the OVA-Alum protocols (Figure 4.36).

As with the BAL, cytokine and chemokine concentrations in the serum were generally low, those detected at the highest concentrations are shown in Figure 4.37. The mean concentration of IL-10 in WT mice immunised with PBS alone was high, although there was marked variance within this group. Concentrations of IL-10 were also relatively high in OVA-Alum mice immunised with OVA and Alum, but were undetectable in the serum of OVA mice. IL-12 was detected in the serum of all mice and was significantly reduced in $ST2^{-/-}$ mice relative to WT

mice in the OVA protocol. KC was present only in the serum of OVA or OVA-Alum WT mice, but despite the absence of KC in $ST2^{-/-}$ mice the difference was not significant due to the high variance. MIG was present in the serum of all OVA and OVA-Alum mice with the exception of $ST2^{-/-}$ mice immunised with OVA alone, but this difference was also not significant. Eotaxin-1 was detected at high concentrations and eotaxin-2 at lower concentrations. Both were detected at similar concentrations in the serum of all mice. Concentrations of IL-1, IL-2, IL-4, IL-5, IL-6, IL-13, IL-17, IFNγ, TNFα, GM-CSF, FGF, IP-10, MCP-1, MIP-1α and RANTES in the serum were below 50 pg/ml.

Antigen specific IgE is a critical component of any model of atopic asthma. While OVA specific IgE was unaffected by ST2 gene deletion in the OVA-Alum model, there was a significant reduction in IgE production in the OVA alone model (Figure 4.38A). The other Th2 associated antibody, IgG1 was unaffected by ST2 gene deletion in either model (Figure 4.38B). OVA specific IgG2a was also not significantly different between WT and $ST2^{-/-}$ mice (Figure 4.38C).

Given that isotype switching is likely to occur in the local draining lymph nodes under the influence of IL-4 or IL-13(120) I went on to examine the production of Th2 cytokines by draining lymph node cells when restimulated with OVA. When draining lymph node cells from mice immunised with OVA and alum were restimulated with OVA there was no difference in IL-4, IL-5 and IL-13 concentrations between WT and $ST2^{-/-}$ mice (Figure 4.39). In contrast when mice were immunised with OVA alone there was a significant reduction in IL-5 and IL-13 in lymph node cultures from $ST2^{-/-}$ mice, and a trend towards reduced IL-4 ($p= 0.06$). There was no significant cytokine production when lymph node cells were cultured with medium alone (data not shown).

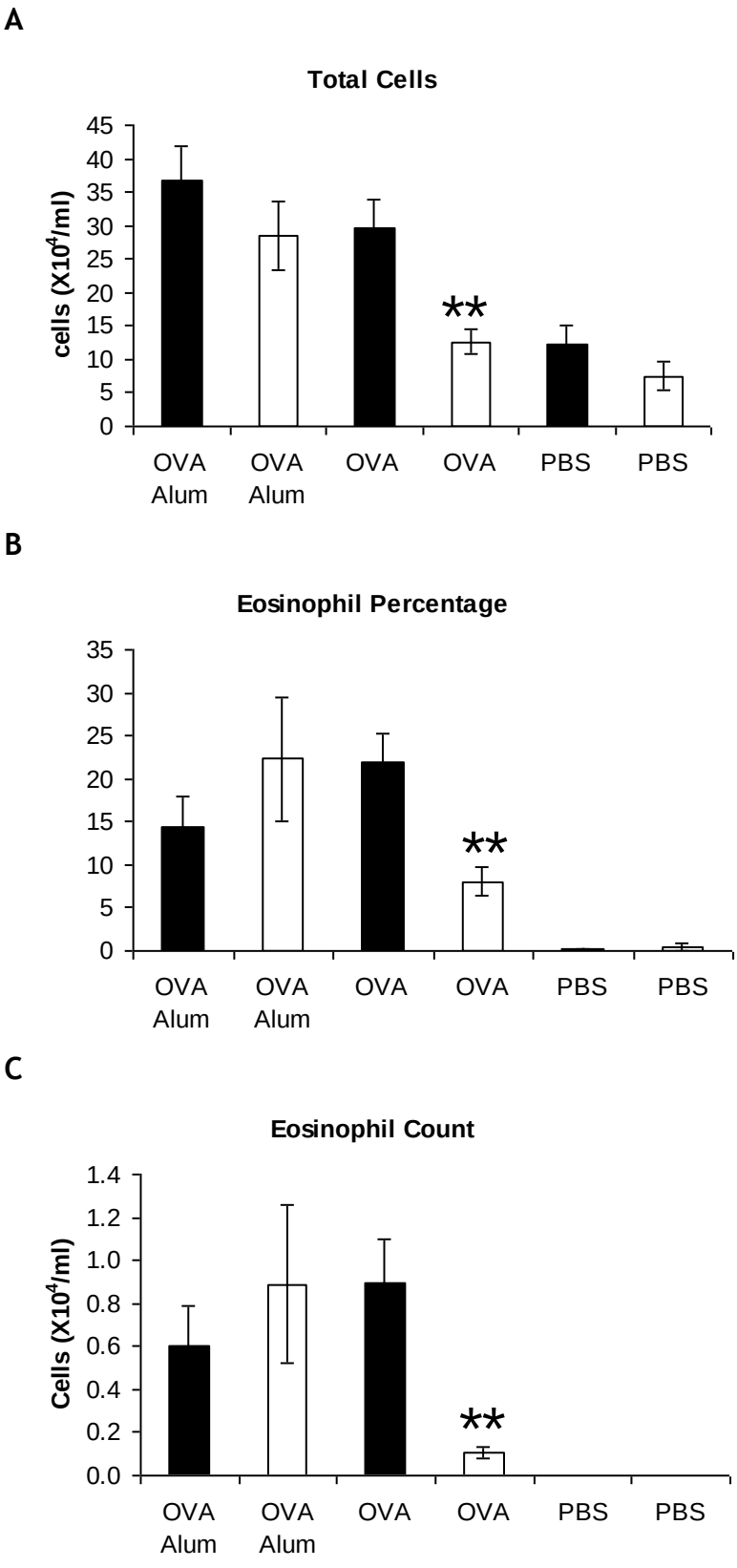


Figure 4.33 - BAL eosinophils are reduced in ST2$^{-/-}$ mice in the absence of alum

WT (■) and ST2$^{-/-}$ (□) mice were immunised with OVA (10µg) and 1% alum (OVA Alum), OVA (10 µg) alone (OVA) or PBS (PBS) on days 0 and 14. All mice were challenge with intranasal OVA (200 µg) on days 25, 26 and 27. Mice were culled on day 29. (**A**) Total cell counts were performed upon BAL. (**B**,**C**) BAL differential counts were performed. Results are shown as the mean of each group of mice +/- SEM. n=8 mice. ** $p<0.01$ compared with WT.

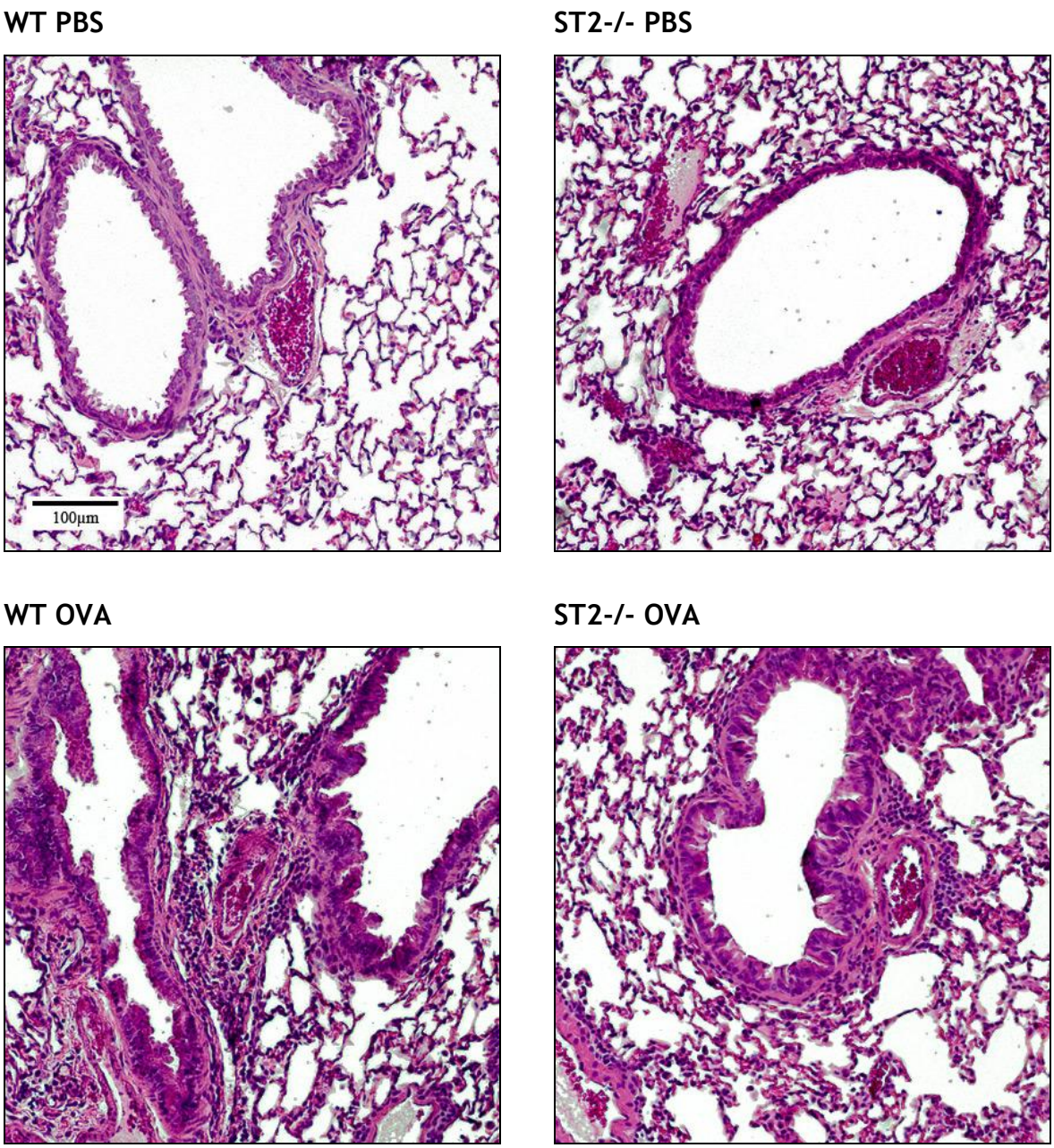


Figure 4.34 – Tissue eosinophilia is reduced in ST2$^{-/-}$ mice in the absence of alum

WT and ST2$^{-/-}$ mice were immunised and challenged and culled as in Figure 4.33. Lungs were fixed in formalin and stained with H&E. Panels shown are representative sections.

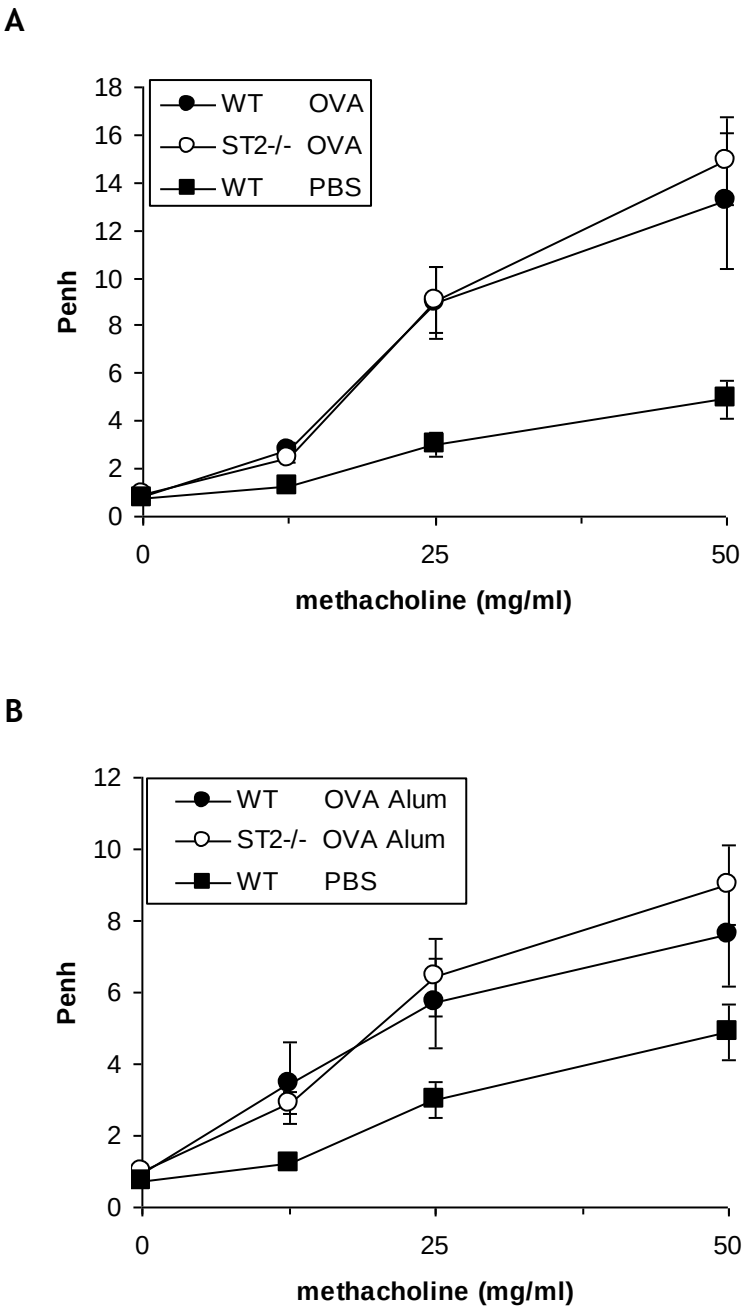


Figure 4.35 - Airways hyperresponsiveness is preserved in ST2$^{-/-}$ mice

WT (■) and ST2$^{-/-}$ (□) mice were immunised and challenged as in Figure 4.33. Penh was measured on day 28. Results are shown as the mean of each group of mice +/- SEM. n=8 mice.

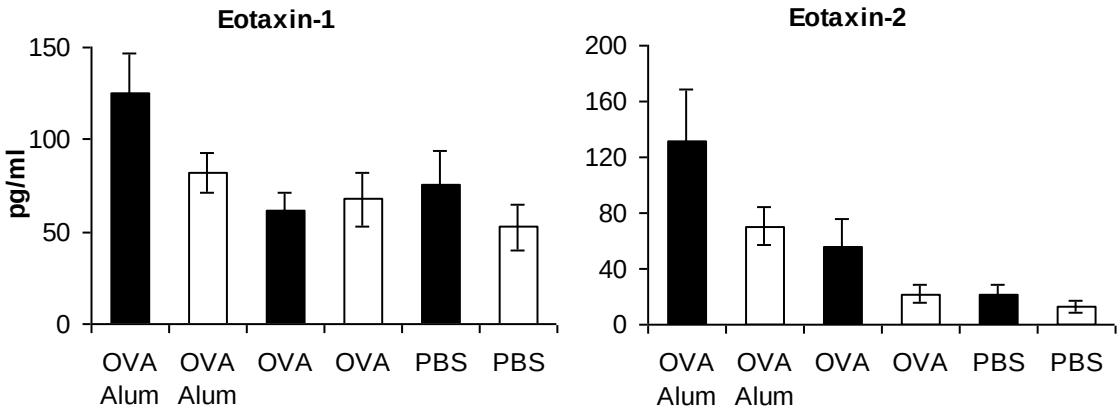


Figure 4.36 – BAL cytokines and chemokines are not significantly altered in ST2$^{-/-}$ mice

WT (■) and ST2$^{-/-}$ (□) mice were immunised and challenged as in Figure 4.33. BAL was collected and cytokine and chemokine concentrations measured by ELISA and multiplexed immunoassay. Results are shown as the mean of each group of mice +/- SEM. n=8 mice.

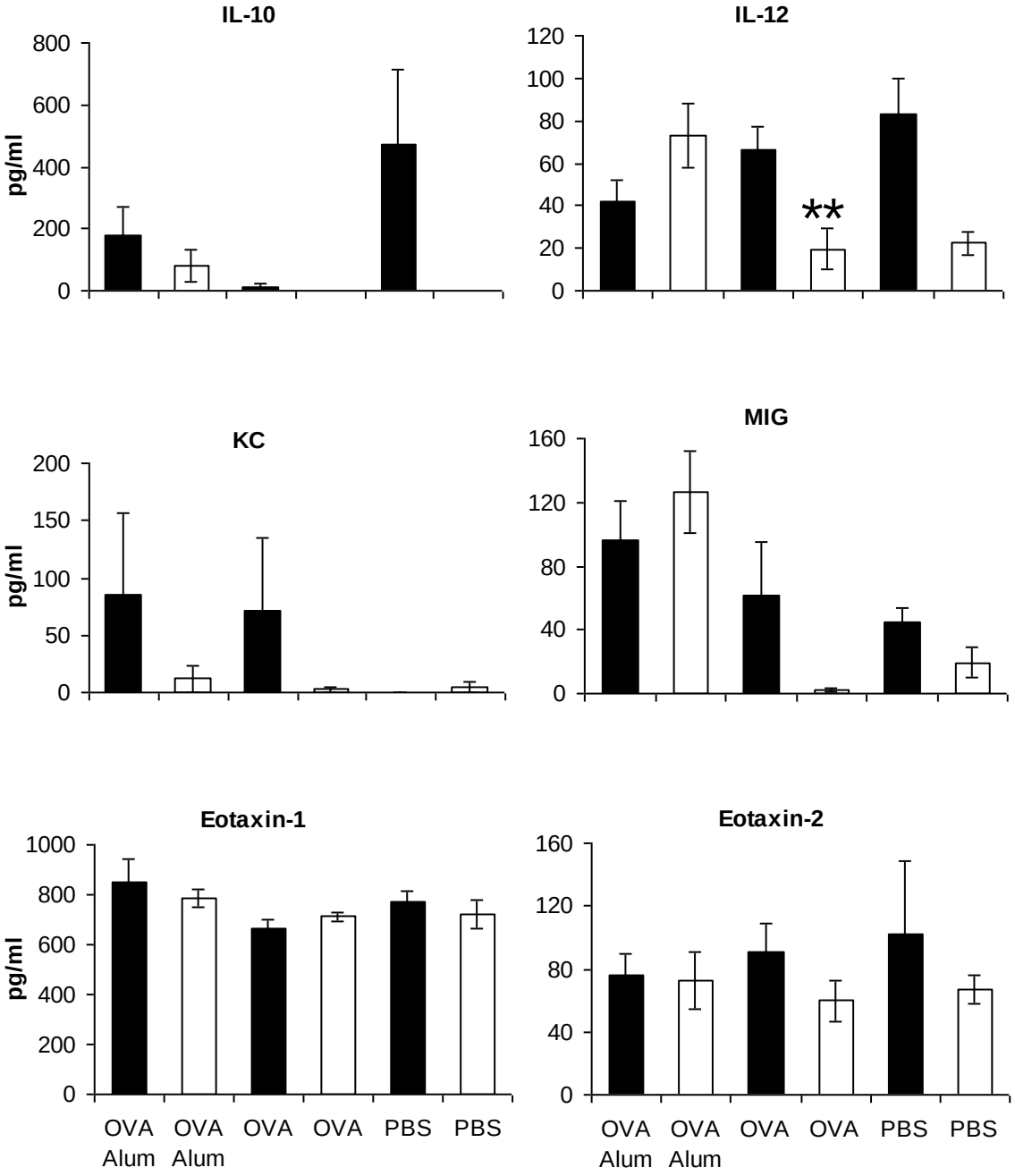


Figure 4.37 – Serum cytokines and chemokines are not significantly altered in ST2$^{-/-}$ mice

WT (■) and ST2$^{-/-}$ (□) mice were immunised and challenged as in Figure 4.33. Serum was collected and cytokine and chemokine concentrations measured by ELISA and multiplexed immunoassay. Results are shown as the mean of each group of mice +/- SEM. n=8 mice. ** $p<0.01$ compared with WT

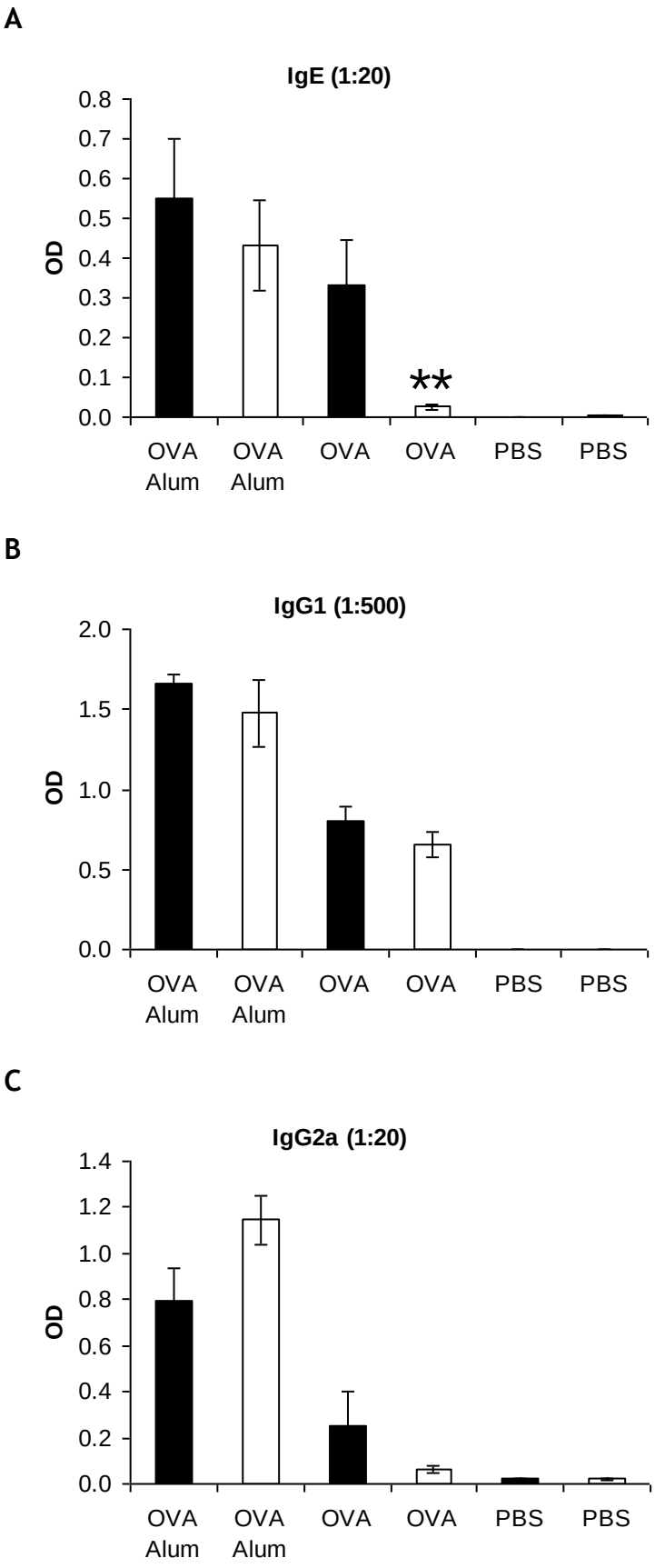


Figure 4.38 – Antigen specific IgE is reduced in ST2$^{-/-}$ mice in the absence of alum

WT (■) and ST2$^{-/-}$ (□) mice were immunised and challenged as in Figure 4.33 Serum was collected and OVA specific immunoglobulins measured by ELISA. Results are shown as the mean of each group of mice +/- SEM. n=8 mice. ** $p<0.01$ compared with WT

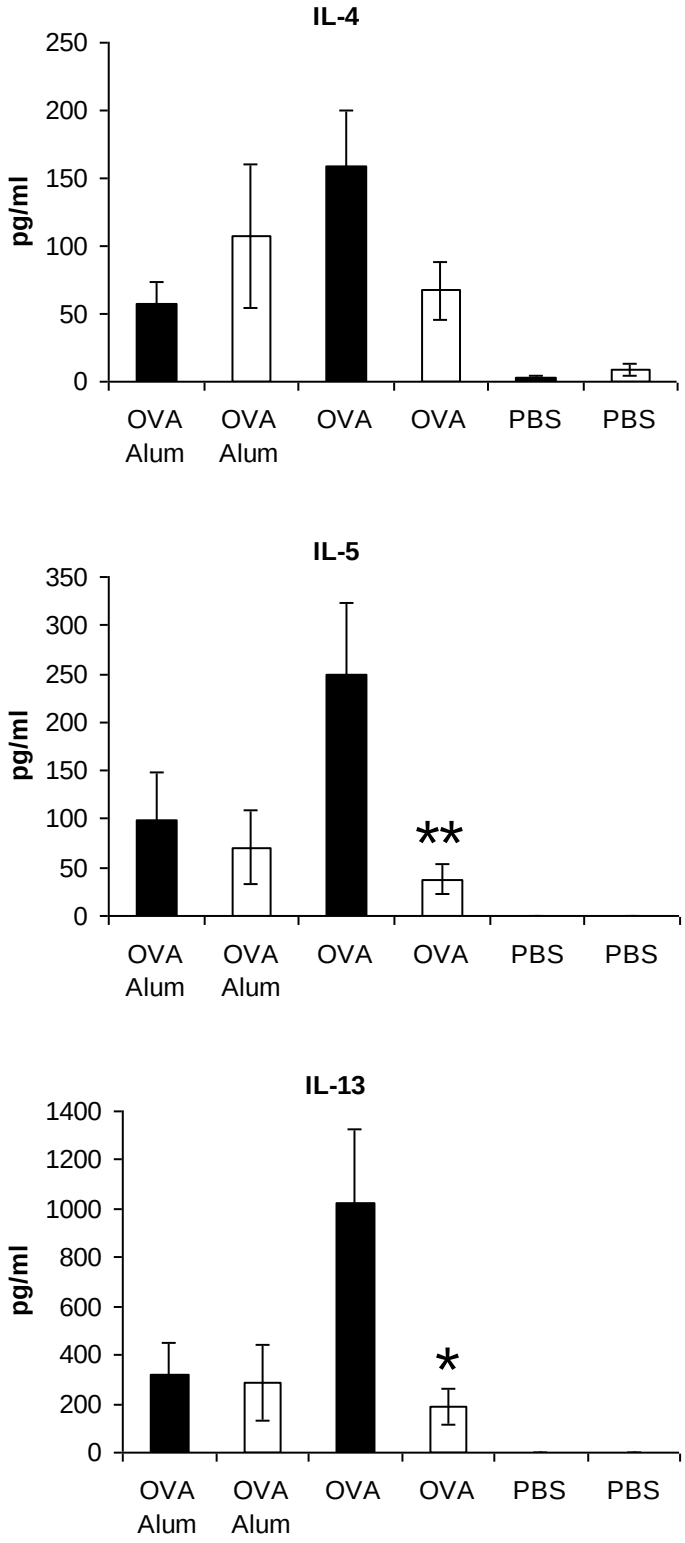


Figure 4.39 – Lymph node Th2 cytokines are reduced in ST2$^{-/-}$ mice in the absence of alum

WT (■) and ST2$^{-/-}$ (□) mice were immunised and challenged as in Figure 4.33. Single cell suspensions from draining lymph nodes were stimulated with OVA 1 mg/ml. Supernatants were collected after 96 hours and cytokines and chemokines measured by ELISA. Results are shown as the mean of each group of mice +/- SEM. n=8 mice. * $p<0.05$, ** $p<0.01$ compared with WT.

4.6 Conclusions

The experiments in this chapter initially served to develop a murine model of allergic airways inflammation for later use in the study of the role of ST2 in experimental asthma. There were several stages in the development and validation of this model. A crucial step in the induction of airways inflammation is the introduction of antigen to the lungs. Ideally the dose delivered should be repeatable and equally distributed throughout the bronchial tree. One solution is to allow conscious animals to inhale a nebulised solution of antigen. While, with careful engineering(482), the concentration of particles within the chamber can be uniform, the dose delivered will be affected by the ventilatory pattern of each animal. Distribution throughout the airways may also be uneven with particle deposition in the upper airways(483) and significant variation in the dose delivered to different parts of the lung(484). An alternative method is to use intranasal administration, which can ensure that the same total dose is delivered on each occasion. However there is concern as to how the delivered antigen is distributed, with evidence that a significant proportion of delivered agent is localised to the upper GI tract and upper respiratory tract(485). By instilling antigen directly into the lungs via cannulation of the trachea these problems may be overcome. I found that there was no difference in the magnitude of the inflammatory response or intra-group variance between intranasal and intratracheal dosing. In addition when Evan's blue was instilled via the intratracheal route under terminal anaesthesia a significant quantity of dye was found within the upper respiratory tract and stomach (data not shown). It is likely that all routes of administration result in delivery of antigen to the upper respiratory tract, but given that this alone can result in pulmonary inflammation and AHR(486) the model is unlikely to be adversely affected. While in theory ingestion of antigen may result in oral tolerance(487), in practice all of the described delivery methods result in a robust recall immune response.

Information on the nature of the T cell response upon antigen challenge can be obtained by culturing cells from the draining lymph nodes. I have found that the culture of lymph nodes from each mouse individually, as opposed to pooled from all mice, is practical and not prohibitively time consuming. This technique also allows for variance to be calculated and statistical comparisons to be made

between groups. The data also suggest that there may be a problem of high responders skewing the response in pooled cultures.

I also set out to establish a method to measure AHR. While invasive measurement of airways resistance and compliance remains the gold standard it was out with the scope of this project to establish this technique within laboratory. Considerable technical skill is required to perform cut-down and tracheal intubation in live anaesthetised mice and such expertise was not available locally. A significant investment in equipment is also required. Within the group there was already the equipment and experience to measure Penh. The principles of this investigation have been discussed in Chapter 2. There are however concerns regarding the use of the dimensionless parameter Penh. These are in part due to the fact that although Penh measurements generally correlate well with direct measures of airways resistance, it is not clear exactly what Penh is a measure of(488,489). Although Penh is acknowledged to be affected by changes in airways resistance there is concern over the contribution of other factors such as nasal resistance, ambient temperature and humidity within the chamber and tidal volume(488,490). I found that, provided sufficiently large groups were used, all three models of asthma utilised in this chapter generated reproducible methacholine-induced changes in Penh which were significantly greater than in control mice. Furthermore Penh continues to be utilised by leading investigators in the field of experimental asthma(31-34).

Having optimised and validated a short model of asthma I was able to study the kinetics of the immune response to antigen challenge. This led to two key observations. Firstly, it would appear that antigen challenge induces an inflammatory response within the airways which continues to develop in the absence of ongoing antigen dosing. One might predict that during the challenge period eosinophil numbers rise following each successive challenge, but the changes following a single antigen challenge were more unexpected. While it is recognised that by 7 to 14 days after challenge eosinophil numbers have fallen significantly(491-493), there is little data on earlier time points. In a 31 day model of asthma in which mice were sensitised twice with OVA and alum before being challenged with OVA on three consecutive days, eosinophil numbers peaked 48 hours after the final challenge, and began to fall by 72 hours(493). It was therefore surprising that in mice which had been sensitised once, a single

antigen challenge resulted in an inflammatory response which continued to develop for up to 72 hours.

In light of this ongoing inflammation it was also unexpected that Th2 cytokines were produced only transiently. It is well established that IL-4, IL-5 and IL-13 are detected within the BAL and serum in murine models of asthma, usually measured 24 hours after the final challenge, and often at relatively low concentrations. BAL IL-4 was detected at high (ng/ml) concentrations 24 hours after a single antigen challenge, with IL-5 and IL-13 at lower concentrations. In the serum IL-5 and IL-13 were also elevated 24 hours after challenge. Surprisingly even when further challenges were given Th2 cytokine concentrations decreased and were at much lower or undetectable concentrations 24 hours after the third challenge. While there is some data available for the kinetics of BAL Th2 cytokine concentrations after the final challenge(493) I could find no data regarding changes during the challenge period.

While Th2 cytokine concentrations in the BAL and serum decreased after the first challenge, eosinophil numbers in the airways continued to rise. The sustained chemokine release seen may account for this, with eotaxin-2 concentrations rising regardless of whether or not further challenges were given. In fact BAL eotaxin-2 concentrations correlated closely with eosinophil numbers. In asthmatic patients eotaxin-1 and eotaxin-2 expression rises after a single antigen challenge(494,495) and eotaxin-2 also correlates closely with eosinophil numbers in bronchial biopsies(495). The close relationship between eosinophilic inflammation and eotaxin-2 is in keeping with the key role of the CCR3-eotaxin pathway in eosinophil recruitment during experimental asthma(268-270).

These data suggest that initial exposure to allergen results in a Th2 cytokine response, which then appears to be down-regulated despite ongoing allergen exposure. This is followed by local eotaxin-2 production which drives eosinophil recruitment. Even though Th2 cytokine expression is fairly transient, IL-4, IL-5 and IL-13 are required for eosinophilic inflammation in experimental asthma, as discussed in Chapter 1. It may be that inflammation is initiated by Th2 cytokine release but then perpetuated by ongoing eotaxin-2 production. IL-5 can induce eosinophils to mobilise from the bone marrow and express CCR3(496,497)

whereas IL-4 can induce eotaxin-2 production by macrophages(498), which are the most abundant cells within the airways.

The other key finding was regarding the requirement for ST2 in allergic airways inflammation. In the 12 day model of asthma airways inflammation was significantly reduced in ST2 deficient mice. This is in contrast to published data from different asthma models. While the study by Hoshino *et al* found no effect of ST2 gene deletion on airways inflammation, they utilised a protocol in which C57Bl/6 mice were used rather than BALB/c mice, and 15 challenges were administered(381). However the study by Mangan *et al*, which also found no requirement for ST2 in the generation of eosinophilic inflammation, not only used BALB/c mice, but also a more conventional 30 day model in which mice were challenged three times(383).

The reason for the dramatic difference between the 12 day and 30 day models is unclear. Subtle differences in models, such as the route of sensitisation, can have significant impact on the pathways required. For example when sensitisation is carried out by the epicutaneous rather than the intraperitoneal route, the requirement for IL-4 is bypassed(213). As there have been few studies using the short model little is know about how it differs functionally from other models. However a very similar model, in which mice were sensitised with OVA and alum on days 1 and 5 before being challenged twice on day 12, was unusual in showing a requirement for mast cells in the generation of eosinophilic inflammation(159).

In light of these data and the role of ST2 on mast cells demonstrated in Chapter 3, I went on to show that ST2 was required in an alum-free, mast cell dependent model of asthma. Eosinophilic inflammation was dramatically reduced in $ST2^{-/-}$ mice in the alum-free model while AHR was preserved. This was associated with a marked reduction in antigen specific IgE and reduced Th2 cytokine production by antigen stimulated lymph node cells. However when alum was used as an adjuvant during sensitisation ST2 gene deletion had no effect on airways inflammation or AHR.

While the results from the 28 day alum model are in keeping with the published literature(381,383), the results from the alum-free model are novel. My hypothesis prior to performing the experiment was that mast cell ST2 is

necessary for allergic airways inflammation provided that the models of asthma used has a requirement for mast cells. Given that the use of alum confers mast cell independence and the omission of alum mast cell dependence(162), the data would support this hypothesis, ST2 was only required in the mast cell dependent model of asthma. This is of course not the only possible interpretation of the data. While the alum-free model of asthma is a mast cell dependent model, this may not be the sole effect of omitting alum. To appreciate other possible explanations it is important to consider the mechanisms by which alum functions as an adjuvant.

In 1926 Glenny *et al* discovered that precipitating antigen onto insoluble aluminium potassium sulphate improved antibody responses(45). It was initially thought that alum worked simply by effecting a slow release of antigen over time. This theory persisted for many years but more recent data suggests that alum works by other methods.

Following intraperitoneal administration of OVA alone antigen appears to travel passively to the draining lymph nodes where it is taken up and presented by resident DCs(499). When OVA is administered with alum it induces an influx of peritoneal monocytes that take up the OVA before maturing to become DCs and migrating to lymph nodes where they activate T cells. This process is dependent on urate production, and results in more effective activation of CD4 T cells than with OVA alone. One of the reasons why alum is able to recruit DCs in this manner appears to be by activation of the inflammasome.

The innate immune system has a collection of receptors to recognise conserved molecules associated with pathogens or pathogen-associated molecular patterns (PAMPs). The best characterised of the PAMP receptors are the predominantly surface expressed TLRs, but there also the intracellular nucleotide binding and oligmerization domain (NOD)-like receptors (NLRs). They function in association with other proteins including pro-caspases, to form a complex referred to as the inflammasome(500). The NLRP3 containing inflammasome is associated with pro-caspase-1 which is converted to active caspase-1 upon triggering and in turn cleaves IL-1β and IL-18 to their active forms(501).

It has recently been demonstrated that alum synergises with LPS *in vitro* to induce IL-1β and IL-18 release from macrophages via the NLRP3

inflammasome(502-505). While IL-18 is expressed constitutively, LPS may be required *in vitro* to induce pro-IL-1β which is then cleaved by alum-mediated activation of the inflammasome(506,507). The NLRP3 inflammasome is also required for the adjuvant effect of alum administered with OVA *in vivo*, including antigen uptake and maturation of peritoneal DCs, IgG1 and IgE production and eosinophilic inflammation in experimental asthma(502,503,505). Despite these findings it is in alum-free models of asthma that IL-1 is indispensable(508,509).

These data demonstrate that the use of alum results in alternative antigen transport and processing, activation of the NLRP3 inflammasome and generation IL-1β and IL-18. In an alum-free model of asthma there is a requirement for TNFα producing mast cells(33,162,163), IgE(125) and IL-1. I have demonstrated that in alum-free models there is also a requirement for ST2.

The question remains as to whether or not ST2 is required at the time of sensitisation, perhaps as part of the pathway which is activated in the absence of alum, or whether in the absence of alum a pathway is utilised which then requires ST2, perhaps on mast cells, at the time of antigen challenge.

In the following chapter I set out establish whether the activation of ST2 in the lungs with IL-33 induces the features of experimental asthma.

Chapter 5

The effect of IL-33 in the airways

In this chapter I will study the effect of activating ST2 in the airways by the administration of IL-33.

5.1 Introduction

In Chapter 3 I demonstrated that IL-33 activated mast cells via the receptor ST2 to release pro-inflammatory cytokines and chemokines *in vitro*. In particular IL-6, IL-13, MIP-1α and MCP-1; all of which have the potential to play a pathological role in clinical and experimental asthma.

In Chapter 4, I found that ST2 contributes to airways inflammation and IgE generation, but not AHR, in an alum-free model of asthma. A key difference between models of asthma which utilise alum and those that do not is that the use of alum appears to eliminate the requirement for mast cells in the development of airways inflammation and AHR(162).

Taken together these data support the possibility that activating ST2 on mast cells plays an important role in experimental asthma.

Schmitz *et al*(323) found that the systemic activation of ST2 by the administration of IL-33 induced blood eosinophilia, the generation of serum IgE and IgA, Th2 cytokine production and some perivascular and epithelial changes in the lungs.

I hypothesised that the activation of ST2 on mast cells within the airways by IL-33 would induce the features of experimental asthma.

5.2 The effect of IL-33 on airways inflammation

In the first instance I set out to establish whether or not the intranasal administration of IL-33 would induce inflammation within the lungs. Schmitz *et al* administered 0.4 or 4 μg of recombinant human IL-33 intraperitoneally to C57BL/6 mice daily for seven days and culled the animals the following day(323). I based the protocol for intranasal administration upon these methods. Others within the lab had found in studies of murine T cells *in vitro* that human recombinant IL-33 has approximately half the bioactivity of recombinant murine IL-33 (M. Kurowska-Stolarska, personal communication). Consequently I selected

a dose of 2 μg of murine IL-33 for initial studies. C57BL/6 mice were chosen given the proven efficacy of systemic IL-33 administration in this strain. In addition this would allow for future investigation of the contribution of mast cells to any effect observed, as mast cell deficient mice are available on the C57Bl/6 background.

5.2.1 The effect of intranasal IL-33

Recombinant murine IL-33 (2 μg) was administered via the intranasal route to C57Bl/6 mice each day for seven days. The day after the final dose mice were culled and BAL fluid and lungs collected.

IL-33 administration resulted in a dramatic rise in cell numbers within the BAL, with a mean total cell count of 54 $x10^5$ cells/ml (Figure 5.1A). This compares with counts in the region of 2 – 5 $x10^5$ cells/ml in the models of asthma presented previously. There was an associated dramatic rise in the proportion of eosinophils in the BAL from 0% to 79% (Figure 5.1B). Consequently there was a marked rise in absolute eosinophil numbers from 0 to 43 $x10^5$ cells/ml (Figure 5.1C). While the proportion of macrophages fell from 93% to 16% there was still a significant rise in absolute numbers from 1 to 8 $x10^5$ cells/ml. The proportion of neutrophils was low in both groups at 6% in the PBS group and 5% in the IL-33 group, this did however translate to a rise in absolute neutrophil numbers from 0.2 to 2.5 $x10^5$ cells/ml in the IL-33 group.

Histological examination of the lungs revealed extensive eosinophilic inflammation throughout (Figure 5.2). The degree of inflammation was much more pronounced than that seen with the murine models of asthma in Chapter 4.

Concentrations of the Th2 cytokines IL-4 and IL-13 were slightly elevated in the BAL at 34 pg/ml and 29 pg/ml respectively (Figure 5.3). The eosinophil chemoattractants eotaxin 1 and 2 were induced at relatively high concentrations by IL-33, 116 pg/ml and 397 pg/ml respectively.

These data confirm that the intranasal administration of IL-33 induces inflammatory changes in the lungs similar to those seen in experimental asthma, the most striking of which is a profound eosinophilia.

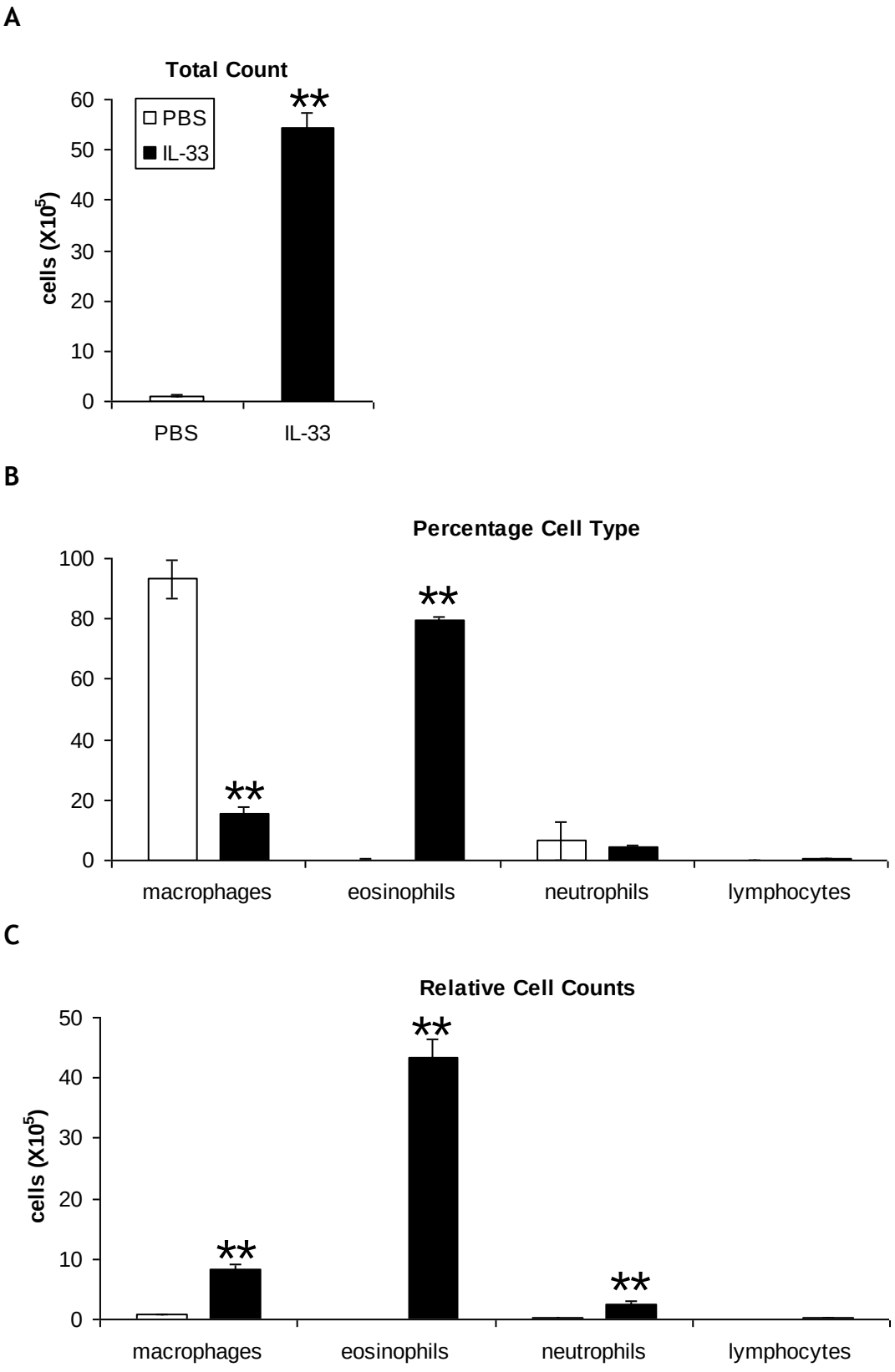


Figure 5.1 – Intranasal IL-33 induces BAL eosinophilia

C57BL/6 mice received PBS or 2 µg of recombinant murine IL-33 via the intranasal route on days 1 to 7 before being culled on day 8 at which point BAL was collected. (**A**) Total cell counts were performed upon BAL. (**B**,**C**) BAL differential counts were performed. Results are shown as the mean of each group of mice +/- SEM. n=7 mice. ** $p<0.01$ compared with PBS

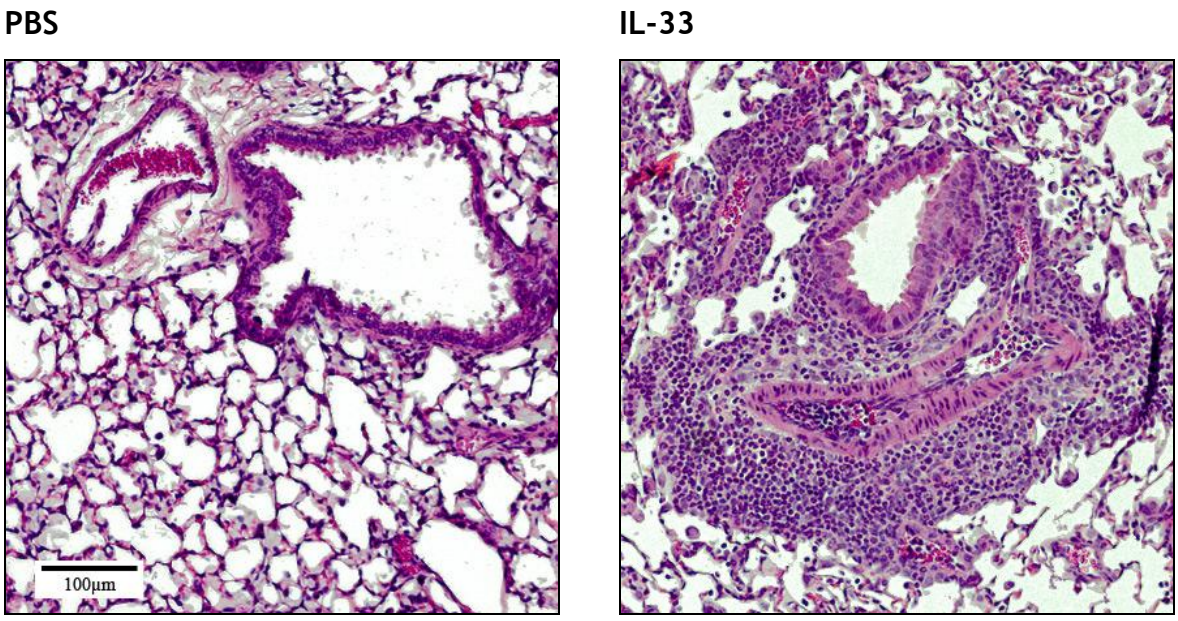


Figure 5.2 – Intranasal IL-33 induces inflammation within the lung

PBS or IL-33 was administered to mice as in Figure 5.1. Lungs were fixed in formalin and stained with H&E. Panels shown are representative sections.

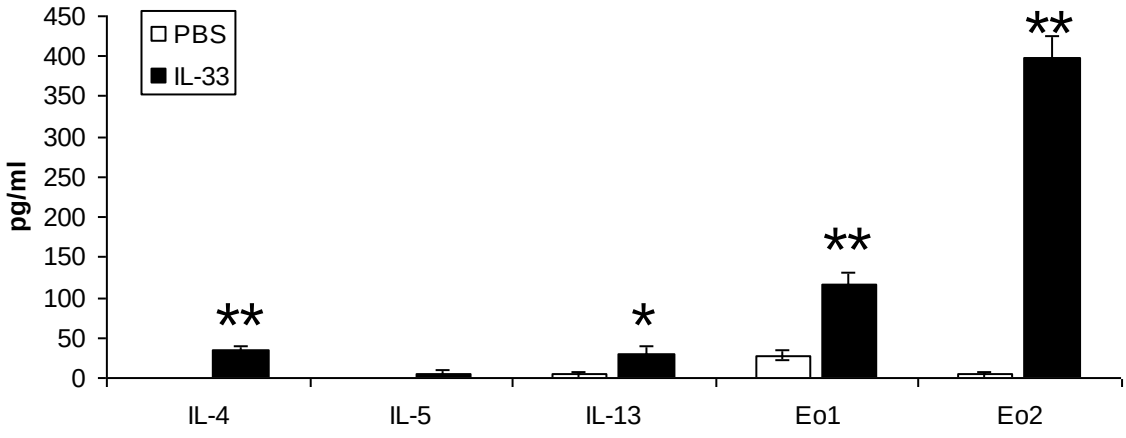


Figure 5.3 – Intranasal IL-33 induces BAL cytokines and chemokines

PBS or IL-33 was administered to mice as in Figure 5.1. BAL was collected and cytokines assayed by ELISA. Results are shown as the mean of each group of mice +/- SEM. n=7 mice. * $p<0.05$, ** $p<0.01$ compared with PBS

5.2.2 Kinetics during serial IL-33 administration

To better understand the changes that lead to the development of airways inflammation in mice administered repeated doses of IL-33, particularly the cytokines and chemokines which may be responsible for the recruitment of eosinophils, I undertook a study of the dynamic response to IL-33. I designed a protocol to study the kinetics of cytokines, chemokines and cell numbers within the lungs over the course of the eight day treatment period. As detailed in Figure 5.4, mice received up to 7 daily doses of intranasal IL-33 and groups of mice were culled at 24 hour intervals throughout this period.

Total cell counts began to rise on day three, 24 hours after the third dose. Numbers continued to rise with time and repeated dosing to a maximum of 50 $x10^5$ after the seventh dose (Figure 5.5A). Differential cell changes are shown in Figure 5.5B. Macrophage numbers rose gradually over the time course from 1.4 $x10^5$ in untreated mice to 9.5 $x10^5$ after seven doses. Neutrophils were first detected on day 3 and rose thereafter to 8.4 $x10^5$ after seven doses. As predicted the most striking change was in eosinophils, which first appeared in the BAL at low numbers (0.5 $x10^5$) 24 hours after the third dose. Numbers rose sharply thereafter reaching 31.5 $x10^5$ on day seven, at which point they comprised 59% of the BAL cell population.

Locally within the BAL IL-13 was the cytokine detected at the highest concentration (Figure 5.6). It was first detectable on day four, peaked at 770 pg/ml on day five and fell thereafter. IL-5 followed a similar pattern but at a much lower concentration, peaking at 175 pg/ml. The other Th2 cytokine IL-4 was never detected at a concentration greater than 82 pg/ml. Eotaxin-1 and eotaxin-2 were first detected on day three, earlier than IL-5 and IL-13. Eotaxin-1 remained at a similar concentration over time while eotaxin-2 then fell slightly. IL-12 and IP-10 also rose modestly to peak on day four and three respectively. Concentrations of IL-1, IL-2, IL-6, IL-10, IL-17, IFNγ, TNFα, GM-CSF, KC, FGF, MCP-1, MIP-1α, RANTES and MIG were below 50 pg/ml.

Within the serum the most marked change was a rise in IL-13 over time to a peak concentration of 614 pg/ml on day seven (Figure 5.7). The other Th2 cytokines IL-4 and IL-5 were detected at much lower concentrations, with IL-4 never rising

above 50 pg/ml and IL-5 peaking at 157 pg/ml after 3 doses. IP-10 and FGF were the only chemokines which changed significantly over time, rising and falling respectively from day zero to day seven. Concentrations of IFNγ, IL-6, GM-CSF, IL-17, MCP-1, MIP-1α, RANTES and MIG within the BAL were below 50 pg/ml. IL-1, IL-2, IL-12 and TNFα were detected but did not change significantly over the time course.

These data demonstrate that with repeated consecutive administrations of IL-33 to the airways an eosinophilia is generated after four days which rises thereafter and is associated with elevated Th2 cytokine and eotaxin concentrations.

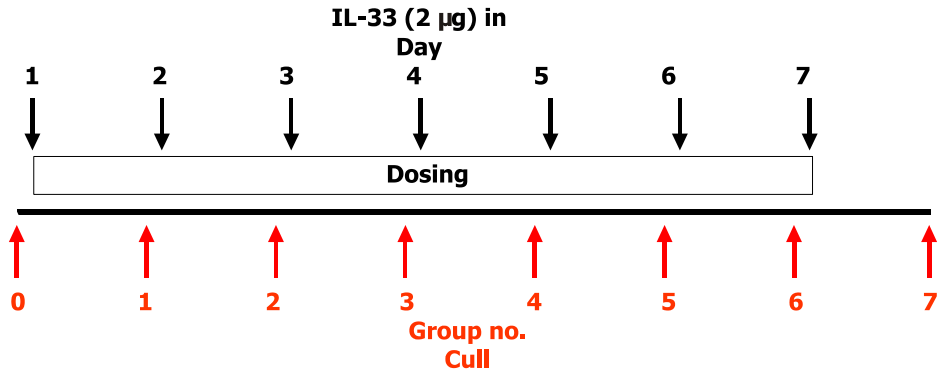


Figure 5.4 - Protocol for studying the kinetics of the response to IL-33

2 µg of recombinant murine IL-33 was administered via the intranasal route once daily on days 1 to 7. Groups of mice were culled 24 hours after each dose. For example Group 0 was culled prior to the first administration, Group 1 culled 24 hours after the first dose and Group 7 culled 24 hours after the seventh and final dose.

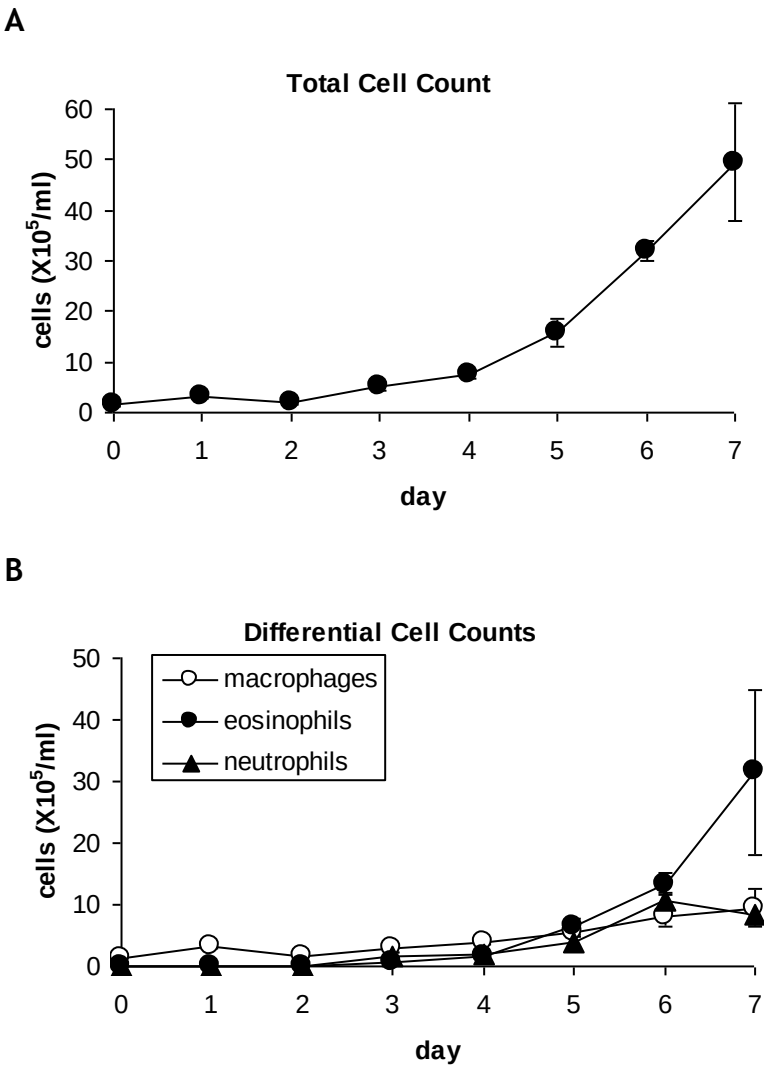


Figure 5.5 - Eosinophils rise with successive IL-33 doses

IL-33 was administered to mice as in Figure 5.4. (**A**) Total cell counts were performed upon BAL. (**B**) BAL differential counts were performed. Results are shown as the mean of each group of mice +/- SEM. n=4-5 mice. One-way ANOVA for the effect of IL-33 dose on macrophages $p<0.001$, eosinophils $p<0.001$, neutrophils $p<0.001$.

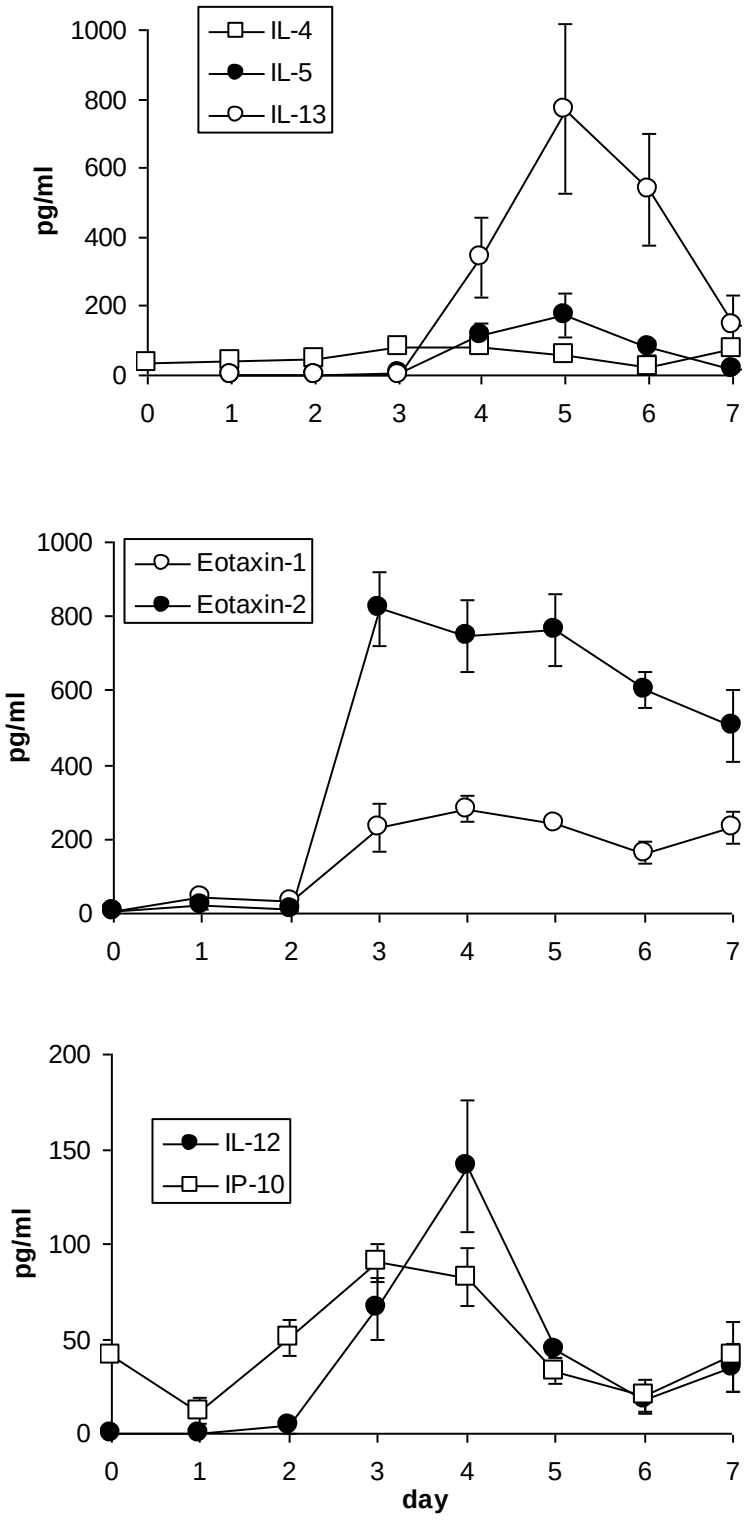


Figure 5.6 - Change in BAL cytokines and chemokines with repeated IL-33 dosing

IL-33 was administered to mice as in Figure 5.4. BAL was collected and cytokines and chemokines assayed by multiplexed immunoassay or ELISA. Results are shown as the mean of each group of mice +/- SEM. n=4-5 mice. One-way ANOVA for the effect of IL-33 dose on IL-13 $p=0.005$, Eotaxin-1 $p<0.001$, Eotaxin-2 $p<0.001$.

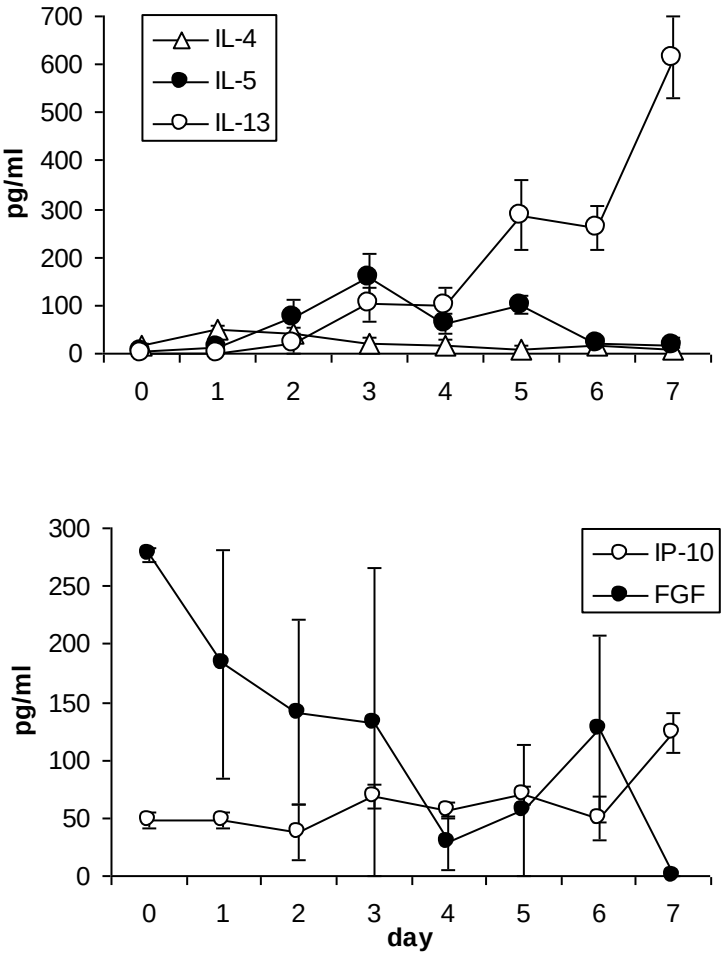


Figure 5.7 - Change in serum cytokines and chemokines with repeated IL-33 dosing

IL-33 was administered to mice as in Figure 5.4. Serum was collected and cytokines and chemokines assayed by multiplexed immunoassay or ELISA. Results are shown as the mean of each group of mice +/- SEM. n=4-5 mice. One-way ANOVA for the effect of IL-33 dose on IL-4 n.s., IL-5 $p<0.05$, IL-13 $p<0.001$, FGF n.s., IP-10 $p<0.05$.

5.2.3 Kinetics following IL-33 administration

Having shown that in the context of repeated IL-33 administration BAL eosinophil counts rise continuously I was interested to establish whether or not an inflammatory process had been initiated which would be perpetuated in the absence of ongoing ST2 stimulation. We had previously found that a single dose of IL-33 is insufficient to generate an appreciable airways eosinophilia at any point up to seven days after administration (P. Kewin *et al* unpublished), and so would need a protocol with multiple doses.

Additionally, as I was interested in studying the contribution of mast cells to the response to IL-33 I wanted to establish an endpoint at which the response was not so great as to mask any contribution by individual cells and pathways. With seven doses of IL-33 inducing a profound eosinophilia I chose a point in the middle of the IL-33 dose response curve shown in Figure 5.5. Having shown that 24 hours after 4 doses of IL-33 a moderate eosinophilia of $1.6x10^5$/ml is generated I chose to study the changes within the BAL and serum for up to eight days after 4 consecutive IL-33 administrations (Figure 5.8).

Following four intranasal doses of IL-33 total cell numbers continued to rise steadily, reaching a peak four days after dosing, before falling again (Figure 5.9). Eosinophil numbers followed an identical pattern peaking at $24x10^5$/ml four days after dosing, at which point they comprised 68% of BAL cells. Macrophages similarly rose to a peak at the earlier time point of three days post dosing, although the relative change was less dramatic with a maximum of $12x10^5$/ml and a minimum of $5.3x10^5$/ml. Neutrophils were also induced, peaking at $4.2x10^5$/ml on day two. While numbers had fallen significantly by day eight, with virtually no detectable neutrophils, there were still a significant number of macrophages and eosinophils present at this late time point. Macrophages are the default cell found on BAL, where generally they make up 100% of the total cell count. While numbers are higher than those seen in naïve mice, at $5.3x10^5$/ml this is perhaps less striking than the persistence of eosinophils seen over a week after IL-33 administration. At day eight the eosinophil count was still $6x10^5$/ml, making up 50% of all BAL cells, levels which are often not even achieved in standard asthma models immediately after antigen challenge.

Perhaps unsurprisingly Th2 cytokine levels within the BAL fell with the cessation of IL-33 dosing (Figure 5.10). IL-13 was present at high concentrations the day after final dosing, but fell rapidly thereafter. IL-4 and IL-5 began at more modest levels and also fell rapidly. Eotaxin-1 and eotaxin-2 followed a similar pattern, although with a more gradual decline, not beginning until day three in the case of eotaxin-2. IFNγ and IL-10 followed a different pattern, rising from zero to peak briefly two days after the last IL-33 administration. IL-12 was initially detected at low levels then also fell rapidly (data not shown). Concentrations of IL-1, IL-2, IL-6, TNFα, GM-CSF, KC, FGF, MCP-1, MIP-1α, RANTES, IP-10 and MIG were below 50 pg/ml.

Serum cytokine concentrations were all very low. IL-4 and IL-5 concentrations remained below 100 pg/ml, where IL-13 fell gradually from 160 pg/ml at day one to below the limit of detection at day eight (Figure 5.11). Concentrations of IL-1, IL-2, IL-6, IL-10, IL-12, IL-17, IFNγ, TNFα, eotaxin-1, eotaxin-2, GM-CSF, KC, FGF, MCP-1, MIP-1α, RANTES, IP-10 and MIG were below 50 pg/ml.

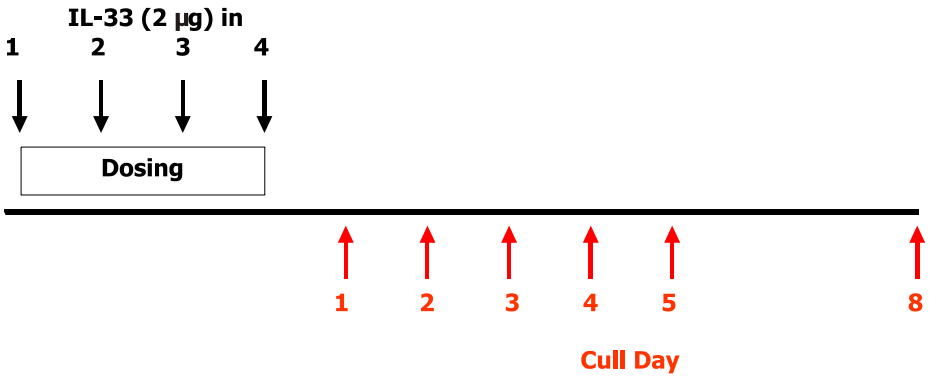


Figure 5.8 - Protocol for studying the kinetics following four doses of IL-33

2 µg of recombinant murine IL-33 was administered via the intranasal route once daily on days 1 to 4. Groups of mice were culled 1, 2, 3, 4, 5 and 8 days after the final dose.

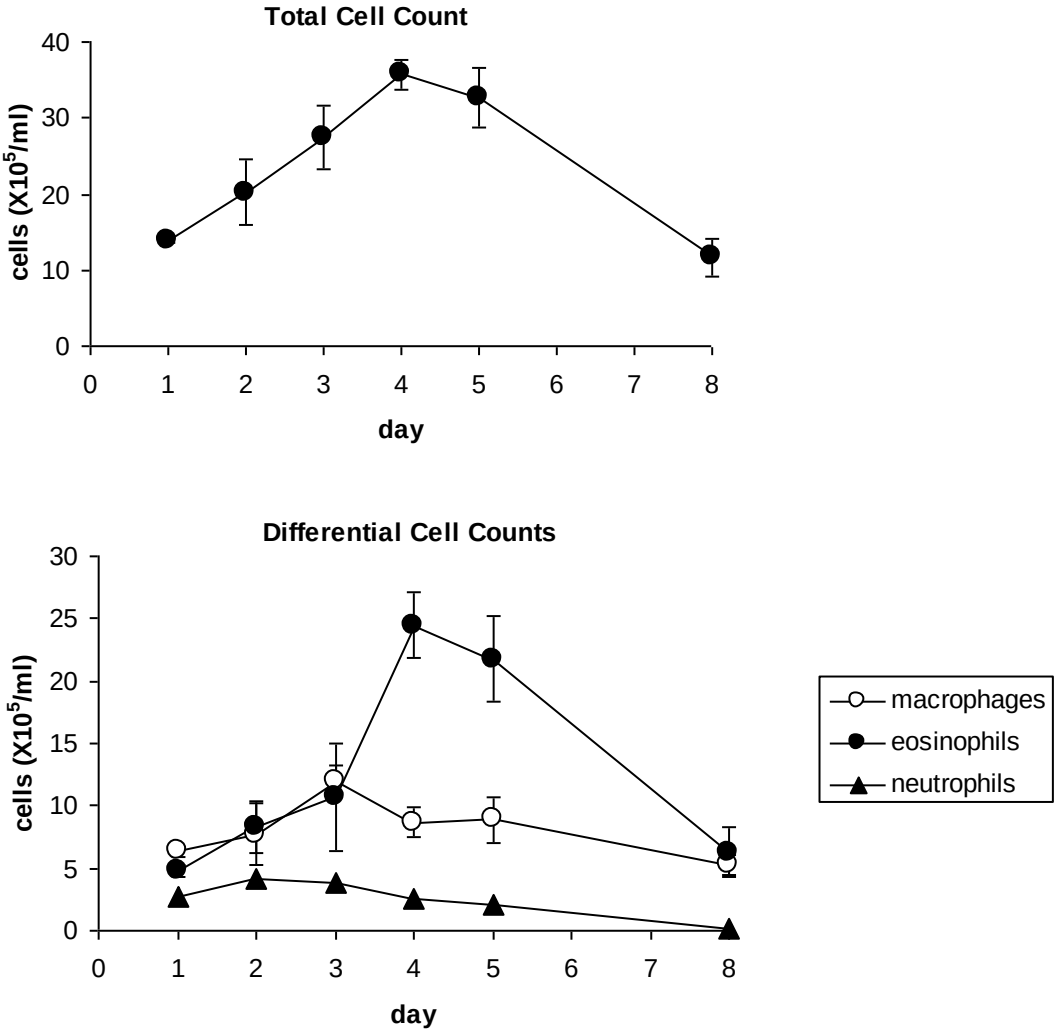


Figure 5.9 - Change in BAL cell numbers with repeated IL-33 dosing

IL-33 was administered to mice as in Figure 5.8.Total and differential cell counts were performed upon BAL. Results are shown as the mean of each group of mice +/- SEM. n=5 mice.

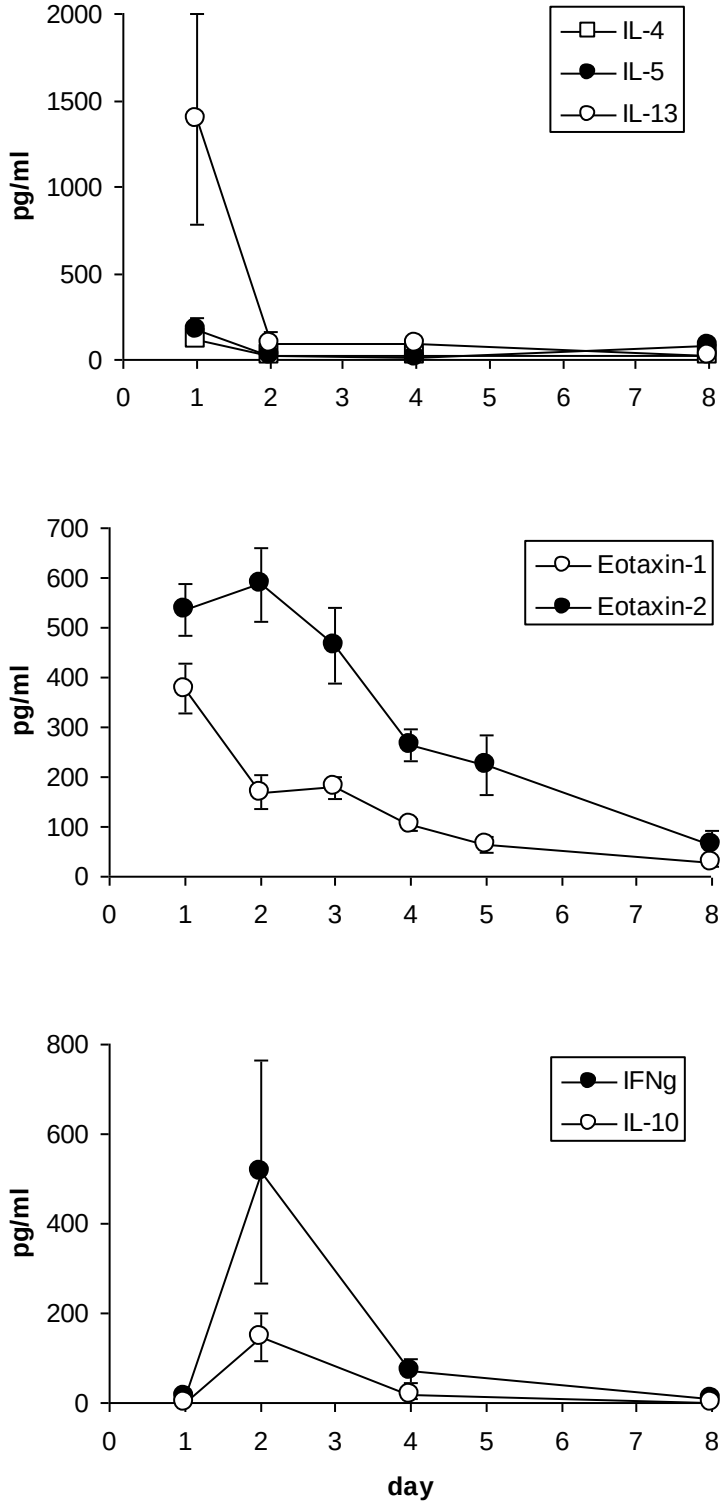


Figure 5.10 - Change in BAL cytokines and chemokines with repeated IL-33 dosing

IL-33 was administered to mice as in Figure 5.8. BAL was collected and cytokines and chemokines assayed by multiplexed immunoassay or ELISA. Results are shown as the mean of each group of mice +/- SEM. n=5 mice.

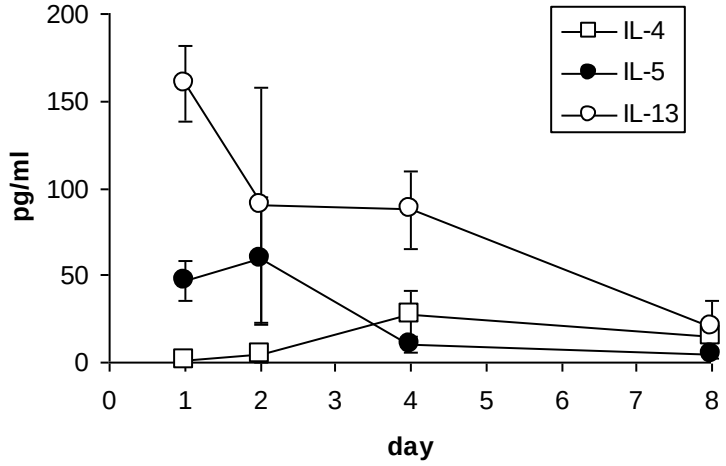


Figure 5.11 - Change in serum cytokines and chemokines with repeated IL-33 dosing

IL-33 was administered to mice as in Figure 5.8. Serum was collected and cytokines and chemokines assayed by multiplexed immunoassay or ELISA. Results are shown as the mean of each group of mice +/- SEM. n=5 mice.

5.2.4 A three dose protocol for the study of IL-33 in C57Bl/6 mice

Using the data from the previous dose response and time course experiments we designed a model to utilise for subsequent experiments. In Figure 5.9 the midpoint of the eosinophil response occurred three days after the last intranasal IL-33 dose. Given that eosinophil numbers continue to rise after dosing is completed we reduced the number of doses from four to three. This also had advantages in terms of reducing both the demands upon the limited supply of IL-33 and the number of procedures mice were subjected to, improving animal welfare. The final protocol of three doses followed by a cull three days later is shown in Figure 5.12.

Figure 5.13 demonstrated that this protocol did induce a moderate eosinophilia of $10x10^5$/ml or 60% (Figure 5.13). The magnitude of this is not dramatically greater than that seen in the asthma models in Chapter 4, but is significantly less than that seen with seven doses of IL-33 (Figure 5.1). Lung eosinophilia was also evident on histology (Figure 5.14). This protocol was then used for subsequent studies.

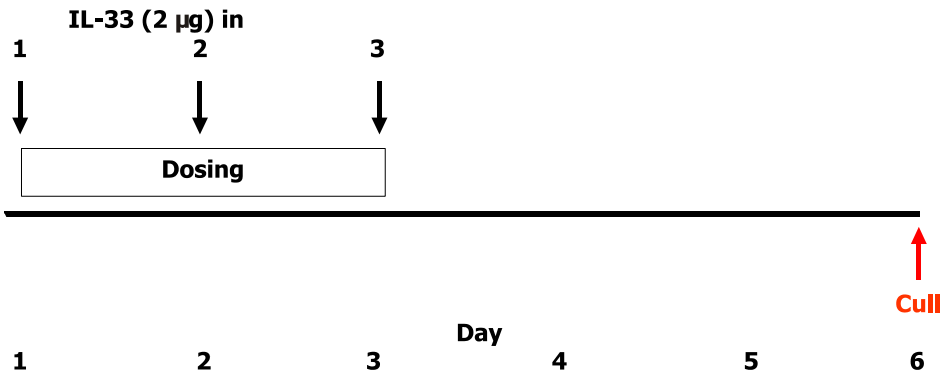


Figure 5.12 - Final protocol for intranasal IL-33 administration

2 µg of recombinant murine IL-33 was administered via the intranasal route once daily on days 1 to 3. Mice were culled on day 6.

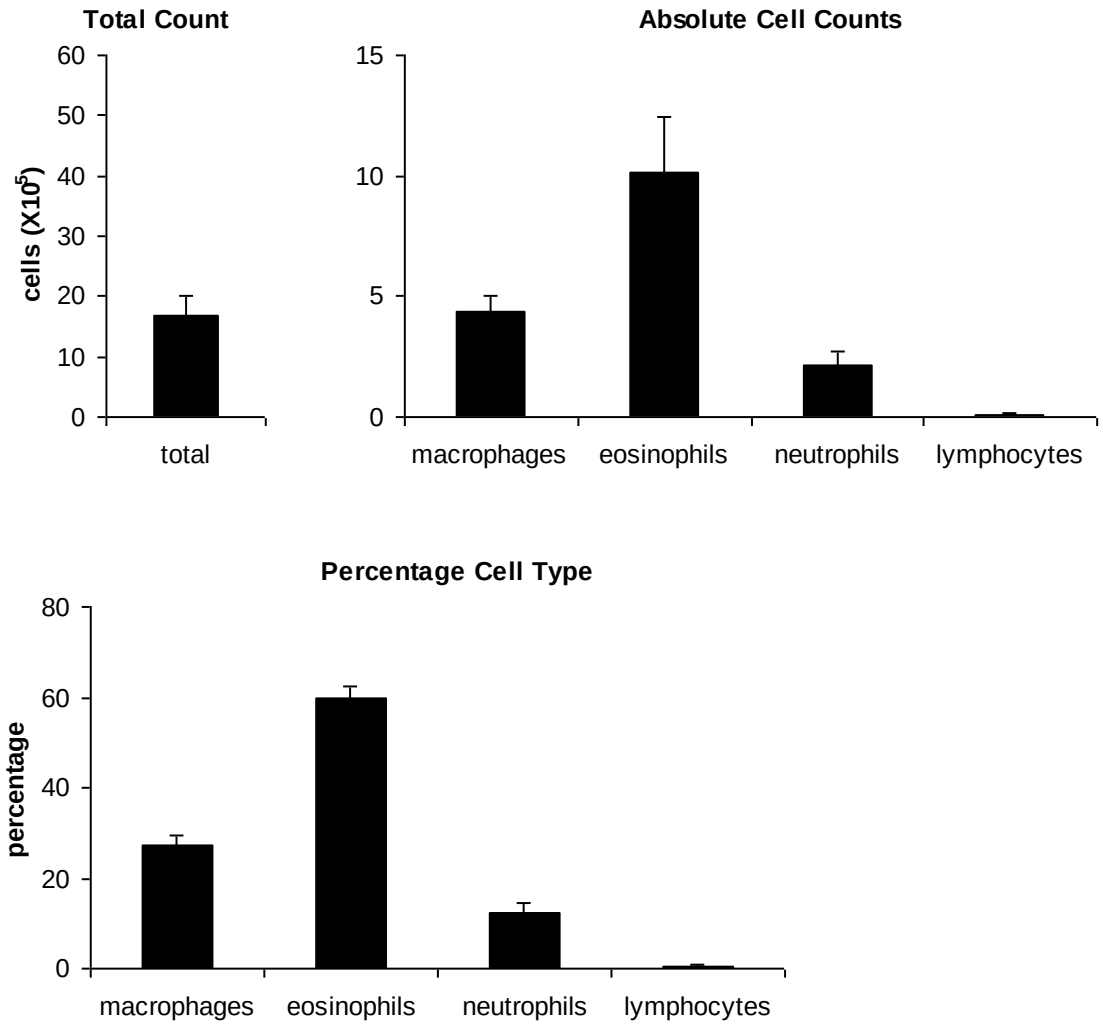


Figure 5.13 - BAL eosinophilia is induced by the modified IL-33 administration protocol

C57BL/6 mice received recombinant murine IL-33 as in Figure 5.12. (**A**) Total cell counts were performed upon BAL. (**B**,**C**) BAL differential counts were performed. Results are shown as the mean of each group of mice +/- SEM. n=5 mice.

PBS **IL-33**

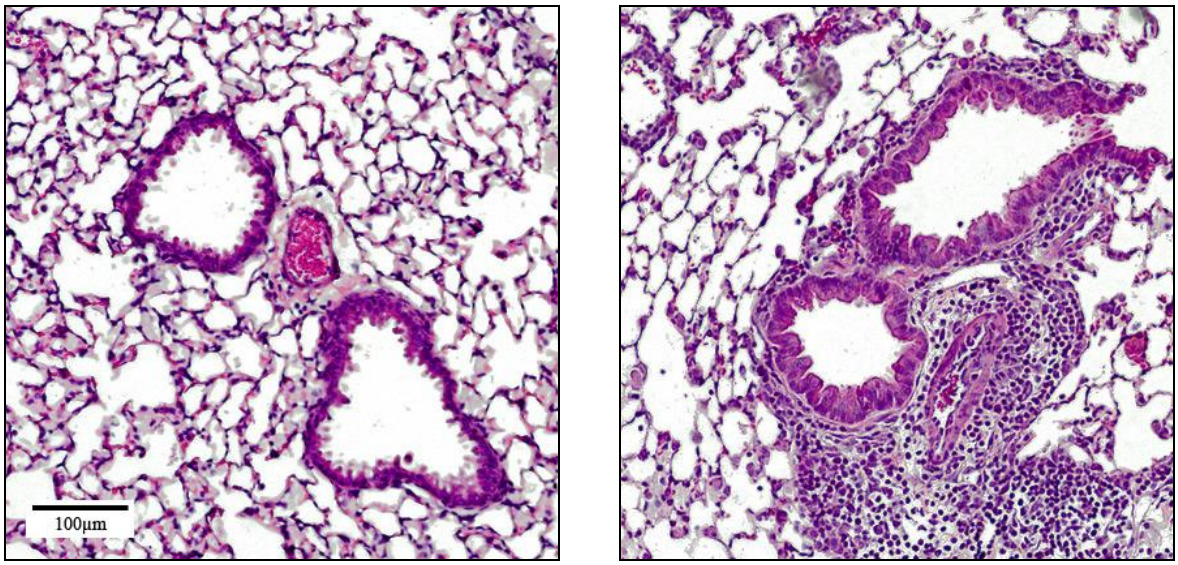


Figure 5.14 –Lung Inflammation is induced by the modified IL-33 administration protocol

PBS or IL-33 was administered to mice as in Figure 5.12. Lungs were fixed in formalin and stained with H&E. Panels shown are representative sections.

5.2.5 The effect of IL-33 in BALB/c mice

As discussed in Chapter 1 there can often be significant differences between different strains of mice. I initially chose to study the effect of IL-33 on the airways of C57Bl/6 mice for two reasons. This was the strain proven to respond to intraperitoneal IL-33, and mast cell deficient mice are available on this background.

Before proceeding further it was important to determine the specificity of the observed effects of IL-33 for ST2. In Chapter 3 I demonstrated that the effect of IL-33 on mast cells *in vitro* was completely ST2 dependent. To confirm that the *in vivo* effects are mediated via ST2 I planned to utilise $ST2^{-/-}$ mice. The $ST2^{-/-}$ mice I had access to were on the BALB/c background. Therefore I needed to test the effect of IL-33 on mice from this background.

The administration of IL-33 into the lungs of BALB/c mice resulted in a rise in total cell numbers within the BAL (Figure 5.15A), as well as eosinophil numbers (Figure 5.15B). The other cell types detected in the BAL were predominantly macrophages, with a very small proportion of neutrophils (Figure 5.15C).

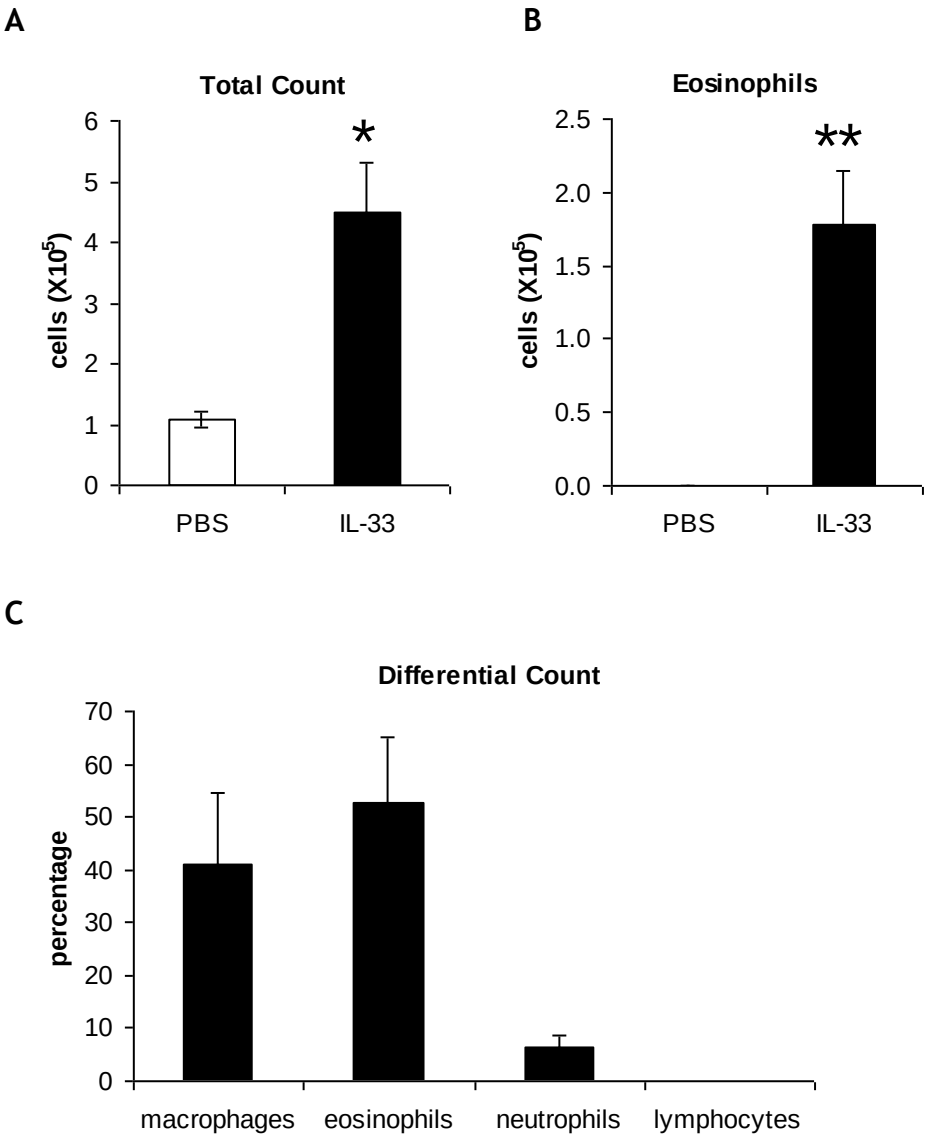


Figure 5.15 – IL-33 induces airways eosinophilia in BALB/c mice

IL-33 (■) or PBS (□) was administered to mice as in Figure 5.12. (**A**) Total cell counts were performed upon BAL. (**B**,**C**) BAL differential counts were performed. Results are shown as the mean of each group of mice +/- SEM. n=5 mice. * $p<0.05$, ** $p<0.01$ compared with PBS

5.2.6 The role of ST2 in IL-33 induced inflammation

In order to confirm the specificity of IL-33 for ST2 *in vivo* I employed $ST2^{-/-}$ mice on a BALB/c background.

IL-33 was administered to wild type (WT) and $ST2^{-/-}$ mice. As expected a robust eosinophilia was generated in the BAL of WT mice (Figure 5.17). In $ST2^{-/-}$ mice the total cell count was similar to that in PBS treated mice, and there was a complete absence of eosinophils. The same was true of lung histology, with eosinophilic inflammation induced in WT but not $ST2^{-/-}$ mice (Figure 5.17).

This confirms that IL-33 is acting exclusively through the ST2 receptor to mediate the inflammatory effects seen in the lungs *in vivo*. It also serves to verify that the phenomena observed are not due to contamination by other bio-active material of the IL-33, which is produced within our laboratory.

A

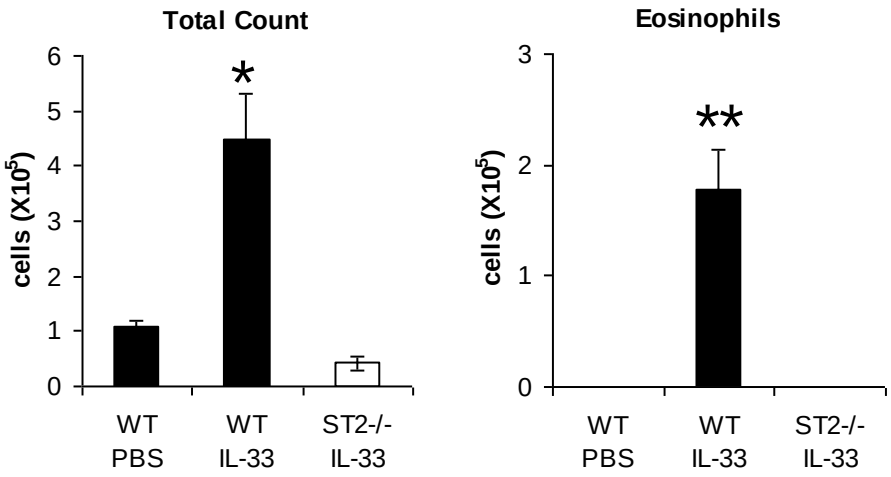
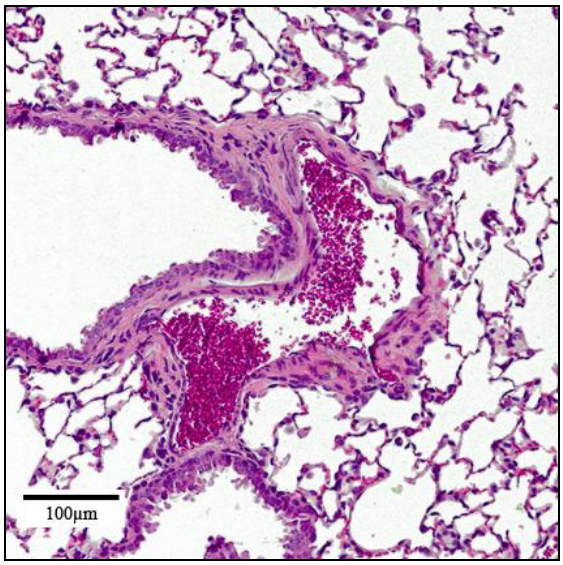


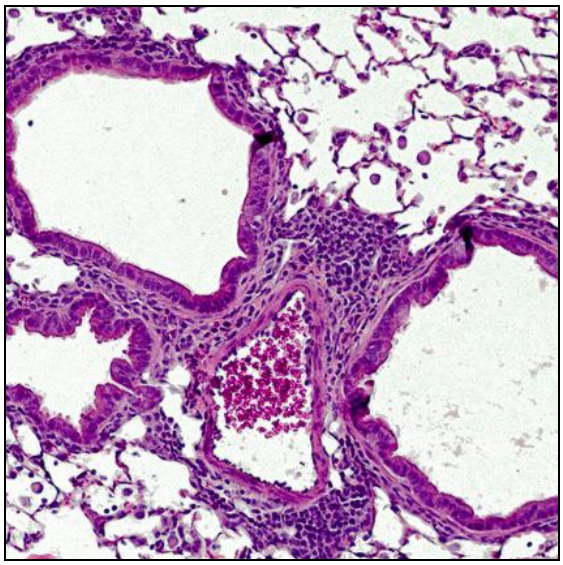
Figure 5.16 – IL-33 induced BAL eosinophilia is ST2 specific

IL-33 or PBS was administered to wild type (WT) or $ST2^{-/-}$ mice as in Figure 5.12. Total and differential cell counts were performed upon BAL. Results are shown as the mean of each group of mice +/- SEM. n=5 mice. * $p<0.05$, ** $p<0.01$ compared with PBS.

WT PBS



WT IL-33



ST2$^{-/-}$ IL-33

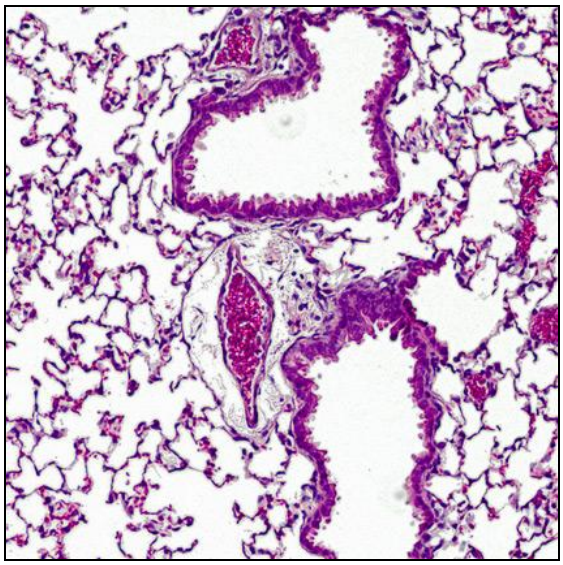


Figure 5.17 – IL-33 induced lung inflammation is ST2 specific

IL-33 or PBS was administered to wild type (WT) or ST2$^{-/-}$ mice as in Figure 5.12. Lungs were fixed in formalin and stained with H&E. Panels shown are representative sections.

5.3 The effect of IL-33 on airways hyperresponsiveness

The data here demonstrate that IL-33 can induce eosinophilic airways inflammation similar in nature to that seen in models of asthma, although of a greater magnitude than is generally seen in such models. One of the other key features of experimental asthma is the development of AHR. As discussed in Chapter 1 eosinophilia and AHR do not always accompany each other and are regulated by different mechanisms. I therefore wanted to establish if IL-33 could induce airways eosinophilia alone, or both eosinophilia and AHR.

5.3.1 Airways hyperresponsiveness following three doses of IL-33

Given that I planned to assess the contribution of mast cells play to the effects of IL-33 *in vivo* I began by testing the attenuated three dose protocol I had developed for use in C57Bl/6 mice (Figure 5.12).

As shown in Figure 5.18A there was a complete failure of IL-33 to generate AHR. While it is certainly possible to induce AHR in C57Bl/6 mice with a number of asthma protocols, it is well know that they have a tendency to display reduced spontaneous(50,53) and induced AHR(21,50,54). Consequently I went on to examine the effect of IL-33 on AHR in BALB/c mice.

While IL-33 did appear to enhance AHR in BALB/c mice this result was not statistically significant (Figure 5.18B). $ST2^{-/-}$ mice were also used to confirm the specificity of any observed effect. The dose response curve to methacholine for $ST2^{-/-}$ mice mirrored that seen with PBS in wild type mice, supporting the hypothesis that the effects of IL-33 are once again ST2 dependent.

The failure to demonstrate statistical significance in the wild type BALB/c mice treated with IL-33 may be in part due to the small group size used. It is also possible that the magnitude of the response is attenuated by the fact that the protocol involves a delay of three days from the last intranasal IL-33 administration. While I have demonstrated that eosinophilia persists for many days after treatment this may not be true of AHR.

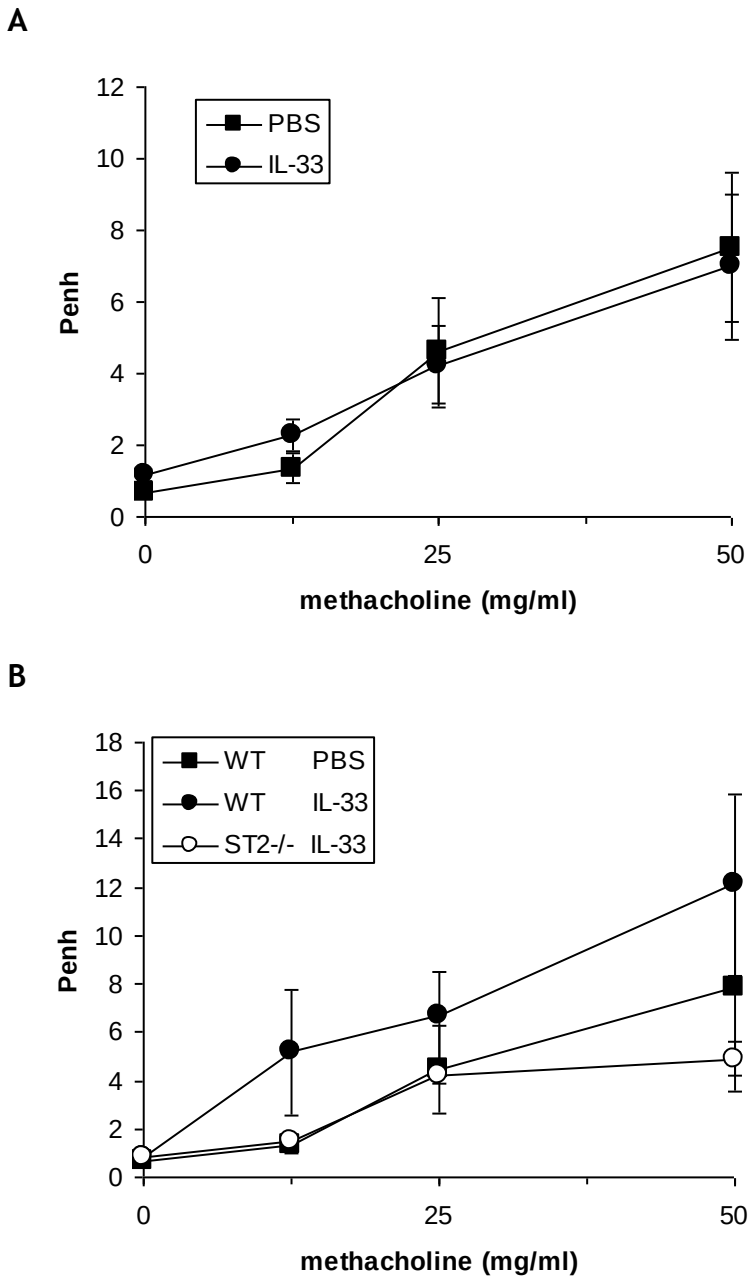


Figure 5.18 – IL-33 induces a trend towards AHR in BALB/c but not C57Bl/6 mice

(**A**) IL-33 or PBS was administered to wild type C57Bl/6 mice as in Figure 5.12. AHR was measured by Penh on day 6 (**B**) Wildtype or $ST2^{-/-}$ BALB/c mice were similarly treated. Results are shown as the mean of each group of mice +/- SEM. n=4-5 mice.

5.3.2 Airways hyperresponsiveness following seven doses of IL-33

Given the failure to generate statistically significant AHR in either strain of mice using the attenuated protocol I investigated whether seven consecutive administrations of IL-33, which induces a profound BAL eosinophilia (see Figure 5.1A), would be capable of inducing AHR, measured the day after the final dose. I chose to study both C57Bl/6 mice, due to the background of the mast cell deficient strain, and BALB/c mice, due to the encouraging results in the three dose protocol. Based on the data from the three day protocol a power calculation with a power of 0.95 for a result with a significance of 0.05 recommended a group size of 11 and group sizes were increased accordingly.

Once again AHR in C57Bl/6 mice was unchanged between the PBS group and the treatment group despite the increased IL-33 dose, reduced time from dosing to AHR measurement, and increased group size (Figure 5.19A).

In BALB/c mice AHR was induced by treatment with IL-33, and this became statistically significant at all does of methacholine (Figure 5.19B). Also shown in the graph is the response curve for mice in which experimental asthma had been induced using the 12 day protocol (Figure 4.8) which I had previously found to generate AHR as effectively as the more traditional 28 day protocol (Figure 4.11). This serves to demonstrate that the degree of AHR generated by IL-33 is very similar to that seen in allergic airways disease.

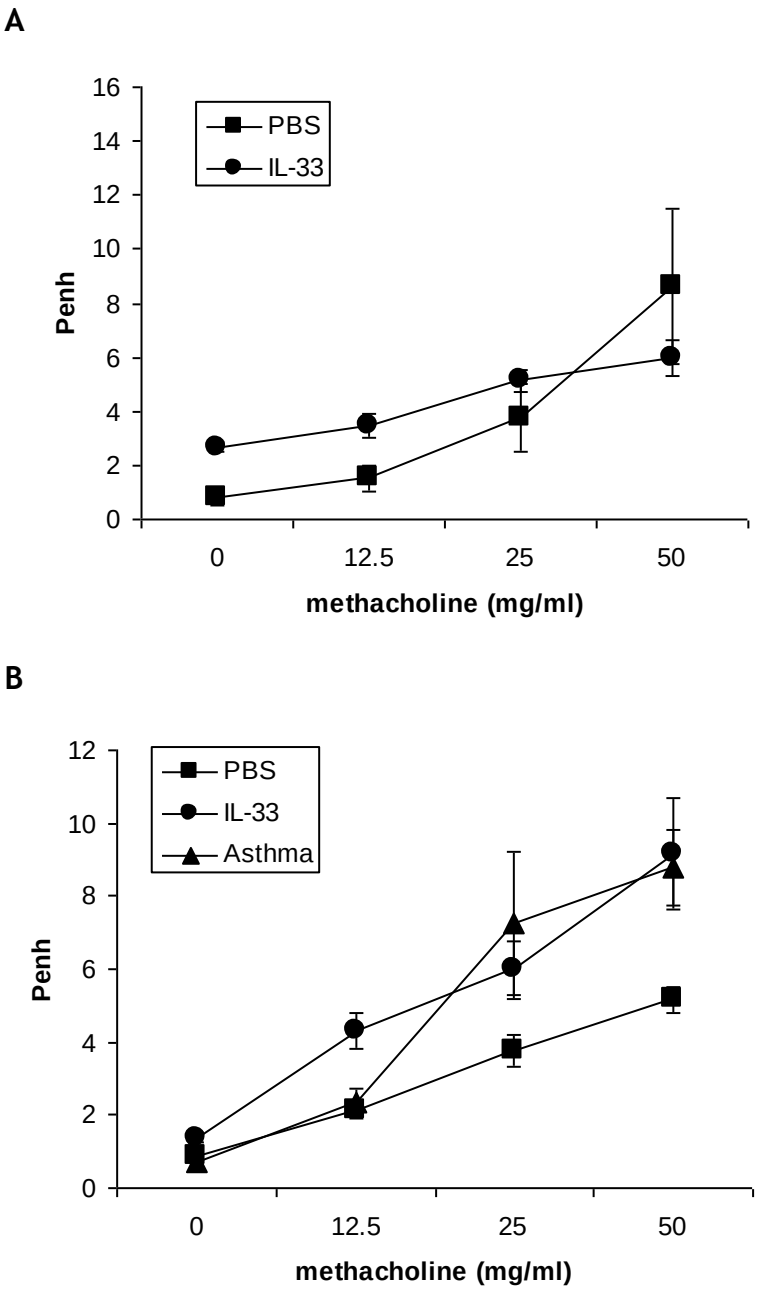


Figure 5.19 – IL-33 induces significant AHR in BALB/c but not C57Bl/6 mice

(**A**) IL-33 or PBS was administered to wild type C57Bl/6 mice as in Figure 5.1. AHR was measured by Penh on day 6 (**B**) Wild type BALB/c mice were similarly treated. Asthma was also induced in wild type BALB/c mice as in Figure 4.8. Results are shown as the mean of each group of mice +/- SEM. n=8-12 mice. Two-way ANOVA $p<0.001$ BALB/c PBS vs IL-33.

5.3.3 Study of the seven day protocol in BALB/c and C57Bl/6 mice

Although eosinophilia and AHR are not always associated, as discussed in Chapter 1, it seemed possible that the magnitude of the response to IL-33 in BALB/c mice, which are predisposed towards Th2 responses(510,511), might be greater in terms of both eosinophilic inflammation and AHR. Given that the difference in AHR between the two strains was most marked following seven doses of IL-33 I performed a direct comparison between BALB/c and C57Bl/6 mice using this protocol.

Figure 5.20 shows that the magnitude and nature of the inflammatory response generated in the BAL is identical in BALB/c and C57Bl/6 mice. Therefore there is a marked dissociation between AHR and eosinophilia.

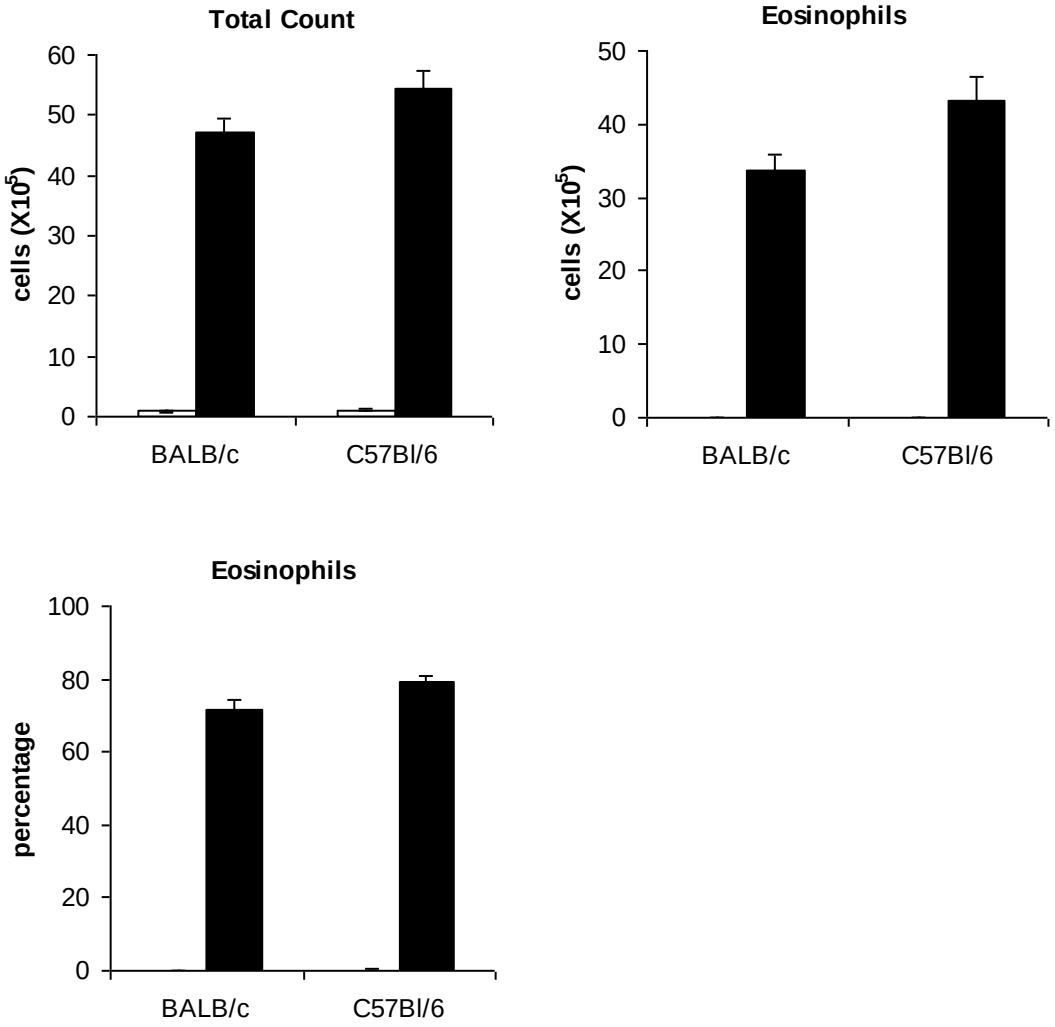


Figure 5.20 – IL-33 induced inflammation is equal in BALB/c and C57Bl/6 mice

IL-33 (■) or PBS (□) was administered to BALB/c and C57Bl/6 mice mice as in Figure 5.1. Total and differential cell counts were performed upon BAL. Results are shown as the mean of each group of mice +/- SEM. n=8 mice.

5.4 The role of mast cells in the response to IL-33

It is unclear why IL-33 failed to generate AHR in C57Bl/6 mice, despite a robust eosinophil response identical to that seen in the BALB/c mice, in which AHR is generated. While the failure may be due to the intrinsically reduced AHR seen in C57Bl/6 mice rather than a specific difference in the response to IL-33, it is generally possible to induce AHR in these mice with the appropriate protocols. Therefore I hypothesised that there may be a difference in the response to IL-33 at a cellular level. Given the response of BALB/c mast cells to IL-33 demonstrated in Chapter 3 I asked whether this response was affected by mouse strain. There is already evidence of functional differences in mast cells between these strains, including a deficiency in mast cell protease 7 in C57Bl/6 mice(56) and differences in mediator release upon FcεR cross-linking(512).

5.4.1 Strain differences in bone marrow mast cell development

Bone marrow mast cells were cultured in parallel from wild type C57Bl/6 and BALB/c mice for 7 weeks with SCF and IL-3 as detailed previously. Before studying the effect of IL-33 I looked for phenotypical differences between BALB/c and C57Bl/6 BMMCs. Due to the prolonged culture time I was unable to make robust comparisons of growth rates, but there was a suggestion that the C57Bl/6 population grew more slowly (data not shown).

C57Bl/6 mast cells appeared to mature more rapidly than BALB/c mast cells. At 5 weeks, 93% of C57Bl/6 cells were double positive for both c-kit and FcεRIα, compared with 83% of BALB/c cells at the same time point (Figure 5.21). The level of c-kit expression was similar at 98% and 99% respectively. ST2 expression was also similar for both strains. By 8 weeks of culture BMMCs from both strains were at least 95% c-kit^{+} and 90% FcεRIα^{+}.

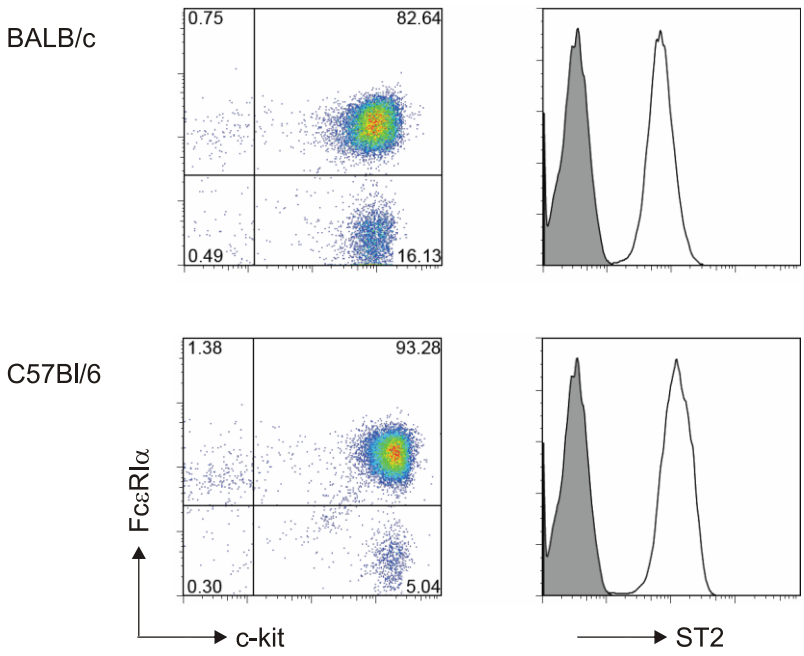


Figure 5.21 – C57Bl/6 BMMCs mature more rapidly than those from BALB/c mice

The surface expression of c-kit, FcεRIα and ST2 on 5 week old BALB/c and C57Bl/6 BMMCs was analysed by FACS.

5.4.2 Strain differences in the effect of IL-33 on mast cells

To assess whether mature BMMCs from these two strains of mice responded similarly to IL-33 C57Bl/6 and BALB/c BMMCs were simultaneously stimulated and the following cytokines and chemokines measured. MIP-1α was measured as I had consistently detected it at the highest concentrations from IL-33 stimulated BALB/c BMMCs. IL-13 was selected as it was also released by BALB/c BMMCs, was detected at the highest concentrations in the BAL of IL-33 treated mice, and plays a key role in the generation of AHR in asthma, as discussed in Chapter 1. Despite the previous lack of induction from BALB/c BMMCs eotaxin-1 and eotaxin-2 were also measured as they are potently induced by intranasal IL-33, and are important chemokines in eosinophil migration.

IL-13 was induced at low levels from BALB/c BMMCs stimulated with IL-33, but at higher levels from C57Bl/6 BMMCs, particularly when IL-33 was used at a concentration of 100 ng/ml (Figure 5.22). MIP-1α was more potently induced from both strains of mast cells, but was again released at markedly higher concentrations from C57Bl/6 than BALB/c BMMCs. Eotaxin-1 and eotaxin-2 were undetectable in supernatants of IL-33 stimulated BMMCs of either strain (data not shown). Given the failure to demonstrate a difference in the cytokines or chemokines produced which might account for the enhanced AHR in BALB/c mice a small number of samples were screened by multiplexed immunoassay. IL-6 and GM-CSF were detected at high concentrations, but as with IL-13 and MIP-1α, were higher from C57Bl/6 than BALB/c BMMCs. None of the cytokines or chemokines assayed were released at higher concentrations from BALB/c than C57Bl/6 BMMCs (data not shown).

These data do not support a strain difference in mast cell chemokine or cytokine release as the explanation for the enhanced AHR in BALB/c mice.

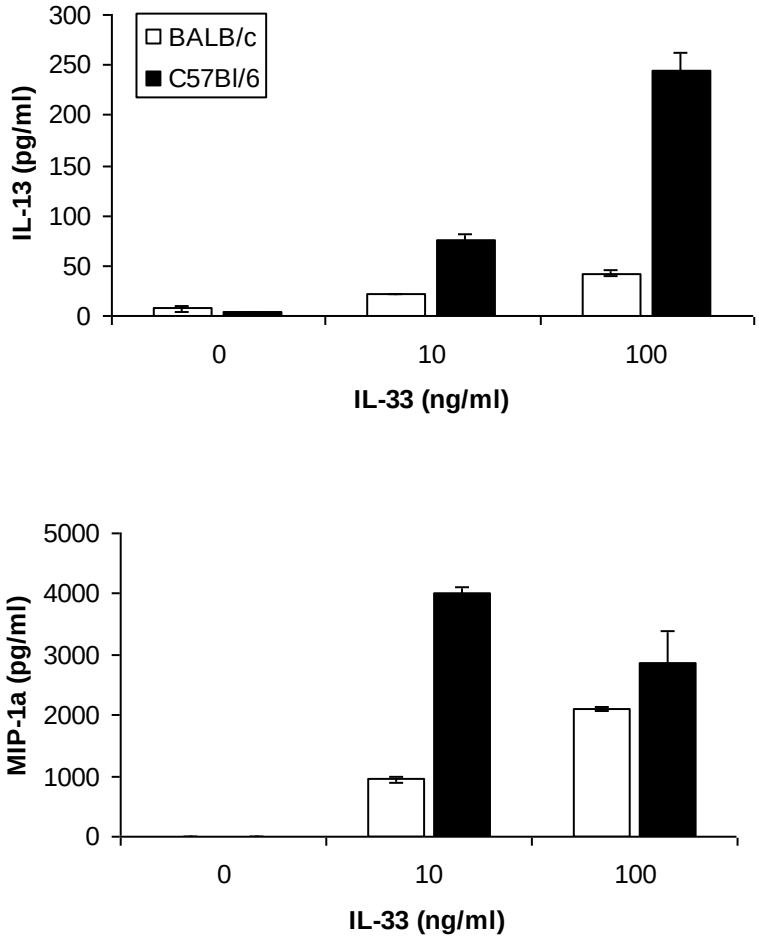


Figure 5.22 – C57Bl/6 BMMCs are more responsive to IL-33 than those from BALB/c mice

Mature WT BMMCs from C57Bl/6 and BALB/c mice at a concentration of $1x10^6$/ml were cultured in duplicate with mIL-33 at between 10 and 100 ng/ml and aliquots of the supernatant were harvested at 24 hours. MIP-1α and IL-13 were measured by ELISA. Results are shown as the mean of duplicate culture wells +/- SEM. Two-way ANOVA for the effect of strain on IL-13 $p<0.001$, on MIP-1α $p<0.001$.

5.4.3 The role of mast cells in the response to intranasal IL-33

I have demonstrated that IL-33 has potent activity within the lung, generating a marked eosinophilia in addition to AHR in BALB/c mice, two key features of asthma. While I could find no evidence of a mast cell basis for the difference in AHR between strains of mouse, mast cells are still a strong candidate as the first line cells responding to IL-33 administered to the lungs. They are the most strongly ST2 positive of all cells studied thus far. I have shown they produce pro-inflammatory cytokines and chemokines in response to IL-33, including the Th2 cytokine linked to AHR, IL-13. They are essential for AHR and eosinophilia in adjuvant free models of asthma(162) where I have found that ST2 is also essential.

This led me to the hypothesis that in asthma IL-33 is released locally and acts on mast cells which in turn release mediators to induce AHR and eosinophil recruitment.

Having demonstrated that IL-33 can induce the features of experimental asthma when delivered to the lungs the question remained as to whether these effects are mediated by ST2 on mast cells. In order to answer this question I administered IL-33 to the lungs of mast cell deficient, W-Sh mice. As discussed in Chapter 2 these mice are profoundly mast cell deficient with no mast cells detectable in the lungs(426,427). Having developed an attenuated dosing schedule to improve the chances of detecting any contribution by mast cells to the inflammatory response to IL-33, I used this three dose protocol in C57Bl/6 wild type and W-Sh mice.

When IL-33 was administered intranasally mast cell deficiency had no impact on the total cell count or the extent of eosinophilia generated in the BAL (Figure 5.23). The accumulation of other cell types was similarly unaffected.

Since I had only been able generate AHR in BALB/c mice, a background on which there are no mast cell deficient strains, it was not possible to assess the importance of masts cells in IL-33 induced AHR.

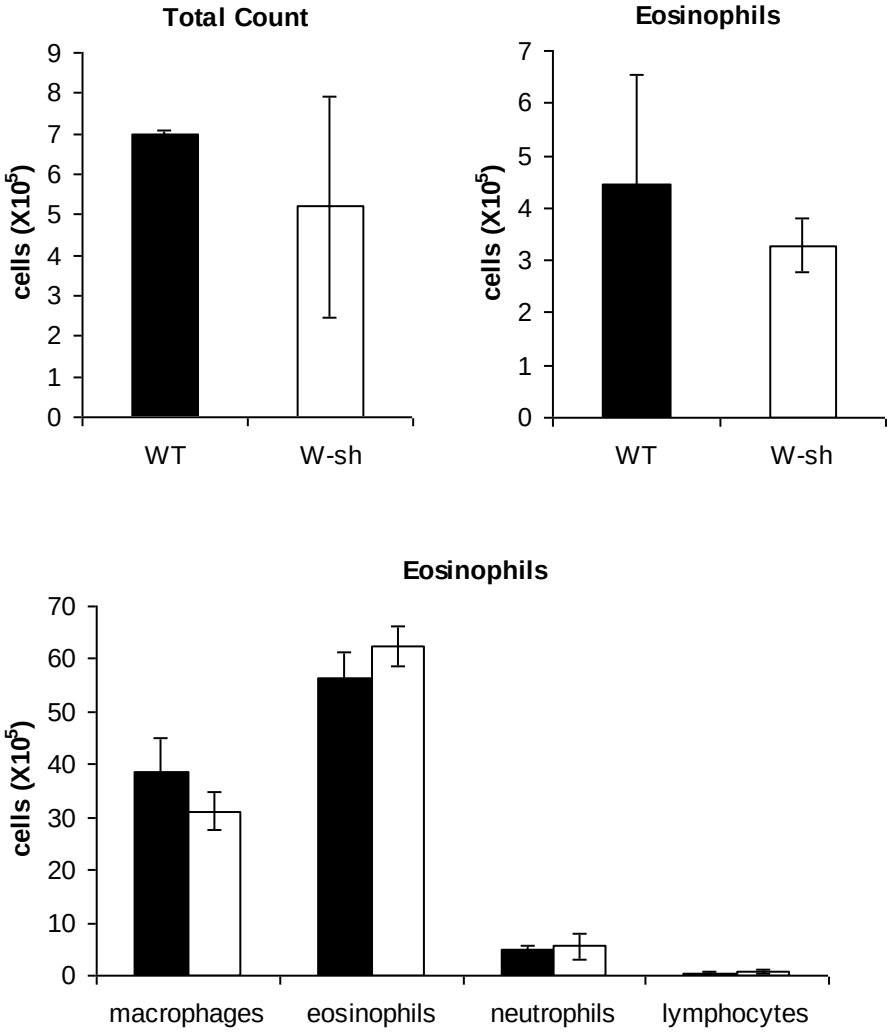


Figure 5.23 - IL-33 induced inflammation is unaffected by mast cell deficiency.

IL-33 was administered to C57Bl/6 mice wild type (■) or W-sh (□) mice as in Figure 5.12. Total and differential cell counts were performed upon BAL. Results are shown as the mean of each group of mice +/- SEM. n=5 mice.

5.5 Conclusion

The data in this chapter demonstrate that IL-33 has a profound effect when administered directly into the airways, inducing the key features of experimental asthma. Based on the initial dosing regime utilised by Schmitz *et al* for intraperitoneal administration(323) I demonstrated that following seven doses of intranasal IL-33 a marked eosinophilia is generated within the BAL and lung parenchyma.

In order to better understand the events which led to pulmonary eosinophil accumulation time course experiments were carried out. When repeated doses of IL-33 were administered eosinophils were not detected in the BAL until three days after the initial dose, but increased steadily to a very high cell count after seven days. In models of asthma, eosinophils accumulate within 24 hours of initial challenge, as demonstrated in Chapter 4. Although it is possible that antigen sensitisation in these models somehow primes or mobilises eosinophils, in studies administering recombinant cytokines such as IL-5 and eotaxin-1 to naïve animals, eosinophils also begin to appear in the BAL within 24 hours(513,514). The three day delay I observed in accumulation within the lungs suggests that the effect of IL-33 on eosinophils is indirect. In the time course experiments eotaxin-1 and eotaxin-2 are also undetectable until after the third dose. The correlation between eotaxin detection and initial eosinophil accumulation suggests that this release of eotaxin-1 and eotaxin-2 may be responsible for recruiting the eosinophils to the lungs. The eotaxins certainly have the potential to recruit eosinophils, and play an important part in the eosinophilia that is seen is experimental asthma(179,260,266-268,514). Given that eosinophils themselves can produce eotaxin(515) one other possibility would be that the eosinophils are recruited by another mechanism and then they are responsible for the release of the eotaxins detected. The Th2 cytokines IL-5 and IL-13 were not present within the BAL until 24 hours after eosinophils and eotaxins were detected suggesting that Th2 cytokine production is not the initiating factor responsible for eosinophil recruitment. It is possible that the converse is true and that eosinophil recruitment is required for Th2 cytokine production, and there is evidence from eosinophil deficient mice that this is the case in asthma models(116).

When four doses of IL-33 were given there were still a high number of eosinophils within the BAL eight days after the final administration. In models of asthma eosinophil numbers are generally very low by seven days after cessation of antigen challenge(492,493). While one must be cautious in interpreting the limited time course data available they raise the possibility that an initial release of IL-33 may be sufficient to initiate an inflammatory process which becomes self-sustaining. To test the hypothesis that a limited number of administrations of IL-33 induces persistent inflammation, and perhaps airway remodelling, it would be necessary to obtain BAL and histological samples at time points many days after initial IL-33 treatment.

After initially studying C57Bl/6 mice I went on to demonstrate that airways eosinophilia was also generated in BALB/c mice, and that the extent of inflammation was unaffected in by strain, despite the preponderance for Th2 responses generally seen in BALB/c mice(510,511). By using BALB/c mice it was also possible to confirm that IL-33 had no effect in $ST2^{-/-}$ mice. This demonstrated for the first time that the effects of IL-33 *in vivo* are mediated via ST2 alone. It also served to confirm that all the phenomena observed were due to a specific effect of IL-33 rather than any possible contaminant.

Using BALB/c mice I have demonstrated that the administration of IL-33 to the lungs induces AHR. When studied initially in a protocol where 3 doses of IL-33 where followed by a delay of 3 days AHR appeared to be induced, but the effect did not reach statistical significance. This failure to reach significance may be due to the delay between the final administration and measurement of AHR, although in models of asthma in BALB/c mice AHR can persist for up to seven days after antigen challenge(492). Alternatively this failure may have been due to the small group size, with only four to five mice in each group. When AHR was studied in BALB/c mice 24 hours after a seventh dose of IL-33, using eight to twelve mice per group, there was a statistically significant increase with methacholine doses as low as 12.5 mg/ml. The magnitude of AHR generated was similar to that seen in a model of asthma.

In C57Bl/6 mice there was no evidence of IL-33 induced AHR regardless of the protocol used. Given that the extent of eosinophilia induced is the same in both strains it seems unlikely to be due to a fundamental inability of C57Bl/6 mice to

respond to IL-33. The dissociation of eosinophilia and AHR is not unprecedented as AHR occurs alone in some IL-5 knock-out asthma studies and eosinophilia alone in some IL-13 knock-out studies(100,225,228).

I could find no evidence that the strain difference in the response to IL-33 was due to different responses of mast cells to IL-33. However as mentioned previously there are reported functional differences in mast cells between these strains (56,512). Having been unable to induce BALB/c mast cell degranulation with IL-33 I did not study this in C57Bl/6 mice, although building on the data in Chapter 3 others within our research group have gone on to demonstrate that IL-33 can induce the degranulation of IgE primed mast cells *in vitro*(449).

Another possible reason for the failure to generate AHR in C57Bl/6 mice might relate to the use of Penh. It has been demonstrated that the correlation between Penh and other invasive measures of AHR is not as strong in C57Bl/6 mice as it is in BALB/c mice(516,517). There is now one published report in which IL-33 appeared to generate AHR in C57Bl/6 mice as measured by Penh, however the was no measure of variance reported and no attempt at statistical analysis(447). There has since been data presented at the recent American Thoracic Society conference in 2009 showing that IL-33 can induce AHR in C57Bl/6 mice(518). IL-33 was used at a higher dose than I used (5 µg) and AHR was measured invasively.

My hypothesis was that the activation of mast cells via ST2 would be responsible for the effects if IL-33 in the lungs *in vivo*. Using mast cell deficient W-sh mice I have demonstrated that there is no requirement for mast cells in IL-33 induced pulmonary eosinophilia. Due to the failure to generate AHR in C57Bl/6 mice I was unable to utilise W-sh mice to assess the contribution of mast cells. However in the recent abstract mentioned above not only did IL-33 treatment result in increased concentrations of mast cell protease within the BAL, but AHR was induced in wild type but not mast cell deficient mice(518).

In summary I have demonstrated that IL-33 acts in an ST2-dependent manner to induce two of the key features of asthma; pulmonary eosinophilia and AHR. This provides further evidence that the ST2-IL-33 axis may play an important role in clinical asthma.

Chapter 6

General Discussion

While the contribution of Th2 inflammation in asthma is accepted, the function of different cells and cytokines at different stages of disease development and resolution remains unclear. Key questions include the role of mast cells, the relationship between structural cells and inflammatory cells and the factors involved in the initiation of asthma. In the context of the emerging role of ST2 in allergic inflammation and the discovery of IL-33, the ligand for ST2 and an inducer of Th2 inflammation, at the outset of this thesis I set out the hypothesis that 'the activation of ST2 on mast cells by IL-33 plays an important role in allergic airways inflammation'.

My aims were to test this hypothesis by meeting three objectives; to assess the effect of IL-33 on mast cells *in vitro*, to study the requirement for ST2 in a murine model of asthma, and to examine the effect of IL-33 administration to the airways. A summary of my findings and their interpretation is below.

6.1 Summary of results

I addressed the first of these aims in Chapter 3, where I demonstrated that ST2 was expressed at high levels from a very early stage of mast cell development *in vitro* and correlated closely with expression of the early mast cell marker c-kit. Despite this, the absence of ST2 did not alter the growth or maturation of mast cells from the bone marrow of ST2 gene-deleted mice *in vitro*, implying that IL-33 does not play an essential role in mast cell development. Mast cells stimulated with IL-33 released a number of pro-inflammatory mediators, specifically the pro-inflammatory cytokine IL-6, the Th2 cytokine IL-13 and the chemokines MIP-1α and MCP-1, and at times the growth factors GM-CSF and VEGF. I was also able to utilise $ST2^{-/-}$ mast cells to confirm the ST2 specificity of this effect.

Having demonstrated the pro-inflammatory effect of IL-33 *in vitro* I went on to study the role if ST2 in experimental asthma in Chapter 4. I began by optimising and validating a short model of asthma in mice and used this to demonstrate for the first time that ST2 gene deficiency could attenuate allergic inflammation. To try and understand the discrepancy between this finding and those published using other models of asthma(381,383) I went on to compare the requirement for ST2 between a mast cell dependent and a mast cell independent model of

asthma. As others had reported(381,383), I demonstrated that ST2 gene deficiency did not attenuate AHR or inflammation in a mast cell independent model of asthma in which mice were immunised with OVA and alum. However when mice were immunised with OVA alone, a protocol which has been shown to confer mast cell dependence(162,163), ST2 deficiency had no impact on AHR but resulted in a significant reduction in eosinophilic inflammation. This was associated with a specific reduction in IgE and reduced Th2 cytokine production by draining lymph node cells.

In Chapter 5, I went on to study the effect of ST2 activation in the airways by the direct administration of IL-33. Intranasal IL-33 induced a dramatic and long lasting airways eosinophilia as well as generating AHR, two of the key features of murine models of asthma. Using $ST2^{-/-}$ mice it was possible to demonstrate that these effects were specific for ST2. Based on the effect of IL-33 on mast cells demonstrated in Chapter 3 and the requirement for ST2 in a mast cell dependent model of asthma reported in Chapter 4 I formulated the hypothesis that the effect of IL-33 administration to the airways was mediated by the activation of ST2 on mast cells. However when I tested this hypothesis using mast cell deficient mice, there was no impact on eosinophil recruitment, demonstrating that this component of inflammation was induced by IL-33 in a mast cell independent manner. Due to the fact that AHR was only generated in BALB/c mice it was not possible to assess the contribution of mast cells to this phenomenon.

In order to interpret these findings in the context of asthma it is necessary to consider the potential source of biologically active IL-33 and the effects it is likely to have at a cellular level.

6.2 The cellular source of IL-33

While Schmitz *et al* published data on the expression of IL-33 RNA in a number of cells and tissues in their initial report(323), any data on the detection of IL-33 protein was limited until very recently. This is due in part to the lack of available reagents but primarily to the apparent scarcity of extracellular IL-33 protein.

The first studies to detect IL-33 protein did so by immunohistochemistry, with the earliest report identifying nuclear localisation within epithelial cells of tonsillar HEVs(417), specialized blood vessels which mediate lymphocyte recruitment into lymphoid organs. The investigators referred to the protein as nuclear factor from HEV (NF-HEV) as this report predated the formal characterisation of the protein as IL-33 by two years. This finding has since been confirmed by others(418,519) and IL-33 has been identified by immunohistochemistry in other vascular endothelial cells(520-523) as well as fibroblasts(407,521,523) and epithelial cells(521).

Despite these findings doubt remained as to whether IL-33 was ever released from cells. IL-33 contains a DNA-binding domain and both naturally occurring and transfected IL-33 localises to the nucleus (417,418,519,521). Also, even though a commercial IL-33 ELISA eventually became available we and others struggled to detect IL-33 in a number of *in vivo* and *in vitro* settings. It was originally proposed that as IL-33 was an IL-1 family member it would be processed for secretion in a similar manner to IL-1ß(323), which is activated by the cleavage of pro-IL-ß by caspase-1(524). However, as discussed in Chapter 1, while Schmitz *et al* showed that IL-33 could be cleaved by caspase-1 *in vitro* it does not have a classical caspase-1 cleavage site.

Four papers published within the last few months have greatly enhanced our understanding of IL-33 processing. While three of these studies confirmed that IL-33 could be cleaved by caspase-1, cleavage was shown to be inefficient as compared with the cleavage of IL-1ß, and very limited when physiological concentrations of caspase-1 were used(525-527). Furthermore in a stringently controlled setting where all other proteases were eliminated caspase-1 failed to cleave IL-33(526), suggesting that any cleavage by caspase-1 may be due to the ability of caspase-1 to activate other proteases(528). However IL-33 did appear to be cleaved more effectively by caspase-7 and to a lesser extent caspase-3(525,527). A separate study using inhibitors suggested that LPS induced IL-33 cleavage was mediated by calpain rather than caspase-1(529). The other novel finding was that the cleavage site for these caspases was within the IL-1 like domain, therefore neither of the cleavage products corresponded to so called “mature IL-33” and both were biologically inactive(525,527). This cleavage of IL-33 by proteases occurs during apoptosis(525,527) and is presumably a mechanism

to avoid the release of potentially bio-active IL-33 during programmed cell death.

These data do not support the theory that active IL-33 is released upon the cleavage of “pro-IL-33” to “mature IL-33” by caspase-1 and therefore led investigators to study the properties of full length “pro-IL-33”. It has now been convincingly demonstrated that full length IL-33 is biologically active *in vitro* (525-527). These recent findings have led to a revision of the theory regarding IL-33 processing and release.

Two other molecules with similarities to IL-33 are High Mobility Group Box 1 (HMGB1) and IL-1α. HMGB1 is generally found within the nucleus and affects transcriptional regulation. HMGB1 can also act via pattern recognition receptors such as RAGE(530) and TLRs(531) to mediate a number of pro-inflammatory effects on macrophages, neutrophils, T cells and endothelial cells including cytokine secretion and upregulation of surface receptors(532). Like IL-33, HMGB1 is inactivated during apoptosis(533). While HMGB1 can be secreted by non-classical pathways similarly to IL-1ß(534,535), it is well recognised for being released by necrotic cells(536).

IL-1α is synthesised as a precursor, pro-IL-1α which is targeted to the nucleus by nuclear localisation sequence within the pro-domain(537), although the function of nuclear IL-1α remains unknown. While there is also uncertainty regarding the active secretion of IL-1α(538,539), it is clear that IL-1α is released by cell necrosis(540,541). IL-1α and IL-1ß both act via the IL-1 receptor with identical biological effects and are collectively referred to as IL-1. IL-1 is a classical pro-inflammatory cytokine with wide ranging effects including the induction of nitric oxide and eicosanoid synthesis, triggering of the acute phase response, the release of a wide number of other cytokines and chemokines and the upregulation of adhesion molecules(542,543).

The concept that the specific immune response can be activated by the presence of ‘danger signals’ was formalised with the discovery of receptors for exogenous pathogen-associated molecular patterns (PAMPs), such as the TLRs and NOD-like receptors (NLRs). More recently it has been suggested the endogenous ‘danger signals’ released in response to tissue injury and necrosis be referred to as ‘alarmins’, with PAMPs and alarmins collectively forming a family

of damage-associated molecular patterns, or DAMPs(544,545). Both HMGB1 and IL-1α have been classed as alarmins due to the fact that they are released by necrotic but not apoptotic cells and act to recruit and activate immune cells(544,545).

It now appears that IL-33 is also a member of this family. In addition to the other characteristics shared with HMGB1 and IL-1α, several investigators have been able to detect IL-33 protein release from damaged or necrosed cells(525,527,529). Similarly to HMGB1, there is also data to show that proinflammatory signals such as TLR agonists or cytokine cocktails can induce the release of full length IL-33(503,526,546,547), although the possible contribution of cell necrosis was not assessed in these experiments. The potential role of IL-33 as an alarmin released upon tissue damage may explain why the main sites of IL-33 protein detection have been within structural cells such as endothelial cells, fibroblasts and epithelial cells. This may also account for the recent detection of IL-33 protein in the damaged tissues from diseases such as rheumatoid arthritis(523,548) and dermatitis(449).

6.3 Cellular targets of IL-33

At the outset of this research a number of cells were known to express ST2 on their surface but there was almost no data on the functional effect of IL-33 upon cells *in vitro*. Schmitz *et al* had demonstrated that IL-33 enhanced IL-5 and IL-13 production by Th2 cells and activated signalling pathways in mast cells(323). Since then IL-33 has been shown to have effects on a wide range of different cells.

In Chapter 3 I demonstrated that IL-33 could activate mast cells to release pro-inflammatory cytokines from murine mast cells. This has since been published by us(449) and confirmed by other investigators in both human and murine mast cells(443,445,447,450). Basophils are closely related to mast cells and are also targets of IL-33. Upon IL-33 stimulation basophils produce a number of cytokines and chemokines, particularly IL-4, IL-6 and IL-13, although this is generally at low levels unless pretreated or co-cultured with IL-3, which is required to induce ST2 expression(447,549-551). IL-33 also enhances basophil adhesion, integrin expression, chemotaxis, degranulation and survival(549,551).

While others have confirmed the finding that IL-33 enhances IL-5 and IL-13 production by Th2 cells(550,552), our group have published data showing that IL-33 can polarise naïve T cells to produce IL-5 and IL-13, and that this occurs independently of IL-4, which is usually required for Th2 polarisation(553). In addition data from our laboratory has shown that IL-33 can be chemotactic for Th2 cells(419). IL-33 has a less restricted effect on NK and iNKT cells, enhancing the release of Th2 cytokines, including IL-4, as well as Th1 cytokines(552,554).

Both the original data published by Schmitz *et al*(323) and the data presented here show that IL-33 is a potent inducer of eosinophilia *in vivo*. Eosinophils do not appear to express surface ST2, although it can be induced by GM-CSF(550,555,556). However IL-33 is active on eosinophils, inducing IL-8 and superoxide production, upregulating adhesion molecules, enhancing adhesion and prolonging survival(550,555,556). It does not appear to induce chemotaxis(556) and there was conflicting data regarding the ability of IL-33 to induce degranulation(555,556). The only granulocyte upon which IL-33 does not appear to have any effect is the neutrophil(550,555).

There is also data now to demonstrate that IL-33 has effects upon cells of the monocytes lineage. It enhances the production of TNFα by LPS and LTA treated macrophages, probably by increasing receptor, co-receptor and adapter protein expression(557). Dendritic cells also have no detectable ST2 upon their surface but respond to IL-33 by upregulating surface HMC-II and costimulatory CD86(558). IL-33 can induce the release of IL-6 from pure DCs, and of IL-5 and IL-13 when co-cultured with CD4 T cells(558), possibly due to the ability of DC derived IL-6 to induce Th2 polarisation(453). The lack of detectable ST2 expression on cells responding to IL-33 is likely to represent a limitation of flow cytometry in detecting low level expression on a small subset of cells, as there have been no reports of IL-33 acting in an ST2 independent manner.

In light of all this new information on the effects of IL-33 at a cellular level, and the data presented in this thesis, we can postulate how IL-33 may be involved in allergic airways inflammation.

6.4 The role of IL-33 in airways inflammation

In Chapter 5 I demonstrated that in an alum free model of asthma eosinophilia but not AHR was markedly reduced in the absence of ST2. This may be due to a requirement for ST2 at sensitisation, at challenge, or both.

6.4.1 IL-33 at sensitisation

In support of a role for IL-33 at sensitisation was the finding that antigen specific IgE was dramatically reduced in $ST2^{-/-}$ mice in the alum free model, suggesting a primary failure of allergic sensitisation. As discussed in Chapter 4 the use of alum fundamentally changes the way antigen is taken up, transported and presented(499). The action of alum is also dependent on the activation of the NLRP3 inflammasome(502-505). It is therefore possible that in the absence of alum a pathway is required which is dependent on IL-33 and ST2 for effective antigen presentation and T cell activation.

In the absence of alum, antigen is taken up and presented by resident DCs in the local draining lymph nodes and there may be a requirement for ST2 at this point. IL-33 can activate DCs to upregulate costimulatory molecules(558) and drive CD4 cell Th2 polarisation as well as enhancing Th2 recruitment and activation (323,419,553).

Given the high level of ST2 expression on mast cells and the requirement for mast cells in this model, IL-33 induced activation of mast cells may be required at sensitisation. There is accumulating evidence that mast cells can play an important role during antigen presentation. They play a significant role in lymph node hypertrophy during infection(559) and mast cell activation can enhanced DC recruitment and migration to draining lymph nodes resulting in the generation of antibodies against otherwise innocuous antigens(560). Both these effects were dependent on mast cell derived TNFα. Mast cells are a crucial early source of TNFα, being the only cells capable of releasing pre-stored TNFα(438) and mast cell derived TNFα is essential in alum free models of asthma(33,163).

One could hypothesise that there are two separate pathways for the generation of a Th2 memory response to antigen, as represented in Figure 6.1. These

represent activation of the adaptive immune response due to the effect of two different types of DAMP acting initially on the innate immune system. Pathway A represents the effect of antigen presented with a PAMP, in this case the activation of the inflammasome by alum acting as an NLR agonist. In pathway B Th2 development is driven by the effect of activation by the 'alarmin' IL-33. This may act directly on DCs and Th2 cells or indirectly via the release of TNFα and other mediators from mast cells. Although I found that IL-33 induced TNFα release from mast cells *in vitro* was variable, it has been reported by others(443,450).

Interestingly activation of the NLRP3 inflammasome by LPS and alum resulted in the release of IL-33 protein from monocytic cell lines(503). The mechanism for this is unclear given that the majority of IL-33 detected was full length "pro-IL-33". This may represent some form of crosstalk between the two pathways.

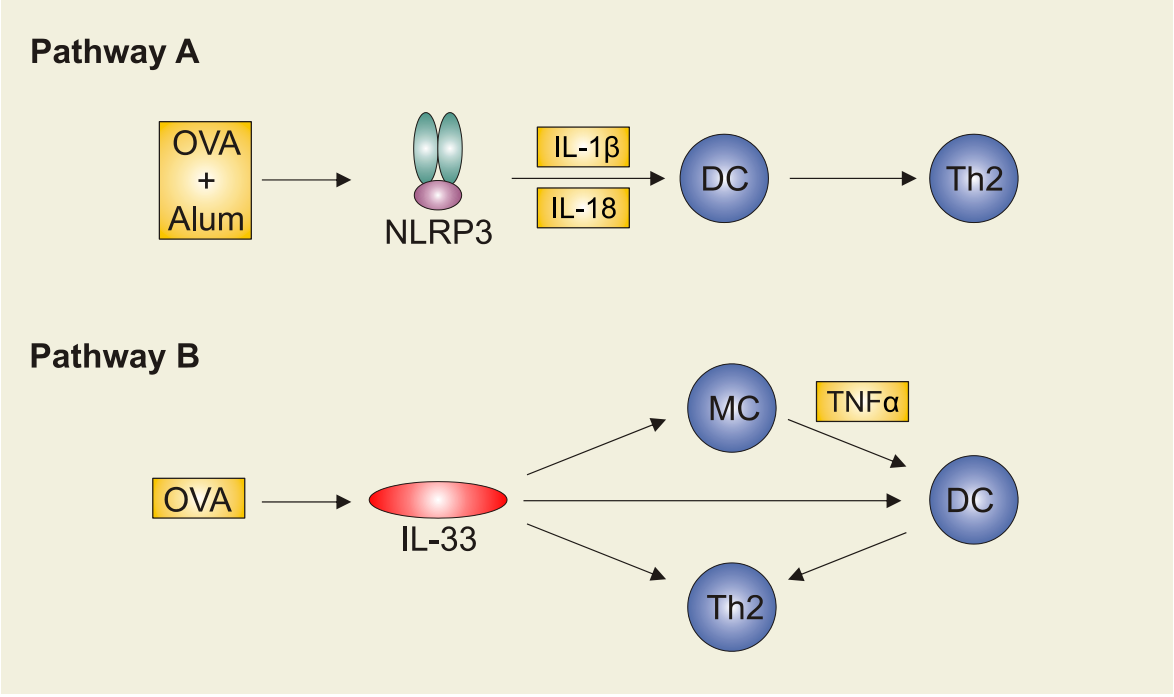


Figure 6.1 - Two pathways of pathogen-associated molecular pattern (PAMP) activation

Pathway A - Alum acts as an NLR agonist to activate the NLRP3 inflammasome which in turn leads to the activation of T cells by dendritic cells (DC).

Pathway B - Tissue damage during the intraperitoneal administration of OVA results in the release of IL-33 which can activate DCs, Th2 cells or mast cells (MCs) directly. IL-3 induced TNFα from mast cells can also activate DCs.

In support of the role of 'alarmins' in models utilising OVA alone is the finding that when mice were injected with intraperitoneal OVA alone on the right side of the abdomen T cell proliferation occurred in the draining lymph node at that side only, but when mice also received a sterile needle puncture on the other side then T cell proliferation also occurred in the draining lymph at that side(499). While the antigen could be readily available it may be that the local needle puncture is required to induce the co-stimulatory 'alarmin' signal. Work from our laboratory has demonstrated that the injection of intraperitoneal IL-33 at the time of sensitisation enhances the inflammatory response in an OVA/alum model of asthma(553).

6.4.2 IL-33 at challenge

Alternatively ST2 and IL-33 may be required at the time of antigen challenge. The fact that AHR is preserved even though eosinophilic inflammation is dramatically reduced suggests that a memory response to OVA has been generated. Studies using anti-ST2 antibodies suggest a requirement for ST2 at challenge. When asthma is induced by adoptive transfer of antigen specific T cells the administration of anti-ST2 at the time of antigen challenge results in a substantial reduction in BAL and lung eosinophilia and AHR(360,372). The data presented in Chapter 5 also demonstrates that the activation of IL-33 in the airways by ST2 can induce the features of experimental asthma. The question remains as to which cells are respond to IL-33 in the airways to elicit airways inflammation and AHR.

Given that I and others have demonstrated that IL-33 can activate mast cell to generate pro-inflammatory cytokines and chemokines *in vitro* I speculated that IL-33 was acting via mast cells to induce eosinophilia and AHR *in vivo*. However I have demonstrated that airways eosinophilia is unaffected in W-sh mice. Although these mice may not be completely mast cell deficient there are no reports of mast cells being detected within the lungs and only one report of mast cells within the skin at 1% of normal levels(426,427). Unfortunately I was only able to generate significant AHR in BALB/c mice and not in C57Bl/6 mice, the background on which the W-sh mutation is present. This meant that it was not possible for me to asses any contribution of mast cells to IL-33 induced AHR. However others have been able to induce AHR, as measured invasively, with IL-

33 in C57Bl/6 mice and found that this was IL-13 dependent and was lost in the absence of mast cells(518).

Th2 cells release IL-5 and IL-13 in response to IL-33, and work from our laboratory has demonstrated that such IL-33 polarised cells can be used induce airways inflammation in an adoptive transfer model of asthma(553). However studies from our group and others have demonstrated that T cells are not required for the induction of eosinophilic inflammation and AHR by intranasal IL-33(G. Murphy *et al* unpublished)(447,518).

It is possible that IL-33 acts directly on eosinophils themselves. Although there are normally no eosinophils present in the lungs, as demonstrated by the absence of eosinophils in control mice in experiments presented here and published by others, it is possible that IL-33 acts to recruit eosinophils directly from the pulmonary circulation. However, as discussed above, IL-33 does not appear to be chemotactic for eosinophils(556). The delay in eosinophil recruitment demonstrated in Chapter 5, with eosinophils first detected three days after IL-33 dosing begins, suggests a less direct effect. The appearance of the eosinophil chemokines eotaxin-1 and eotaxin-2 at the same time suggests that these chemokines drive eosinophil recruitment. We have been able to demonstrate that blocking eotaxin-1 or eotaxin-2 results in a marked reduction in IL-33 induced airways eosinophilia(561).

Macrophages are the most abundant inflammatory cell within the lung and have been shown to respond to respond to IL-33(557). On the basis of the data presented in Chapter 5, work within the laboratory has been carried to look at the possible role of macrophages in this response(562). IL-33 is able to synergise with IL-4 or IL-13 *in vitro* to induce macrophages to differentiate to the alternatively activated macrophage (AAM) phenotype and produce eotaxin-2. Furthermore intranasal IL-33 administration induces AAM development and macrophage depletion attenuates eosinophilic inflammation.

As discussed earlier it appears that, like the other ‘alarmins’ IL-1α and HMGB1, IL-33 is released upon cell breakdown in response to injury. In addition to the data regarding the expression of IL-33 in vascular endothelial cells, IL-33 protein has also been detected in epithelial cells(521) and we have reported the detection of IL-33 protein within the lung parenchyma(553).

Figure 6.2 is a schematic representation of how tissue injury within the lung could result in the release of IL-33 which induces eosinophilic inflammation and AHR by two distinct pathways. IL-33 triggers eotaxin release from alternatively activated macrophages which results in the recruitment of eosinophils, and mast cells are activated by IL-33 to induce AHR.

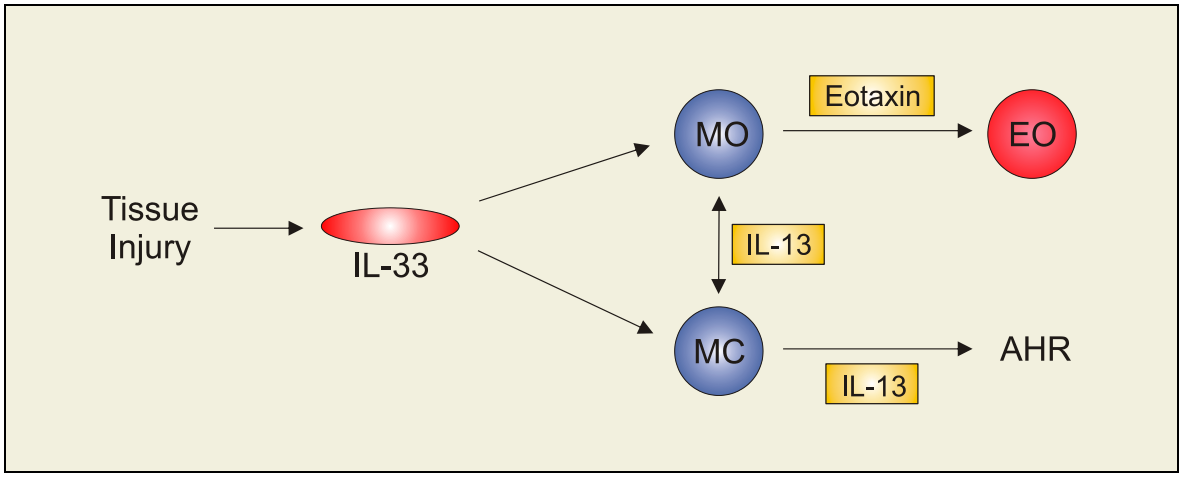


Figure 6.2 – The release of IL-33 as an 'alarmin' inducing the features of asthma

Local tissue injury in the lung results in the release of IL-33 which acts on macrophages (MO) to recruit eosinophils (EO) via eotaxin and on mast cells (MC) to induce airways hyperresponsiveness (AHR) via IL-13.

6.5 IL-33 in clinical asthma

The data presented in this thesis have been derived entirely from *in vitro* studies and *in vivo* animal models. The effect of IL-33 on mast cells described in Chapter 3 has since been repeated within our group using human cord blood derived mast cells(449). However it is important to consider the role of the IL-33 – ST2 axis in the context of the intact organism *in vivo*. Given that it would not be practical or ethical to study the effect of blocking ST2 or administering IL-33 in humans, I have chosen to utilise mice. In Chapter 4 I demonstrated that the requirement for ST2 was dependent on the model of asthma used. One might criticise the validity of a model where such modifications can have such a dramatic impact on the outcome. However one of the limitations of gene-deletion studies is the degree of redundancy which occurs in many biological systems. It is interesting to note that in all studies using anti-ST2 antibodies in intact animals treatment

attenuated experimental asthma(298,360,372,396,492) with similar results in a recent study using anti-IL-33 antibody(563). It may be that the modification to the model is required to overcome such redundancy, perhaps by emphasising the contribution of mast cells. One could argue that the omission of adjuvant makes the model more akin to clinical asthma.

In Chapter 5 I demonstrated that IL-33 is a potent inducer of eosinophilic inflammation and AHR, and this data in combination with the work of others suggests that this occurs via two separate pathways. This raises the possibility that IL-33 has a pivotal place in the cytokine hierarchy. If IL-33 is an alarmin which is released by structural cells in response to tissue damage then it could be a key upstream mediator of inflammation and bronchospasm in asthma. IL-33 release could be induced by bioactive allergens such as those from house dust mite. Such allergens have been shown to induce cytokine release from epithelial cells(564) and there is a preliminary report of house dust mite extract inducing IL-33 release from cultured epithelial cells(547). Viral infections are the commonest cause of exacerbations in asthma(5) and can induce the release of the related alarmin IL-1α from epithelial cells(565-567). It is possible that viral infection in asthmatic induces the release of IL-33 from infected epithelial cells which goes on to trigger eosinophilic inflammation and AHR as represented in Figure 6.2. Once inflammation is established the epithelial damage which characterises asthma(57,69) may result in ongoing IL-33 release and the perpetuation of asthma. IL-33 has recently been detected at elevated concentrations from the lungs in an model of asthma seven days after the antigen challenge(492).

6.6 Future work

IL-33 biology is a fast moving area of research at present, both within our laboratory and internationally, but there are a number of specific lines of investigation which would advance the work presented here.

- In order to definitively establish whether or not the requirement for ST2 in the alum free model is upon mast cells adoptive transfer experiments could be undertaken. Mast cells can be restored in W–sh mice by the adoptive transfer of bone marrow derived mast cells(426). It would

therefore be possible to study the alum free model of asthma in W-sh mice reconstituted with wild type or $ST2^{-/-}$ mast cells. In such reconstituted mice only the mast cells would lack ST2.

- Further work is required to establish the potential source and stimulus of IL-33 release in the context of both experimental and clinical asthma. Studies carried out *in vitro* on murine and human samples could establish whether triggers such as house dust mite and viral infection result in IL-33 release from specific cells, particularly epithelial cells. Further animal studies could also establish whether these triggers resulted in the release of IL-33 *in vivo*.

- To begin to establish a role for IL-33 in clinical asthma it is important to demonstrate that IL-33 is elevated in asthma. We have recently found that IL-33 expression is enhanced within the epithelial cells of asthmatic patients(568). An important next step would be to assay the extracellular IL-33 in clinical samples, particularly sputum or BAL. If IL-33 is released from epithelial cells in response to allergens or infections it would be useful to measure IL-33 in samples collected during asthma exacerbations.

- Building on the data presented in Chapter 3 and some preliminary data not shown, others within the group have characterised the role of IL-33 activation in other models of allergic inflammation(449). IL-33 could induce both passive cutaneous anaphylaxis and systemic anaphylaxis in IgE sensitised mice in the absence of antigen. This was mediated by degranulation of IgE sensitised mast cells. We have also demonstrated that the activation of mast cells by IL-33 can play a role in experimental arthritis(548).

6.7 Conclusions

At the outset of this work there was only a single published report on IL-33 and very little information on the biological function of this novel cytokine. The data presented in this thesis is among the earliest work on IL-33. I have demonstrated that IL-33 administration induces pulmonary eosinophilia and AHR, that IL-33 is a potent activator of mast cells, and that ST2 is crucial in an adjuvant free mast cell dependent model of asthma, perhaps more akin to human asthma. These data position IL-33 as a central cytokine in allergic airways inflammation and hence a novel therapeutic target in clinical asthma.

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REFERENCES

1. Masoli M., Fabian D., Holt S., & Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 2004; 59: 469-478.

2. British Guideline on the Management of Asthma. *Thorax* 2008; 63 Suppl 4: iv1-121.

3. Bateman E.D., Hurd S.S., Barnes P.J., Bousquet J., Drazen J.M., FitzGerald M., Gibson P., Ohta K., O'Byrne P., Pedersen S.E., Pizzichini E., Sullivan S.D., Wenzel S.E., & Zar H.J. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008; 31: 143-178.

4. Dougherty R.H. & Fahy J.V. Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype. *Clin.Exp.Allergy* 2009; 39: 193-202.

5. Nicholson K.G., Kent J., & Ireland D.C. Respiratory viruses and exacerbations of asthma in adults. *BMJ* 1993; 307: 982-986.

6. Green R.H., Brightling C.E., McKenna S., Hargadon B., Parker D., Bradding P., Wardlaw A.J., & Pavord I.D. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002; 360: 1715-1721.

7. Jeffery P.K. Remodeling and inflammation of bronchi in asthma and chronic obstructive pulmonary disease. *Proc.Am Thorac.Soc.* 2004; 1: 176-183.

8. Peat J.K., Woolcock A.J., & Cullen K. Rate of decline of lung function in subjects with asthma. *Eur J Respir Dis.* 1987; 70: 171-179.

9. Lange P., Parner J., Vestbo J., Schnohr P., & Jensen G. A 15-year follow-up study of ventilatory function in adults with asthma. *N.Engl.J Med* 1998; 339: 1194-1200.

10. Park J.W., Hong Y.K., Kim C.W., Kim D.K., Choe K.O., & Hong C.S. High-resolution computed tomography in patients with bronchial asthma: correlation with clinical features, pulmonary functions and bronchial hyperresponsiveness. *J Investig.Allergol.Clin.Immunol.* 1997; 7: 186-192.

11. Niimi A., Matsumoto H., Amitani R., Nakano Y., Mishima M., Minakuchi M., Nishimura K., Itoh H., & Izumi T. Airway wall thickness in asthma assessed by computed tomography. Relation to clinical indices. *Am J Respir Crit Care Med* 2000; 162: 1518-1523.

12. Little S.A., Sproule M.W., Cowan M.D., Macleod K.J., Robertson M., Love J.G., Chalmers G.W., McSharry C.P., & Thomson N.C. High resolution computed tomographic assessment of airway wall thickness in chronic asthma: reproducibility and relationship with lung function and severity. *Thorax* 2002; 57: 247-253.

13. Aysola R.S., Hoffman E.A., Gierada D., Wenzel S., Cook-Granroth J., Tarsi J., Zheng J., Schechtman K.B., Ramkumar T.P., Cochran R., Xueping E., Christie C., Newell J., Fain S., Altes T.A., & Castro M. Airway remodeling measured by multidetector CT is increased in severe asthma and correlates with pathology. *Chest* 2008; 134: 1183-1191.

14. Chu H.W., Halliday J.L., Martin R.J., Leung D.Y., Szefler S.J., & Wenzel S.E. Collagen deposition in large airways may not differentiate severe asthma from milder forms of the disease. *Am J Respir Crit Care Med* 1998; 158: 1936-1944.

15. Bel E.H. Clinical phenotypes of asthma. *Curr.Opin.Pulm.Med* 2004; 10: 44-50.

16. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med* 2000; 162: 2341-2351.

17. Salter H.H. (1864) *On Asthma: Its Pathology and Treatment*, Blanchard & Lea, Philadelphia.

18. Humbert M., Menz G., Ying S., Corrigan C.J., Robinson D.S., Durham S.R., & Kay A.B. The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. *Immunol.Today* 1999; 20: 528-533.

19. Barnes P.J. Intrinsic asthma: not so different from allergic asthma but driven by superantigens? *Clin.Exp.Allergy* 2009; 39: 1145-1151.

20. Haldar P. & Pavord I.D. Noneosinophilic asthma: a distinct clinical and pathologic phenotype. *J Allergy Clin.Immunol*. 2007; 119: 1043-1052.

21. Lloyd C.M., Gonzalo J.A., Coyle A.J., & Gutierrez-Ramos J.C. Mouse models of allergic airway disease. *Adv.Immunol*. 2001; 77: 263-295.

22. Kips J.C., Anderson G.P., Fredberg J.J., Herz U., Inman M.D., Jordana M., Kemeny D.M., Lotvall J., Pauwels R.A., Plopper C.G., Schmidt D., Sterk P.J., Van Oosterhout A.J., Vargaftig B.B., & Chung K.F. Murine models of asthma. *Eur Respir J* 2003; 22: 374-382.

23. Finkelman F.D. & Wills-Karp M. Usefulness and optimization of mouse models of allergic airway disease. *J Allergy Clin.Immunol*. 2008; 121: 603-606.

24. van Rijt L.S., Kuipers H., Vos N., Hijdra D., Hoogsteden H.C., & Lambrecht B.N. A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *J Immunol.Methods* 2004; 288: 111-121.

25. Fisher C.E., Ahmad S.A., Fitch P.M., Lamb J.R., & Howie S.E. FITC-induced murine pulmonary inflammation: CC10 up-regulation and concurrent Shh expression. *Cell Biol.Int*. 2005; 29: 868-876.

26. Kearley J., Barker J.E., Robinson D.S., & Lloyd C.M. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+

regulatory T cells is interleukin 10 dependent. *J Exp.Med* 2005; 202: 1539-1547.

27. Martin T.R., Gerard N.P., Galli S.J., & Drazen J.M. Pulmonary responses to bronchoconstrictor agonists in the mouse. *J Appl.Physiol* 1988; 64: 2318-2323.

28. Glaab T., Taube C., Braun A., & Mitzner W. Invasive and noninvasive methods for studying pulmonary function in mice. *Respir Res*. 2007; 8: 63.

29. Hamelmann E., Schwarze J., Takeda K., Oshiba A., Larsen G.L., Irvin C.G., & Gelfand E.W. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am.J.Respir.Crit Care Med*. 1997; 156: 766-775.

30. Finkelman F.D. Use of unrestrained, single-chamber barometric plethysmography to evaluate sensitivity to cholinergic stimulation in mouse models of allergic airway disease. *J Allergy Clin.Immunol*. 2008; 121: 334-335.

31. Mangan N.E., van R.N., McKenzie A.N., & Fallon P.G. Helminth-modified pulmonary immune response protects mice from allergen-induced airway hyperresponsiveness. *J Immunol*. 2006; 176: 138-147.

32. Gonzalo J.A., Qiu Y., Lora J.M., Al-Garawi A., Villeval J.L., Boyce J.A., Martinez A., Marquez G., Goya I., Hamid Q., Fraser C.C., Picarella D., Cote-Sierra J., Hodge M.R., Gutierrez-Ramos J.C., Kolbeck R., & Coyle A.J. Coordinated involvement of mast cells and T cells in allergic mucosal inflammation: critical role of the CC chemokine ligand 1:CCR8 axis. *J Immunol*. 2007; 179: 1740-1750.

33. Nakae S., Ho L.H., Yu M., Monteforte R., Iikura M., Suto H., & Galli S.J. Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J Allergy Clin.Immunol*. 2007; 120: 48-55.

34. Bartlett N.W., Walton R.P., Edwards M.R., Aniscenko J., Caramori G., Zhu J., Glanville N., Choy K.J., Jourdan P., Burnet J., Tuthill T.J., Pedrick M.S., Hurle M.J., Plumpton C., Sharp N.A., Bussell J.N., Swallow D.M., Schwarze J., Guy B., Almond J.W., Jeffery P.K., Lloyd C.M., Papi A., Killington R.A., Rowlands D.J., Blair E.D., Clarke N.J., & Johnston S.L. Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. *Nat.Med* 2008; 14: 199-204.

35. McMillan S.J. & Lloyd C.M. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin.Exp.Allergy* 2004; 34: 497-507.

36. Kumar R.K., Herbert C., & Foster P.S. Expression of growth factors by airway epithelial cells in a model of chronic asthma: regulation and relationship to subepithelial fibrosis. *Clin.Exp.Allergy* 2004; 34: 567-575.

37. Yu M., Tsai M., Tam S.Y., Jones C., Zehnder J., & Galli S.J. Mast cells can promote the development of multiple features of chronic asthma in mice. *J Clin.Invest* 2006; 116: 1633-1641.

38. Johnson J.R., Wiley R.E., Fattouh R., Swirski F.K., Gajewska B.U., Coyle A.J., Gutierrez-Ramos J.C., Ellis R., Inman M.D., & Jordana M. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am.J Respir Crit Care Med.* 2004; 169: 378-385.

39. Grunig G., Corry D.B., Leach M.W., Seymour B.W., Kurup V.P., & Rennick D.M. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. *J Exp.Med* 1997; 185: 1089-1099.

40. Lukacs N.W., Strieter R.M., Chensue S.W., & Kunkel S.L. Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *Am J Respir Cell Mol.Biol.* 1994; 10: 526-532.

41. Campbell E.M., Kunkel S.L., Strieter R.M., & Lukacs N.W. Temporal role of chemokines in a murine model of cockroach allergen-induced airway hyperreactivity and eosinophilia. *J Immunol.* 1998; 161: 7047-7053.

42. Coyle A.J., Wagner K., Bertrand C., Tsuyuki S., Bews J., & Heusser C. Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody. *J Exp.Med* 1996; 183: 1303-1310.

43. John R.J., Rusznak C., Ramjee M., Lamont A.G., Abrahamson M., & Hewitt E.L. Functional effects of the inhibition of the cysteine protease activity of the major house dust mite allergen Der p 1 by a novel peptide-based inhibitor. *Clin.Exp.Allergy* 2000; 30: 784-793.

44. Hammad H., Chieppa M., Perros F., Willart M.A., Germain R.N., & Lambrecht B.N. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat.Med* 2009; 15: 410-416.

45. Glenny A.T., Pope C.G., Waddington U., & Wallace H. Immunological notes, XXIII. The antigenic value of toxoid precipitated by potassium alum. *J Path Bact* 1926; 29: 38-39.

46. Wilson-Welder J.H., Torres M.P., Kipper M.J., Mallapragada S.K., Wannemuehler M.J., & Narasimhan B. Vaccine adjuvants: current challenges and future approaches. *J Pharm.Sci* 2009; 98: 1278-1316.

47. Grun J.L. & Maurer P.H. Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. *Cell Immunol.* 1989; 121: 134-145.

48. Brewer J.M., Conacher M., Satoskar A., Bluethmann H., & Alexander J. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production. *Eur J Immunol.* 1996; 26: 2062-2066.

49. Stock P., Akbari O., Berry G., Freeman G.J., DeKruyff R.H., & Umetsu D.T. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat.Immunol* 2004.

50. Zhang Y., Lamm W.J., Albert R.K., Chi E.Y., Henderson W.R., Jr., & Lewis D.B. Influence of the route of allergen administration and genetic background on the murine allergic pulmonary response. *Am J Respir Crit Care Med* 1997; 155: 661-669.

51. Brewer J.P., Kisselgof A.B., & Martin T.R. Genetic variability in pulmonary physiological, cellular, and antibody responses to antigen in mice. *Am J Respir Crit Care Med* 1999; 160: 1150-1156.

52. Takeda K., Haczku A., Lee J.J., Irvin C.G., & Gelfand E.W. Strain dependence of airway hyperresponsiveness reflects differences in eosinophil localization in the lung. *Am J Physiol Lung Cell Mol.Physiol* 2001; 281: L394-L402.

53. Drazen J.M., Finn P.W., & De Sanctis G.T. Mouse models of airway responsiveness: physiological basis of observed outcomes and analysis of selected examples using these outcome indicators. *Annu.Rev.Physiol* 1999; 61: 593-625.

54. Corry D.B., Folkesson H.G., Warnock M.L., Erle D.J., Matthay M.A., Wiener-Kronish J.P., & Locksley R.M. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J Exp.Med.* 1996; 183: 109-117.

55. Drazen J.M., Arm J.P., & Austen K.F. Sorting out the cytokines of asthma. *J Exp.Med* 1996; 183: 1-5.

56. Ghildyal N., Friend D.S., Freelund R., Austen K.F., McNeil H.P., Schiller V., & Stevens R.L. Lack of expression of the tryptase mouse mast cell protease 7 in mast cells of the C57BL/6J mouse. *J Immunol.* 1994; 153: 2624-2630.

57. Ellis A.G. The pathological anatomy of bronchial asthma. *Am J Med Sci* 1908; 136: 407-429.

58. Durham S.R. & Kay A.B. Eosinophils, bronchial hyperreactivity and late-phase asthmatic reactions. *Clin.Allergy* 1985; 15: 411-418.

59. Taylor K.J. & Luksza A.R. Peripheral blood eosinophil counts and bronchial responsiveness. *Thorax* 1987; 42: 452-456.

60. Horn B.R., Robin E.D., Theodore J., & Van K.A. Total eosinophil counts in the management of bronchial asthma. *N.Engl.J Med.* 1975; 292: 1152-1155.

61. Petsky H.L., Kynaston J.A., Turner C., Li A.M., Cates C.J., Lasserson T.J., & Chang A.B. Tailored interventions based on sputum eosinophils versus clinical symptoms for asthma in children and adults. *Cochrane.Database.Syst.Rev.* 2007; CD005603.

62. Rankin J.A., Snyder P.E., Schachter E.N., & Matthay R.A. Bronchoalveolar lavage. Its safety in subjects with mild asthma. *Chest* 1984; 85: 723-728.

63. Godard P., Chaintreuil J., Damon M., Coupe M., Flandre O., Crastes de P.A., & Michel F.B. Functional assessment of alveolar macrophages:

comparison of cells from asthmatics and normal subjects. *J Allergy Clin.Immunol*. 1982; 70: 88-93.

64. Flint K.C., Leung K.B., Hudspith B.N., Brostoff J., Pearce F.L., & Johnson N.M. Bronchoalveolar mast cells in extrinsic asthma: a mechanism for the initiation of antigen specific bronchoconstriction. *Br.Med.J (Clin.Res.Ed)* 1985; 291: 923-926.

65. Kirby J.G., Hargreave F.E., Gleich G.J., & O'Byrne P.M. Bronchoalveolar cell profiles of asthmatic and nonasthmatic subjects. *Am.Rev.Respir Dis.* 1987; 136: 379-383.

66. Wardlaw A.J., Dunnette S., Gleich G.J., Collins J.V., & Kay A.B. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am.Rev.Respir Dis.* 1988; 137: 62-69.

67. Walker C., Kaegi M.K., Braun P., & Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin.Immunol*. 1991; 88: 935-942.

68. Bousquet J., Chanez P., Lacoste J.Y., Barneon G., Ghavanian N., Enander I., Venge P., Ahlstedt S., Simony-Lafontaine J., Godard P., & . Eosinophilic inflammation in asthma. *N.Engl.J Med*. 1990; 323: 1033-1039.

69. Laitinen L.A., Heino M., Laitinen A., Kava T., & Haahtela T. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am.Rev.Respir Dis.* 1985; 131: 599-606.

70. Beasley R., Roche W.R., Roberts J.A., & Holgate S.T. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am.Rev.Respir Dis.* 1989; 139: 806-817.

71. Azzawi M., Bradley B., Jeffery P.K., Frew A.J., Wardlaw A.J., Knowles G., Assoufi B., Collins J.V., Durham S., & Kay A.B. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am.Rev.Respir Dis.* 1990; 142: 1407-1413.

72. Bradley B.L., Azzawi M., Jacobson M., Assoufi B., Collins J.V., Irani A.M., Schwartz L.B., Durham S.R., Jeffery P.K., & Kay A.B. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin.Immunol*. 1991; 88: 661-674.

73. Gibson P.G., Dolovich J., Denburg J., Ramsdale E.H., & Hargreave F.E. Chronic cough: eosinophilic bronchitis without asthma. *Lancet* 1989; 1: 1346-1348.

74. Rothenberg M.E. & Hogan S.P. The eosinophil. *Annu.Rev.Immunol*. 2006; 24: 147-174.

75. Frigas E., Loegering D.A., Solley G.O., Farrow G.M., & Gleich G.J. Elevated levels of the eosinophil granule major basic protein in the sputum of patients with bronchial asthma. *Mayo Clin.Proc.* 1981; 56: 345-353.

76. Filley W.V., Holley K.E., Kephart G.M., & Gleich G.J. Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet* 1982; 2: 11-16.

77. Gleich G.J., Frigas E., Loegering D.A., Wassom D.L., & Steinmuller D. Cytotoxic properties of the eosinophil major basic protein. *J Immunol.* 1979; 123: 2925-2927.

78. Flavahan N.A., Slifman N.R., Gleich G.J., & Vanhoutte P.M. Human eosinophil major basic protein causes hyperreactivity of respiratory smooth muscle. Role of the epithelium. *Am.Rev.Respir Dis.* 1988; 138: 685-688.

79. O'Donnell M.C., Ackerman S.J., Gleich G.J., & Thomas L.L. Activation of basophil and mast cell histamine release by eosinophil granule major basic protein. *J Exp.Med.* 1983; 157: 1981-1991.

80. Zheutlin L.M., Ackerman S.J., Gleich G.J., & Thomas L.L. Stimulation of basophil and rat mast cell histamine release by eosinophil granule-derived cationic proteins. *J Immunol.* 1984; 133: 2180-2185.

81. Piliponsky A.M., Pickholtz D., Gleich G.J., & Levi-Schaffer F. Human eosinophils induce histamine release from antigen-activated rat peritoneal mast cells: a possible role for mast cells in late-phase allergic reactions. *J Allergy Clin.Immunol.* 2001; 107: 993-1000.

82. Piliponsky A.M., Gleich G.J., Nagler A., Bar I., & Levi-Schaffer F. Non-IgE-dependent activation of human lung- and cord blood-derived mast cells is induced by eosinophil major basic protein and modulated by the membrane form of stem cell factor. *Blood* 2003; 101: 1898-1904.

83. Denzler K.L., Farmer S.C., Crosby J.R., Borchers M., Cieslewicz G., Larson K.A., Cormier-Regard S., Lee N.A., & Lee J.J. Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma. *J Immunol.* 2000; 165: 5509-5517.

84. Broide D.H., Paine M.M., & Firestein G.S. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J Clin.Invest* 1992; 90: 1414-1424.

85. Moqbel R., Ying S., Barkans J., Newman T.M., Kimmitt P., Wakelin M., Taborda-Barata L., Meng Q., Corrigan C.J., Durham S.R., & Kay A.B. Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. *J Immunol.* 1995; 155: 4939-4947.

86. Woerly G., Roger N., Loiseau S., & Capron M. Expression of Th1 and Th2 immunoregulatory cytokines by human eosinophils. *Int.Arch.Allergy Immunol.* 1999; 118: 95-97.

87. MacKenzie J.R., Mattes J., Dent L.A., & Foster P.S. Eosinophils promote allergic disease of the lung by regulating CD4(+) Th2 lymphocyte function. *J Immunol*. 2001; 167: 3146-3155.

88. Odemuyiwa S.O., Ghahary A., Li Y., Puttagunta L., Lee J.E., Musat-Marcu S., Ghahary A., & Moqbel R. Cutting edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase. *J Immunol*. 2004; 173: 5909-5913.

89. Sanderson C.J., Warren D.J., & Strath M. Identification of a lymphokine that stimulates eosinophil differentiation in vitro. Its relationship to interleukin 3, and functional properties of eosinophils produced in cultures. *J Exp.Med*. 1985; 162: 60-74.

90. Lopez A.F., Begley C.G., Williamson D.J., Warren D.J., Vadas M.A., & Sanderson C.J. Murine eosinophil differentiation factor. An eosinophil-specific colony-stimulating factor with activity for human cells. *J Exp.Med*. 1986; 163: 1085-1099.

91. Wang J.M., Rambaldi A., Biondi A., Chen Z.G., Sanderson C.J., & Mantovani A. Recombinant human interleukin 5 is a selective eosinophil chemoattractant. *Eur J Immunol*. 1989; 19: 701-705.

92. Dent L.A., Strath M., Mellor A.L., & Sanderson C.J. Eosinophilia in transgenic mice expressing interleukin 5. *J Exp.Med*. 1990; 172: 1425-1431.

93. Lee J.J., McGarry M.P., Farmer S.C., Denzler K.L., Larson K.A., Carrigan P.E., Brenneise I.E., Horton M.A., Haczku A., Gelfand E.W., Leikauf G.D., & Lee N.A. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp.Med*. 1997; 185: 2143-2156.

94. Foster P.S., Hogan S.P., Ramsay A.J., Matthaei K.I., & Young I.G. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp.Med*. 1996; 183: 195-201.

95. Corry D.B., Grunig G., Hadeiba H., Kurup V.P., Warnock M.L., Sheppard D., Rennick D.M., & Locksley R.M. Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Mol.Med*. 1998; 4: 344-355.

96. Mathur M., Herrmann K., Li X., Qin Y., Weinstock J., Elliott D., Monahan J., & Padrid P. TRFK-5 reverses established airway eosinophilia but not established hyperresponsiveness in a murine model of chronic asthma. *Am.J Respir Crit Care Med*. 1999; 159: 580-587.

97. Shen H.H., Ochkur S.I., McGarry M.P., Crosby J.R., Hines E.M., Borchers M.T., Wang H., Biechelle T.L., O'Neill K.R., Ansay T.L., Colbert D.C., Cormier S.A., Justice J.P., Lee N.A., & Lee J.J. A causative relationship exists between eosinophils and the development of allergic pulmonary pathologies in the mouse. *J Immunol*. 2003; 170: 3296-3305.

98. Hogan S.P., Koskinen A., & Foster P.S. Interleukin-5 and eosinophils induce airway damage and bronchial hyperreactivity during allergic airway inflammation in BALB/c mice. *Immunol.Cell Biol.* 1997; 75: 284-288.

99. Hamelmann E., Oshiba A., Loader J., Larsen G.L., Gleich G., Lee J., & Gelfand E.W. Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *Am J Respir Crit Care Med* 1997; 155: 819-825.

100. Hogan S.P., Matthaei K.I., Young J.M., Koskinen A., Young I.G., & Foster P.S. A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. *J Immunol.* 1998; 161: 1501-1509.

101. Hamelmann E., Cieslewicz G., Schwarze J., Ishizuka T., Joetham A., Heusser C., & Gelfand E.W. Anti-interleukin 5 but not anti-IgE prevents airway inflammation and airway hyperresponsiveness. *Am.J Respir Crit Care Med.* 1999; 160: 934-941.

102. Blyth D.I., Wharton T.F., Pedrick M.S., Savage T.J., & Sanjar S. Airway subepithelial fibrosis in a murine model of atopic asthma: suppression by dexamethasone or anti-interleukin-5 antibody. *Am.J Respir Cell Mol.Biol.* 2000; 23: 241-246.

103. Mattes J., Yang M., Mahalingam S., Kuehr J., Webb D.C., Simson L., Hogan S.P., Koskinen A., McKenzie A.N., Dent L.A., Rothenberg M.E., Matthaei K.I., Young I.G., & Foster P.S. Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. *J Exp.Med.* 2002; 195: 1433-1444.

104. Hamelmann E., Takeda K., Schwarze J., Vella A.T., Irvin C.G., & Gelfand E.W. Development of eosinophilic airway inflammation and airway hyperresponsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *Am.J Respir Cell Mol.Biol.* 1999; 21: 480-489.

105. Shi H.Z., Xiao C.Q., Zhong D., Qin S.M., Liu Y., Liang G.R., Xu H., Chen Y.Q., Long X.M., & Xie Z.F. Effect of inhaled interleukin-5 on airway hyperreactivity and eosinophilia in asthmatics. *Am.J Respir Crit Care Med.* 1998; 157: 204-209.

106. Leckie M.J., ten Brinke A., Khan J., Diamant Z., O'Connor B.J., Walls C.M., Mathur A.K., Cowley H.C., Chung K.F., Djukanovic R., Hansel T.T., Holgate S.T., Sterk P.J., & Barnes P.J. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000; 356: 2144-2148.

107. O'Byrne P.M., Inman M.D., & Parameswaran K. The trials and tribulations of IL-5, eosinophils, and allergic asthma. *J Allergy Clin.Immunol.* 2001; 108: 503-508.

108. Flood-Page P.T., Menzies-Gow A.N., Kay A.B., & Robinson D.S. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am.J Respir Crit Care Med.* 2003; 167: 199-204.

109. Flood-Page P., Menzies-Gow A., Phipps S., Ying S., Wangoo A., Ludwig M.S., Barnes N., Robinson D., & Kay A.B. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin.Invest* 2003; 112: 1029-1036.

110. Flood-Page P., Swenson C., Faiferman I., Matthews J., Williams M., Brannick L., Robinson D., Wenzel S., Busse W., Hansel T.T., & Barnes N.C. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *Am.J Respir Crit Care Med.* 2007; 176: 1062-1071.

111. Haldar P., Brightling C.E., Hargadon B., Gupta S., Monteiro W., Sousa A., Marshall R.P., Bradding P., Green R.H., Wardlaw A.J., & Pavord I.D. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N.Engl.J Med* 2009; 360: 973-984.

112. Nair P., Pizzichini M.M., Kjarsgaard M., Inman M.D., Efthimiadis A., Pizzichini E., Hargreave F.E., & O'Byrne P.M. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N.Engl.J Med* 2009; 360: 985-993.

113. Lee J.J., Dimina D., Macias M.P., Ochkur S.I., McGarry M.P., O'Neill K.R., Protheroe C., Pero R., Nguyen T., Cormier S.A., Lenkiewicz E., Colbert D., Rinaldi L., Ackerman S.J., Irvin C.G., & Lee N.A. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 2004; 305: 1773-1776.

114. Yu C., Cantor A.B., Yang H., Browne C., Wells R.A., Fujiwara Y., & Orkin S.H. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. *J Exp.Med.* 2002; 195: 1387-1395.

115. Humbles A.A., Lloyd C.M., McMillan S.J., Friend D.S., Xanthou G., McKenna E.E., Ghiran S., Gerard N.P., Yu C., Orkin S.H., & Gerard C. A critical role for eosinophils in allergic airways remodeling. *Science* 2004; 305: 1776-1779.

116. Walsh E.R., Sahu N., Kearley J., Benjamin E., Kang B.H., Humbles A., & August A. Strain-specific requirement for eosinophils in the recruitment of T cells to the lung during the development of allergic asthma. *J Exp.Med.* 2008; 205: 1285-1292.

117. Vaughan W.T. The Biologic Aspects of Hay Fever and Asthma. *Science* 1928; 68: 1-6.

118. Prausnitz C. & Küstner H. Studien über die Überempfindlichkeit. *Zbl Bakt Parasits Infect I Abt Orig* 1921; 86: 160-169.

119. Ishizaka K., Ishizaka T., & Hornbrook M.M. Physico-chemical properties of human reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. *J Immunol.* 1966; 97: 75-85.

120. Gould H.J. & Sutton B.J. IgE in allergy and asthma today. *Nat.Rev.Immunol.* 2008; 8: 205-217.

121. Johansson S.G. Raised levels of a new immunoglobulin class (IgND) in asthma. *Lancet* 1967; 2: 951-953.

122. Martin T.R., Takeishi T., Katz H.R., Austen K.F., Drazen J.M., & Galli S.J. Mast cell activation enhances airway responsiveness to methacholine in the mouse. *J Clin.Invest* 1993; 91: 1176-1182.

123. Oshiba A., Hamelmann E., Takeda K., Bradley K.L., Loader J.E., Larsen G.L., & Gelfand E.W. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *J Clin.Invest* 1996; 97: 1398-1408.

124. Lloyd C.M., Gonzalo J.A., Nguyen T., Delaney T., Tian J., Oettgen H., Coyle A.J., & Gutierrez-Ramos J.C. Resolution of bronchial hyperresponsiveness and pulmonary inflammation is associated with IL-3 and tissue leukocyte apoptosis. *J Immunol*. 2001; 166: 2033-2040.

125. Taube C., Wei X., Swasey C.H., Joetham A., Zarini S., Lively T., Takeda K., Loader J., Miyahara N., Kodama T., Shultz L.D., Donaldson D.D., Hamelmann E.H., Dakhama A., & Gelfand E.W. Mast cells, Fc epsilon RI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. *J Immunol*. 2004; 172: 6398-6406.

126. Fahy J.V., Fleming H.E., Wong H.H., Liu J.T., Su J.Q., Reimann J., Fick R.B., Jr., & Boushey H.A. The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am J Respir Crit Care Med* 1997; 155: 1828-1834.

127. Boulet L.P., Chapman K.R., Cote J., Kalra S., Bhagat R., Swystun V.A., Laviolette M., Cleland L.D., Deschesnes F., Su J.Q., DeVault A., Fick R.B., Jr., & Cockcroft D.W. Inhibitory effects of an anti-IgE antibody E25 on allergen-induced early asthmatic response. *Am J Respir Crit Care Med* 1997; 155: 1835-1840.

128. Milgrom H., Fick R.B., Jr., Su J.Q., Reimann J.D., Bush R.K., Watrous M.L., & Metzger W.J. Treatment of allergic asthma with monoclonal anti-IgE antibody. rhuMAb-E25 Study Group. *N.Engl.J Med* 1999; 341: 1966-1973.

129. Busse W., Corren J., Lanier B.Q., McAlary M., Fowler-Taylor A., Cioppa G.D., van A.A., & Gupta N. Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. *J Allergy Clin.Immunol*. 2001; 108: 184-190.

130. Soler M., Matz J., Townley R., Buhl R., O'Brien J., Fox H., Thirlwell J., Gupta N., & Della C.G. The anti-IgE antibody omalizumab reduces exacerbations and steroid requirement in allergic asthmatics. *Eur Respir J* 2001; 18: 254-261.

131. Holgate S.T., Chuchalin A.G., Hebert J., Lotvall J., Persson G.B., Chung K.F., Bousquet J., Kerstjens H.A., Fox H., Thirlwell J., & Cioppa G.D. Efficacy and safety of a recombinant anti-immunoglobulin E antibody (omalizumab) in severe allergic asthma. *Clin.Exp.Allergy* 2004; 34: 632-638.

132. Humbert M., Beasley R., Ayres J., Slavin R., Hebert J., Bousquet J., Beeh K.M., Ramos S., Canonica G.W., Hedgecock S., Fox H., Blogg M., & Surrey K. Benefits of omalizumab as add-on therapy in patients with severe persistent asthma who are inadequately controlled despite best available therapy (GINA 2002 step 4 treatment): INNOVATE. *Allergy* 2005; 60: 309-316.

133. National Institute for Health and Clinical Excellence. Omalizumab for severe persistent allergic asthma. NICE technology appraisal guidance 133. 2007. London.
Ref Type: Report

134. Hamelmann E., Vella A.T., Oshiba A., Kappler J.W., Marrack P., & Gelfand E.W. Allergic airway sensitization induces T cell activation but not airway hyperresponsiveness in B cell-deficient mice. *Proc.Natl.Acad.Sci U.S.A* 1997; 94: 1350-1355.

135. Korsgren M., Erjefalt J.S., Korsgren O., Sundler F., & Persson C.G. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J Exp.Med* 1997; 185: 885-892.

136. MacLean J.A., Sauty A., Luster A.D., Drazen J.M., & De Sanctis G.T. Antigen-induced airway hyperresponsiveness, pulmonary eosinophilia, and chemokine expression in B cell-deficient mice. *Am J Respir Cell Mol.Biol.* 1999; 20: 379-387.

137. Ehrlich, P. Beiträge zur Theorie und Praxis der Histologischen Färbung. 1878. Leipzig University.
Ref Type: Thesis/Dissertation

138. Dale H.H. & Laidlaw P.P. The physiological action of beta-iminazolylethylamine. *J Physiol* 1910; 41: 318-344.

139. Weiss S., Robb G.P., & Blumgart H.L. The velocity of blood flow in health and disease as measured by the effect of histamine on the minute vessels. *American Heart Journal* 1929; 4: 664-691.

140. RILEY J.F. & WEST G.B. Histamine in tissue mast cells. *J Physiol* 1952; 117: 72P-73P.

141. RILEY J.F. & WEST G.B. The presence of histamine in tissue mast cells. *J Physiol* 1953; 120: 528-537.

142. Tomioka M., Ida S., Shindoh Y., Ishihara T., & Takishima T. Mast cells in bronchoalveolar lumen of patients with bronchial asthma. *Am Rev.Respir Dis.* 1984; 129: 1000-1005.

143. Jeffery P.K., Wardlaw A.J., Nelson F.C., Collins J.V., & Kay A.B. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am.Rev.Respir Dis.* 1989; 140: 1745-1753.

144. Adelroth E., Rosenhall L., Johansson S.A., Linden M., & Venge P. Inflammatory cells and eosinophilic activity in asthmatics investigated by bronchoalveolar lavage. The effects of antiasthmatic treatment with budesonide or terbutaline. *Am Rev.Respir Dis.* 1990; 142: 91-99.

145. Pesci A., Foresi A., Bertorelli G., Chetta A., & Olivieri D. Histochemical characteristics and degranulation of mast cells in epithelium and lamina propria of bronchial biopsies from asthmatic and normal subjects. *Am Rev.Respir Dis.* 1993; 147: 684-689.

146. Laitinen L.A., Laitinen A., & Haahtela T. Airway mucosal inflammation even in patients with newly diagnosed asthma. *Am Rev.Respir Dis.* 1993; 147: 697-704.

147. Gibson P.G., Allen C.J., Yang J.P., Wong B.J., Dolovich J., Denburg J., & Hargreave F.E. Intraepithelial mast cells in allergic and nonallergic asthma. Assessment using bronchial brushings. *Am Rev.Respir Dis.* 1993; 148: 80-86.

148. Djukanovic R., Wilson J.W., Britten K.M., Wilson S.J., Walls A.F., Roche W.R., Howarth P.H., & Holgate S.T. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev.Respir Dis.* 1990; 142: 863-871.

149. Vignola A.M., Chanez P., Campbell A.M., Souques F., Lebel B., Enander I., & Bousquet J. Airway inflammation in mild intermittent and in persistent asthma. *Am J Respir Crit Care Med* 1998; 157: 403-409.

150. Salvato G. Some histological changes in chronic bronchitis and asthma. *Thorax* 1968; 23: 168-172.

151. Brightling C.E., Bradding P., Symon F.A., Holgate S.T., Wardlaw A.J., & Pavord I.D. Mast-cell infiltration of airway smooth muscle in asthma. *N.Engl.J Med* 2002; 346: 1699-1705.

152. Carroll N.G., Mutavdzic S., & James A.L. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. *Eur Respir J* 2002; 19: 879-885.

153. Ammit A.J., Bekir S.S., Johnson P.R., Hughes J.M., Armour C.L., & Black J.L. Mast cell numbers are increased in the smooth muscle of human sensitized isolated bronchi. *Am J Respir Crit Care Med* 1997; 155: 1123-1129.

154. Broide D.H., Gleich G.J., Cuomo A.J., Coburn D.A., Federman E.C., Schwartz L.B., & Wasserman S.I. Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *J Allergy Clin.Immunol.* 1991; 88: 637-648.

155. Nogami M., Suko M., Okudaira H., Miyamoto T., Shiga J., Ito M., & Kasuya S. Experimental pulmonary eosinophilia in mice by Ascaris suum extract. *Am Rev.Respir Dis.* 1990; 141: 1289-1295.

156. Brusselle G.G., Kips J.C., Tavernier J.H., van der Heyden J.G., Cuvelier C.A., Pauwels R.A., & Bluethmann H. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin.Exp.Allergy* 1994; 24: 73-80.

157. Takeda K., Hamelmann E., Joetham A., Shultz L.D., Larsen G.L., Irvin C.G., & Gelfand E.W. Development of eosinophilic airway inflammation

and airway hyperresponsiveness in mast cell-deficient mice. *J Exp.Med* 1997; 186: 449-454.

158. Kobayashi T., Miura T., Haba T., Sato M., Serizawa I., Nagai H., & Ishizaka K. An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *J Immunol*. 2000; 164: 3855-3861.

159. Kung T.T., Stelts D., Zurcher J.A., Jones H., Umland S.P., Kreutner W., Egan R.W., & Chapman R.W. Mast cells modulate allergic pulmonary eosinophilia in mice. *Am J Respir Cell Mol.Biol*. 1995; 12: 404-409.

160. Ogawa K., Kaminuma O., Kikkawa H., Nakata A., Asahina M., Egan R.W., Akiyama K., & Mori A. Transient contribution of mast cells to pulmonary eosinophilia but not to hyper-responsiveness. *Clin.Exp.Allergy* 2002; 32: 140-148.

161. Kim Y.S., Ko H.M., Kang N.I., Song C.H., Zhang X., Chung W.C., Kim J.H., Choi I.H., Park Y.M., Kim G.Y., Im S.Y., & Lee H.K. Mast cells play a key role in the development of late airway hyperresponsiveness through TNF-alpha in a murine model of asthma. *Eur J Immunol*. 2007; 37: 1107-1115.

162. Williams C.M. & Galli S.J. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J Exp.Med* 2000; 192: 455-462.

163. Reuter S., Heinz A., Sieren M., Wiewrodt R., Gelfand E.W., Stassen M., Buhl R., & Taube C. Mast cell-derived tumour necrosis factor is essential for allergic airway disease. *Eur Respir J* 2008; 31: 773-782.

164. Miyajima A., Miyatake S., Schreurs J., De V.J., Arai N., Yokota T., & Arai K. Coordinate regulation of immune and inflammatory responses by T cell-derived lymphokines. *FASEB J* 1988; 2: 2462-2473.

165. Mosmann T.R., Cherwinski H., Bond M.W., Giedlin M.A., & Coffman R.L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986; 136: 2348-2357.

166. Ohashi Y., Motojima S., Fukuda T., & Makino S. Airway hyperresponsiveness, increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma. *Am Rev.Respir Dis.* 1992; 145: 1469-1476.

167. Gerblich A.A., Salik H., & Schuyler M.R. Dynamic T-cell changes in peripheral blood and bronchoalveolar lavage after antigen bronchoprovocation in asthmatics. *Am Rev.Respir Dis.* 1991; 143: 533-537.

168. Corrigan C.J., Hartnell A., & Kay A.B. T lymphocyte activation in acute severe asthma. *Lancet* 1988; 1: 1129-1132.

169. Wilson J.W., Djukanovic R., Howarth P.H., & Holgate S.T. Lymphocyte activation in bronchoalveolar lavage and peripheral blood in atopic asthma. *Am Rev.Respir Dis.* 1992; 145: 958-960.

170. Walker C., Bode E., Boer L., Hansel T.T., Blaser K., & Virchow J.C., Jr. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am.Rev.Respir.Dis.* 1992; 146: 109-115.

171. Robinson D., Hamid Q., Bentley A., Ying S., Kay A.B., & Durham S.R. Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin.Immunol.* 1993; 92: 313-324.

172. Walker C., Virchow J.C., Jr., Bruijnzeel P.L., & Blaser K. T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. *J Immunol.* 1991; 146: 1829-1835.

173. Kon O.M., Sihra B.S., Compton C.H., Leonard T.B., Kay A.B., & Barnes N.C. Randomised, dose-ranging, placebo-controlled study of chimeric antibody to CD4 (keliximab) in chronic severe asthma. *Lancet* 1998; 352: 1109-1113.

174. Gavett S.H., Chen X., Finkelman F., & Wills-Karp M. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol.Biol.* 1994; 10: 587-593.

175. Foster P.S., Yang M., Herbert C., & Kumar R.K. CD4(+) T-lymphocytes regulate airway remodeling and hyper-reactivity in a mouse model of chronic asthma. *Lab Invest* 2002; 82: 455-462.

176. Komai M., Tanaka H., Masuda T., Nagao K., Ishizaki M., Sawada M., & Nagai H. Role of Th2 responses in the development of allergen-induced airway remodelling in a murine model of allergic asthma. *Br.J Pharmacol.* 2003; 138: 912-920.

177. Leigh R., Southam D.S., Ellis R., Wattie J.N., Sehmi R., Wan Y., & Inman M.D. T-cell-mediated inflammation does not contribute to the maintenance of airway dysfunction in mice. *J Appl.Physiol* 2004; 97: 2258-2265.

178. Doherty T.A., Soroosh P., Broide D.H., & Croft M. CD4+ cells are required for chronic eosinophilic lung inflammation but not airway remodeling. *Am J Physiol Lung Cell Mol.Physiol* 2009; 296: L229-L235.

179. Gonzalo J.A., Lloyd C.M., Kremer L., Finger E., Martinez A., Siegelman M.H., Cybulsky M., & Gutierrez-Ramos J.C. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin.Invest* 1996; 98: 2332-2345.

180. Hansen G., Berry G., DeKruyff R.H., & Umetsu D.T. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin.Invest* 1999; 103: 175-183.

181. Randolph D.A., Stephens R., Carruthers C.J., & Chaplin D.D. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J.Clin.Invest* 1999; 104: 1021-1029.

182. Hamelmann E., Oshiba A., Paluh J., Bradley K., Loader J., Potter T.A., Larsen G.L., & Gelfand E.W. Requirement for CD8+ T cells in the development of airway hyperresponsiveness in a marine model of airway sensitization. *J Exp.Med* 1996; 183: 1719-1729.

183. Wierenga E.A., Snoek M., de G.C., Chretien I., Bos J.D., Jansen H.M., & Kapsenberg M.L. Evidence for compartmentalization of functional subsets of CD2+ T lymphocytes in atopic patients. *J Immunol*. 1990; 144: 4651-4656.

184. Ying S., Durham S.R., Corrigan C.J., Hamid Q., & Kay A.B. Phenotype of cells expressing mRNA for TH2-type (interleukin 4 and interleukin 5) and TH1-type (interleukin 2 and interferon gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal control subjects. *Am.J Respir Cell Mol.Biol*. 1995; 12: 477-487.

185. Robinson D.S., Hamid Q., Ying S., Tsicopoulos A., Barkans J., Bentley A.M., Corrigan C., Durham S.R., & Kay A.B. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N.Engl.J.Med*. 1992; 326: 298-304.

186. Del Prete G.F., De C.M., D'Elios M.M., Maestrelli P., Ricci M., Fabbri L., & Romagnani S. Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol*. 1993; 23: 1445-1449.

187. Humbert M., Durham S.R., Ying S., Kimmitt P., Barkans J., Assoufi B., Pfister R., Menz G., Robinson D.S., Kay A.B., & Corrigan C.J. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity. *Am J Respir Crit Care Med* 1996; 154: 1497-1504.

188. Humbert M., Durham S.R., Kimmitt P., Powell N., Assoufi B., Pfister R., Menz G., Kay A.B., & Corrigan C.J. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J Allergy Clin.Immunol*. 1997; 99: 657-665.

189. Gelder C.M., Thomas P.S., Yates D.H., Adcock I.M., Morrison J.F., & Barnes P.J. Cytokine expression in normal, atopic, and asthmatic subjects using the combination of sputum induction and the polymerase chain reaction. *Thorax* 1995; 50: 1033-1037.

190. Komai-Koma M., McKay A., Thomson L., McSharry C., Chalmers G.W., Liew F.Y., & Thomson N.C. Immuno-regulatory cytokines in asthma: IL-15 and IL-13 in induced sputum. *Clin.Exp.Allergy* 2001; 31: 1441-1448.

191. Robinson D.S., Ying S., Bentley A.M., Meng Q., North J., Durham S.R., Kay A.B., & Hamid Q. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J Allergy Clin.Immunol*. 1993; 92: 397-403.

192. Shimoda K., van D.J., Sangster M.Y., Sarawar S.R., Carson R.T., Tripp R.A., Chu C., Quelle F.W., Nosaka T., Vignali D.A., Doherty P.C., Grosveld G., Paul W.E., & Ihle J.N. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 1996; 380: 630-633.

193. Zheng W. & Flavell R.A. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997; 89: 587-596.

194. Nakamura Y., Ghaffar O., Olivenstein R., Taha R.A., Soussi-Gounni A., Zhang D.H., Ray A., & Hamid Q. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. *J Allergy Clin.Immunol.* 1999; 103: 215-222.

195. Christodoulopoulos P., Cameron L., Nakamura Y., Lemiere C., Muro S., Dugas M., Boulet L.P., Laviolette M., Olivenstein R., & Hamid Q. TH2 cytokine-associated transcription factors in atopic and nonatopic asthma: evidence for differential signal transducer and activator of transcription 6 expression. *J Allergy Clin.Immunol.* 2001; 107: 586-591.

196. Szabo S.J., Kim S.T., Costa G.L., Zhang X., Fathman C.G., & Glimcher L.H. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000; 100: 655-669.

197. Finotto S., Neurath M.F., Glickman J.N., Qin S., Lehr H.A., Green F.H., Ackerman K., Haley K., Galle P.R., Szabo S.J., Drazen J.M., De Sanctis G.T., & Glimcher L.H. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 2002; 295: 336-338.

198. Cohn L., Homer R.J., Marinov A., Rankin J., & Bottomly K. Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp.Med* 1997; 186: 1737-1747.

199. Cohn L., Tepper J.S., & Bottomly K. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J Immunol.* 1998; 161: 3813-3816.

200. Mowen K.A. & Glimcher L.H. Signaling pathways in Th2 development. *Immunol.Rev.* 2004; 202: 203-222.

201. Fallon P.G. & Mangan N.E. Suppression of TH2-type allergic reactions by helminth infection. *Nat.Rev.Immunol.* 2007; 7: 220-230.

202. Noma Y., Sideras P., Naito T., Bergstedt-Lindquist S., Azuma C., Severinson E., Tanabe T., Kinashi T., Matsuda F., Yaoita Y., & . Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter. *Nature* 1986; 319: 640-646.

203. Isakson P.C., Pure E., Vitetta E.S., & Krammer P.H. T cell-derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J Exp.Med* 1982; 155: 734-748.

204. Coffman R.L., Ohara J., Bond M.W., Carty J., Zlotnik A., & Paul W.E. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J Immunol*. 1986; 136: 4538-4541.

205. Swain S.L., Weinberg A.D., English M., & Huston G. IL-4 directs the development of Th2-like helper effectors. *J Immunol*. 1990; 145: 3796-3806.

206. Minty A., Chalon P., Derocq J.M., Dumont X., Guillemot J.C., Kaghad M., Labit C., Leplatois P., Liauzun P., Miloux B., & . Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* 1993; 362: 248-250.

207. McKenzie A.N., Culpepper J.A., de Waal M.R., Briere F., Punnonen J., Aversa G., Sato A., Dang W., Cocks B.G., Menon S., & . Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc.Natl.Acad.Sci U.S.A* 1993; 90: 3735-3739.

208. Wills-Karp M. & Finkelman F.D. Untangling the complex web of IL-4- and IL-13-mediated signaling pathways. *Sci Signal*. 2008; 1: e55.

209. Grunig G., Warnock M., Wakil A.E., Venkayya R., Brombacher F., Rennick D.M., Sheppard D., Mohrs M., Donaldson D.D., Locksley R.M., & Corry D.B. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998; 282: 2261-2263.

210. Perkins C., Wills-Karp M., & Finkelman F.D. IL-4 induces IL-13-independent allergic airway inflammation. *J Allergy Clin.Immunol*. 2006; 118: 410-419.

211. Kopf M., Le G.G., Bachmann M., Lamers M.C., Bluethmann H., & Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 1993; 362: 245-248.

212. Kuhn R., Rajewsky K., & Muller W. Generation and analysis of interleukin-4 deficient mice. *Science* 1991; 254: 707-710.

213. Herrick C.A., MacLeod H., Glusac E., Tigelaar R.E., & Bottomly K. Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J Clin.Invest* 2000; 105: 765-775.

214. Coyle A.J., Le G.G., Bertrand C., Tsuyuki S., Heusser C.H., Kopf M., & Anderson G.P. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol.Biol*. 1995; 13: 54-59.

215. Kips J.C., Brusselle G.G., Joos G.F., Peleman R.A., Devos R.R., Tavernier J.H., & Pauwels R.A. Importance of interleukin-4 and interleukin-12 in allergen-induced airway changes in mice. *Int.Arch.Allergy Immunol*. 1995; 107: 115-118.

216. Brusselle G., Kips J., Joos G., Bluethmann H., & Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol.Biol*. 1995; 12: 254-259.

217. Hogan S.P., Mould A., Kikutani H., Ramsay A.J., & Foster P.S. Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyperreactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. *J Clin.Invest* 1997; 99: 1329-1339.

218. Ritz S.A., Cundall M.J., Gajewska B.U., Alvarez D., Gutierrez-Ramos J.C., Coyle A.J., McKenzie A.N., Stampfli M.R., & Jordana M. Granulocyte macrophage colony-stimulating factor-driven respiratory mucosal sensitization induces Th2 differentiation and function independently of interleukin-4. *Am.J Respir Cell Mol.Biol*. 2002; 27: 428-435.

219. Kuperman D., Schofield B., Wills-Karp M., & Grusby M.J. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp.Med* 1998; 187: 939-948.

220. Akimoto T., Numata F., Tamura M., Takata Y., Higashida N., Takashi T., Takeda K., & Akira S. Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J Exp.Med* 1998; 187: 1537-1542.

221. Mattes J., Yang M., Siqueira A., Clark K., MacKenzie J., McKenzie A.N., Webb D.C., Matthaei K.I., & Foster P.S. IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. *J Immunol*. 2001; 167: 1683-1692.

222. Fichtner-Feigl S., Strober W., Kawakami K., Puri R.K., & Kitani A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat.Med* 2006; 12: 99-106.

223. Wills-Karp M., Luyimbazi J., Xu X., Schofield B., Neben T.Y., Karp C.L., & Donaldson D.D. Interleukin-13: central mediator of allergic asthma. *Science* 1998; 282: 2258-2261.

224. Zhu Z., Homer R.J., Wang Z., Chen Q., Geba G.P., Wang J., Zhang Y., & Elias J.A. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin.Invest* 1999; 103: 779-788.

225. Walter D.M., McIntire J.J., Berry G., McKenzie A.N., Donaldson D.D., DeKruyff R.H., & Umetsu D.T. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol*. 2001; 167: 4668-4675.

226. Webb D.C., McKenzie A.N., Koskinen A.M., Yang M., Mattes J., & Foster P.S. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J Immunol*. 2000; 165: 108-113.

227. Eum S.Y., Maghni K., Tolloczko B., Eidelman D.H., & Martin J.G. IL-13 may mediate allergen-induced hyperresponsiveness independently of IL-5 or eotaxin by effects on airway smooth muscle. *Am J Physiol Lung Cell Mol.Physiol* 2005; 288: L576-L584.

228. Ramalingam T.R., Pesce J.T., Sheikh F., Cheever A.W., Mentink-Kane M.M., Wilson M.S., Stevens S., Valenzuela D.M., Murphy A.J., Yancopoulos

G.D., Urban J.F., Jr., Donnelly R.P., & Wynn T.A. Unique functions of the type II interleukin 4 receptor identified in mice lacking the interleukin 13 receptor alpha1 chain. *Nat.Immunol*. 2008; 9: 25-33.

229. Munitz A., Brandt E.B., Mingler M., Finkelman F.D., & Rothenberg M.E. Distinct roles for IL-13 and IL-4 via IL-13 receptor alpha1 and the type II IL-4 receptor in asthma pathogenesis. *Proc.Natl.Acad.Sci U.S.A* 2008; 105: 7240-7245.

230. Cembrzynska-Nowak M., Szklarz E., Inglot A.D., & Teodorczyk-Injeyan J.A. Elevated release of tumor necrosis factor-alpha and interferon-gamma by bronchoalveolar leukocytes from patients with bronchial asthma. *Am Rev.Respir Dis*. 1993; 147: 291-295.

231. Krug N., Madden J., Redington A.E., Lackie P., Djukanovic R., Schauer U., Holgate S.T., Frew A.J., & Howarth P.H. T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am J Respir Cell Mol.Biol*. 1996; 14: 319-326.

232. Cho S.H., Stanciu L.A., Holgate S.T., & Johnston S.L. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. *Am J Respir Crit Care Med* 2005; 171: 224-230.

233. Truyen E., Coteur L., Dilissen E., Overbergh L., Dupont L.J., Ceuppens J.L., & Bullens D.M. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* 2006; 61: 202-208.

234. Shannon J., Ernst P., Yamauchi Y., Olivenstein R., Lemiere C., Foley S., Cicora L., Ludwig M., Hamid Q., & Martin J.G. Differences in airway cytokine profile in severe asthma compared to moderate asthma. *Chest* 2008; 133: 420-426.

235. Cohn L., Homer R.J., Niu N., & Bottomly K. T helper 1 cells and interferon gamma regulate allergic airway inflammation and mucus production. *J Exp.Med* 1999; 190: 1309-1318.

236. Randolph D.A., Carruthers C.J., Szabo S.J., Murphy K.M., & Chaplin D.D. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J Immunol*. 1999; 162: 2375-2383.

237. Ying S., Robinson D.S., Varney V., Meng Q., Tsicopoulos A., Moqbel R., Durham S.R., Kay A.B., & Hamid Q. TNF alpha mRNA expression in allergic inflammation. *Clin.Exp.Allergy* 1991; 21: 745-750.

238. Bradding P., Roberts J.A., Britten K.M., Montefort S., Djukanovic R., Mueller R., Heusser C.H., Howarth P.H., & Holgate S.T. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol.Biol*. 1994; 10: 471-480.

239. Howarth P.H., Babu K.S., Arshad H.S., Lau L., Buckley M., McConnell W., Beckett P., Al A.M., Chauhan A., Wilson S.J., Reynolds A., Davies D.E., &

Holgate S.T. Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005; 60: 1012-1018.

240. Berry M.A., Hargadon B., Shelley M., Parker D., Shaw D.E., Green R.H., Bradding P., Brightling C.E., Wardlaw A.J., & Pavord I.D. Evidence of a role of tumor necrosis factor alpha in refractory asthma. *N.Engl.J Med* 2006; 354: 697-708.

241. Morjaria J.B., Chauhan A.J., Babu K.S., Polosa R., Davies D.E., & Holgate S.T. The role of a soluble TNFalpha receptor fusion protein (etanercept) in corticosteroid refractory asthma: a double blind, randomised, placebo controlled trial. *Thorax* 2008; 63: 584-591.

242. Erin E.M., Leaker B.R., Nicholson G.C., Tan A.J., Green L.M., Neighbour H., Zacharasiewicz A.S., Turner J., Barnathan E.S., Kon O.M., Barnes P.J., & Hansel T.T. The effects of a monoclonal antibody directed against tumor necrosis factor-alpha in asthma. *Am J Respir Crit Care Med* 2006; 174: 753-762.

243. Rouhani F.N., Meitin C.A., Kaler M., Miskinis-Hilligoss D., Stylianou M., & Levine S.J. Effect of tumor necrosis factor antagonism on allergen-mediated asthmatic airway inflammation. *Respir Med* 2005; 99: 1175-1182.

244. Wenzel S.E., Barnes P.J., Bleecker E.R., Bousquet J., Busse W., Dahlen S.E., Holgate S.T., Meyers D.A., Rabe K.F., Antczak A., Baker J., Horvath I., Mark Z., Bernstein D., Kerwin E., Schlenker-Herceg R., Lo K.H., Watt R., Barnathan E.S., & Chanez P. A randomized, double-blind, placebo-controlled study of tumor necrosis factor-alpha blockade in severe persistent asthma. *Am J Respir Crit Care Med* 2009; 179: 549-558.

245. Aggarwal S., Ghilardi N., Xie M.H., de Sauvage F.J., & Gurney A.L. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol.Chem.* 2003; 278: 1910-1914.

246. Langrish C.L., Chen Y., Blumenschein W.M., Mattson J., Basham B., Sedgwick J.D., McClanahan T., Kastelein R.A., & Cua D.J. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp.Med* 2005; 201: 233-240.

247. Molet S., Hamid Q., Davoine F., Nutku E., Taha R., Page N., Olivenstein R., Elias J., & Chakir J. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin.Immunol.* 2001; 108: 430-438.

248. Barczyk A., Pierzchala W., & Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 2003; 97: 726-733.

249. Laan M., Cui Z.H., Hoshino H., Lotvall J., Sjostrand M., Gruenert D.C., Skoogh B.E., & Linden A. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol.* 1999; 162: 2347-2352.

250. Park H., Li Z., Yang X.O., Chang S.H., Nurieva R., Wang Y.H., Wang Y., Hood L., Zhu Z., Tian Q., & Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat.Immunol.* 2005; 6: 1133-1141.

251. Yang X.O., Chang S.H., Park H., Nurieva R., Shah B., Acero L., Wang Y.H., Schluns K.S., Broaddus R.R., Zhu Z., & Dong C. Regulation of inflammatory responses by IL-17F. *J Exp.Med* 2008; 205: 1063-1075.

252. Schnyder-Candrian S., Togbe D., Couillin I., Mercier I., Brombacher F., Quesniaux V., Fossiez F., Ryffel B., & Schnyder B. Interleukin-17 is a negative regulator of established allergic asthma. *J Exp.Med* 2006; 203: 2715-2725.

253. Nakae S., Komiyama Y., Nambu A., Sudo K., Iwase M., Homma I., Sekikawa K., Asano M., & Iwakura Y. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity.* 2002; 17: 375-387.

254. Hellings P.W., Kasran A., Liu Z., Vandekerckhove P., Wuyts A., Overbergh L., Mathieu C., & Ceuppens J.L. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol.Biol.* 2003; 28: 42-50.

255. Song C., Luo L., Lei Z., Li B., Liang Z., Liu G., Li D., Zhang G., Huang B., & Feng Z.H. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol.* 2008; 181: 6117-6124.

256. Sallusto F., Mackay C.R., & Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 1997; 277: 2005-2007.

257. Bisset L.R. & Schmid-Grendelmeier P. Chemokines and their receptors in the pathogenesis of allergic asthma: progress and perspective. *Curr.Opin.Pulm.Med* 2005; 11: 35-42.

258. Alam R., York J., Boyars M., Stafford S., Grant J.A., Lee J., Forsythe P., Sim T., & Ida N. Increased MCP-1, RANTES, and MIP-1alpha in bronchoalveolar lavage fluid of allergic asthmatic patients. *Am.J Respir Crit Care Med.* 1996; 153: 1398-1404.

259. Hartl D., Griese M., Nicolai T., Zissel G., Prell C., Konstantopoulos N., Gruber R., Reinhardt D., Schendel D.J., & Krauss-Etschmann S. Pulmonary chemokines and their receptors differentiate children with asthma and chronic cough. *J Allergy Clin.Immunol.* 2005; 115: 728-736.

260. Rothenberg M.E., MacLean J.A., Pearlman E., Luster A.D., & Leder P. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J Exp.Med* 1997; 185: 785-790.

261. Lamkhioued B., Renzi P.M., bi-Younes S., Garcia-Zepada E.A., Allakhverdi Z., Ghaffar O., Rothenberg M.D., Luster A.D., & Hamid Q. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J Immunol.* 1997; 159: 4593-4601.

262. Ying S., Robinson D.S., Meng Q., Rottman J., Kennedy R., Ringler D.J., Mackay C.R., Daugherty B.L., Springer M.S., Durham S.R., Williams T.J., & Kay A.B. Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells. *Eur J Immunol*. 1997; 27: 3507-3516.

263. Ying S., Meng Q., Zeibecoglou K., Robinson D.S., Macfarlane A., Humbert M., & Kay A.B. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (Intrinsic) asthmatics. *J Immunol*. 1999; 163: 6321-6329.

264. Nakamura H., Weiss S.T., Israel E., Luster A.D., Drazen J.M., & Lilly C.M. Eotaxin and impaired lung function in asthma. *Am J Respir Crit Care Med* 1999; 160: 1952-1956.

265. Wenzel S.E., Trudeau J.B., Barnes S., Zhou X., Cundall M., Westcott J.Y., McCord K., & Chu H.W. TGF-beta and IL-13 synergistically increase eotaxin-1 production in human airway fibroblasts. *J Immunol*. 2002; 169: 4613-4619.

266. Gonzalo J.A., Lloyd C.M., Wen D., Albar J.P., Wells T.N., Proudfoot A., Martinez A., Dorf M., Bjerke T., Coyle A.J., & Gutierrez-Ramos J.C. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp.Med* 1998; 188: 157-167.

267. Yang Y., Loy J., Ryseck R.P., Carrasco D., & Bravo R. Antigen-induced eosinophilic lung inflammation develops in mice deficient in chemokine eotaxin. *Blood* 1998; 92: 3912-3923.

268. Pope S.M., Zimmermann N., Stringer K.F., Karow M.L., & Rothenberg M.E. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J Immunol*. 2005; 175: 5341-5350.

269. Humbles A.A., Lu B., Friend D.S., Okinaga S., Lora J., Al-Garawi A., Martin T.R., Gerard N.P., & Gerard C. The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc.Natl.Acad.Sci U.S.A* 2002; 99: 1479-1484.

270. Ma W., Bryce P.J., Humbles A.A., Laouini D., Yalcindag A., Alenius H., Friend D.S., Oettgen H.C., Gerard C., & Geha R.S. CCR3 is essential for skin eosinophilia and airway hyperresponsiveness in a murine model of allergic skin inflammation. *J Clin.Invest* 2002; 109: 621-628.

271. Wegmann M., Goggel R., Sel S., Sel S., Erb K.J., Kalkbrenner F., Renz H., & Garn H. Effects of a low-molecular-weight CCR-3 antagonist on chronic experimental asthma. *Am J Respir Cell Mol.Biol*. 2007; 36: 61-67.

272. Morokata T., Suzuki K., Masunaga Y., Taguchi K., Morihira K., Sato I., Fujii M., Takizawa S., Torii Y., Yamamoto N., Kaneko M., Yamada T., Takahashi K., & Shimizu Y. A novel, selective, and orally available

antagonist for CC chemokine receptor 3. *J Pharmacol.Exp.Ther*. 2006; 317: 244-250.

273. Chen Y., Kuchroo V.K., Inobe J., Hafler D.A., & Weiner H.L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 1994; 265: 1237-1240.

274. Groux H., O'Garra A., Bigler M., Rouleau M., Antonenko S., de Vries J.E., & Roncarolo M.G. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; 389: 737-742.

275. Sakaguchi S., Sakaguchi N., Asano M., Itoh M., & Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J.Immunol*. 1995; 155: 1151-1164.

276. Xu D., Liu H., Komai-Koma M., Campbell C., McSharry C., Alexander J., & Liew F.Y. CD4+CD25+ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, Leishmania major infection, and colitis in mice. *J.Immunol* 2003; 170: 394-399.

277. Hartl D., Koller B., Mehlhorn A.T., Reinhardt D., Nicolai T., Schendel D.J., Griese M., & Krauss-Etschmann S. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin.Immunol*. 2007; 119: 1258-1266.

278. Lin Y.L., Shieh C.C., & Wang J.Y. The functional insufficiency of human CD4+CD25 high T-regulatory cells in allergic asthma is subjected to TNF-alpha modulation. *Allergy* 2008; 63: 67-74.

279. Lewkowich I.P., Herman N.S., Schleifer K.W., Dance M.P., Chen B.L., Dienger K.M., Sproles A.A., Shah J.S., Kohl J., Belkaid Y., & Wills-Karp M. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp.Med* 2005; 202: 1549-1561.

280. Kearley J., Robinson D.S., & Lloyd C.M. CD4+CD25+ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. *J Allergy Clin.Immunol*. 2008; 122: 617-624.

281. Jaffar Z., Sivakuru T., & Roberts K. CD4+CD25+ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. *J.Immunol*. 2004; 172: 3842-3849.

282. Leech M.D., Benson R.A., De V.A., Fitch P.M., & Howie S.E. Resolution of Der p1-induced allergic airway inflammation is dependent on CD4+CD25+Foxp3+ regulatory cells. *J Immunol*. 2007; 179: 7050-7058.

283. Akbari O., Freeman G.J., Meyer E.H., Greenfield E.A., Chang T.T., Sharpe A.H., Berry G., DeKruyff R.H., & Umetsu D.T. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat.Med*. 2002; 8: 1024-1032.

284. Hume D.A. Macrophages as APC and the dendritic cell myth. *J Immunol*. 2008; 181: 5829-5835.

285. Lohmann-Matthes M.L., Steinmuller C., & Franke-Ullmann G. Pulmonary macrophages. *Eur Respir J* 1994; 7: 1678-1689.

286. Geissmann F., Jung S., & Littman D.R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003; 19: 71-82.

287. Janssen E.M., Wauben M.H., Nijkamp F.P., van E.W., & Van Oosterhout A.J. Immunomodulatory effects of antigen-pulsed macrophages in a murine model of allergic asthma. *Am J Respir Cell Mol.Biol*. 2002; 27: 257-264.

288. Vissers J.L., van Esch B.C., Hofman G.A., & Van Oosterhout A.J. Macrophages induce an allergen-specific and long-term suppression in a mouse asthma model. *Eur Respir J* 2005; 26: 1040-1046.

289. Tang C., Inman M.D., van R.N., Yang P., Shen H., Matsumoto K., & O'Byrne P.M. Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol*. 2001; 166: 1471-1481.

290. Careau E. & Bissonnette E.Y. Adoptive transfer of alveolar macrophages abrogates bronchial hyperresponsiveness. *Am J Respir Cell Mol.Biol*. 2004; 31: 22-27.

291. Moon K.A., Kim S.Y., Kim T.B., Yun E.S., Park C.S., Cho Y.S., Moon H.B., & Lee K.Y. Allergen-induced CD11b+ CD11c(int) CCR3+ macrophages in the lung promote eosinophilic airway inflammation in a mouse asthma model. *Int.Immunol*. 2007; 19: 1371-1381.

292. Vermaelen K.Y., Carro-Muino I., Lambrecht B.N., & Pauwels R.A. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp.Med* 2001; 193: 51-60.

293. Eisenbarth S.C., Piggott D.A., Huleatt J.W., Visintin I., Herrick C.A., & Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp.Med* 2002; 196: 1645-1651.

294. Tsitoura D.C., DeKruyff R.H., Lamb J.R., & Umetsu D.T. Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled CD4+ T cells. *J Immunol*. 1999; 163: 2592-2600.

295. Akbari O., DeKruyff R.H., & Umetsu D.T. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat.Immunol*. 2001; 2: 725-731.

296. Kuipers H. & Lambrecht B.N. The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma. *Curr.Opin.Immunol*. 2004; 16: 702-708.

297. Jahnsen F.L., Moloney E.D., Hogan T., Upham J.W., Burke C.M., & Holt P.G. Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge. *Thorax* 2001; 56: 823-826.

298. Lambrecht B.N., De V.M., Coyle A.J., Gutierrez-Ramos J.C., Thielemans K., & Pauwels R.A. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin.Invest* 2000; 106: 551-559.

299. Sung S., Rose C.E., & Fu S.M. Intratracheal priming with ovalbumin- and ovalbumin 323-339 peptide-pulsed dendritic cells induces airway hyperresponsiveness, lung eosinophilia, goblet cell hyperplasia, and inflammation. *J Immunol*. 2001; 166: 1261-1271.

300. Lambrecht B.N., Salomon B., Klatzmann D., & Pauwels R.A. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. *J Immunol*. 1998; 160: 4090-4097.

301. van Rijt L.S., Jung S., Kleinjan A., Vos N., Willart M., Duez C., Hoogsteden H.C., & Lambrecht B.N. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp.Med* 2005; 201: 981-991.

302. Holgate S.T., Davies D.E., Lackie P.M., Wilson S.J., Puddicombe S.M., & Lordan J.L. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin.Immunol*. 2000; 105: 193-204.

303. Davies D.E., Wicks J., Powell R.M., Puddicombe S.M., & Holgate S.T. Airway remodeling in asthma: new insights. *J.Allergy Clin.Immunol*. 2003; 111: 215-225.

304. Richter A., Puddicombe S.M., Lordan J.L., Bucchieri F., Wilson S.J., Djukanovic R., Dent G., Holgate S.T., & Davies D.E. The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol.Biol*. 2001; 25: 385-391.

305. Mitzner W. Airway smooth muscle: the appendix of the lung. *Am J Respir Crit Care Med* 2004; 169: 787-790.

306. Joubert P. & Hamid Q. Role of airway smooth muscle in airway remodeling. *J Allergy Clin.Immunol*. 2005; 116: 713-716.

307. Lazaar A.L. & Panettieri R.A., Jr. Airway smooth muscle: a modulator of airway remodeling in asthma. *J Allergy Clin.Immunol*. 2005; 116: 488-495.

308. Howarth P.H., Knox A.J., Amrani Y., Tliba O., Panettieri R.A., Jr., & Johnson M. Synthetic responses in airway smooth muscle. *J Allergy Clin.Immunol*. 2004; 114: S32-S50.

309. Johnson S.R. & Knox A.J. Synthetic functions of airway smooth muscle in asthma. *Trends Pharmacol.Sci* 1997; 18: 288-292.

310. Spanevello A., Confalonieri M., Sulotto F., Romano F., Balzano G., Migliori G.B., Bianchi A., & Michetti G. Induced sputum cellularity. Reference values and distribution in normal volunteers. *Am J Respir Crit Care Med* 2000; 162: 1172-1174.

311. Belda J., Leigh R., Parameswaran K., O'Byrne P.M., Sears M.R., & Hargreave F.E. Induced sputum cell counts in healthy adults. *Am J Respir Crit Care Med* 2000; 161: 475-478.

312. Pavord I.D., Pizzichini M.M., Pizzichini E., & Hargreave F.E. The use of induced sputum to investigate airway inflammation. *Thorax* 1997; 52: 498-501.

313. Turner M.O., Hussack P., Sears M.R., Dolovich J., & Hargreave F.E. Exacerbations of asthma without sputum eosinophilia. *Thorax* 1995; 50: 1057-1061.

314. Sur S., Crotty T.B., Kephart G.M., Hyma B.A., Colby T.V., Reed C.E., Hunt L.W., & Gleich G.J. Sudden-onset fatal asthma. A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? *Am Rev.Respir Dis.* 1993; 148: 713-719.

315. Fahy J.V., Kim K.W., Liu J., & Boushey H.A. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin.Immunol.* 1995; 95: 843-852.

316. Wenzel S.E., Szefler S.J., Leung D.Y., Sloan S.I., Rex M.D., & Martin R.J. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med* 1997; 156: 737-743.

317. Gibson P.G., Simpson J.L., & Saltos N. Heterogeneity of airway inflammation in persistent asthma : evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001; 119: 1329-1336.

318. Berry M., Morgan A., Shaw D.E., Parker D., Green R., Brightling C., Bradding P., Wardlaw A.J., & Pavord I.D. Pathological features and inhaled corticosteroid response of eosinophilic and non-eosinophilic asthma. *Thorax* 2007; 62: 1043-1049.

319. Simpson J.L., Grissell T.V., Douwes J., Scott R.J., Boyle M.J., & Gibson P.G. Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax* 2007; 62: 211-218.

320. Ito K., Herbert C., Siegle J.S., Vuppusetty C., Hansbro N., Thomas P.S., Foster P.S., Barnes P.J., & Kumar R.K. Steroid-resistant neutrophilic inflammation in a mouse model of an acute exacerbation of asthma. *Am J Respir Cell Mol.Biol.* 2008; 39: 543-550.

321. Werenskiold A.K., Hoffmann S., & Klemenz R. Induction of a mitogen-responsive gene after expression of the Ha-ras oncogene in NIH 3T3 fibroblasts. *Mol.Cell Biol.* 1989; 9: 5207-5214.

322. Tominaga S. A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett.* 1989; 258: 301-304.

323. Schmitz J., Owyang A., Oldham E., Song Y., Murphy E., McClanahan T.K., Zurawski G., Moshrefi M., Qin J., Li X., Gorman D.M., Bazan J.F., & Kastelein R.A. IL-33, an interleukin-1-like cytokine that signals via the IL-1

receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005; 23: 479-490.

324. Klemenz R., Hoffmann S., & Werenskiold A.K. Serum- and oncoprotein-mediated induction of a gene with sequence similarity to the gene encoding carcinoembryonic antigen. *Proc.Natl.Acad.Sci.U.S.A* 1989; 86: 5708-5712.

325. Yanagisawa K., Takagi T., Tsukamoto T., Tetsuka T., & Tominaga S. Presence of a novel primary response gene ST2L, encoding a product highly similar to the interleukin 1 receptor type 1. *FEBS Lett*. 1993; 318: 83-87.

326. Tominaga S., Yokota T., Yanagisawa K., Tsukamoto T., Takagi T., & Tetsuka T. Nucleotide sequence of a complementary DNA for human ST2. *Biochim.Biophys.Acta* 1992; 1171: 215-218.

327. Yanagisawa K., Tsukamoto T., Takagi T., & Tominaga S. Murine ST2 gene is a member of the primary response gene family induced by growth factors. *FEBS Lett*. 1992; 302: 51-53.

328. Tominaga S., Jenkins N.A., Gilbert D.J., Copeland N.G., & Tetsuka T. Molecular cloning of the murine ST2 gene. Characterization and chromosomal mapping. *Biochim.Biophys.Acta* 1991; 1090: 1-8.

329. Sims J.E., Painter S.L., & Gow I.R. Genomic organization of the type I and type II IL-1 receptors. *Cytokine* 1995; 7: 483-490.

330. Werenskiold A.K. Characterization of a secreted glycoprotein of the immunoglobulin superfamily inducible by mitogen and oncogene. *Eur J Biochem*. 1992; 204: 1041-1047.

331. Takagi T., Yanagisawa K., Tsukamoto T., Tetsuka T., Nagata S., & Tominaga S. Identification of the product of the murine ST2 gene. *Biochim.Biophys.Acta* 1993; 1178: 194-200.

332. Tominaga S., Kuroiwa K., Tago K., Iwahana H., Yanagisawa K., & Komatsu N. Presence and expression of a novel variant form of ST2 gene product in human leukemic cell line UT-7/GM. *Biochem.Biophys.Res.Commun*. 1999; 264: 14-18.

333. Iwahana H., Hayakawa M., Kuroiwa K., Tago K., Yanagisawa K., Noji S., & Tominaga S. Molecular cloning of the chicken ST2 gene and a novel variant form of the ST2 gene product, ST2LV. *Biochim.Biophys.Acta* 2004; 1681: 1-14.

334. Li H., Tago K., Io K., Kuroiwa K., Arai T., Iwahana H., Tominaga S., & Yanagisawa K. The cloning and nucleotide sequence of human ST2L cDNA. *Genomics* 2000; 67: 284-290.

335. Kalousek M.B., Trub T., Schuermann M., & Klemenz R. T1 is a c-Fos- and FosB-responsive gene which is induced by growth factors through multiple signal transduction pathways. *J Biol.Chem*. 1994; 269: 6866-6873.

336. Lanahan A., Williams J.B., Sanders L.K., & Nathans D. Growth factor-induced delayed early response genes. *Mol.Cell Biol*. 1992; 12: 3919-3929.

337. Hess J., Angel P., & Schorpp-Kistner M. AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci* 2004; 117: 5965-5973.

338. Superti-Furga G., Bergers G., Picard D., & Busslinger M. Hormone-dependent transcriptional regulation and cellular transformation by Fos-steroid receptor fusion proteins. *Proc.Natl.Acad.Sci.U.S.A* 1991; 88: 5114-5118.

339. Bergers G., Reikerstorfer A., Braselmann S., Graninger P., & Busslinger M. Alternative promoter usage of the Fos-responsive gene Fit-1 generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor. *EMBO J* 1994; 13: 1176-1188.

340. Kessler R., Zacharova-Albinger A., Laursen N.B., Kalousek M., & Klemenz R. Attenuated expression of the serum responsive T1 gene in ras transformed fibroblasts due to the inhibition of c-fos gene activity. *Oncogene* 1999; 18: 1733-1744.

341. Laursen N.B., Kessler R., Frohli E., & Klemenz R. Effects of ras transformation on the induction of the IL-1 receptor related T1 gene in response to mitogens, anisomycin, IL-1 and TNFalpha. *Oncogene* 1998; 16: 575-586.

342. Tominaga S., Kato-Yamazaki M., Yanagisawa K., Kawakami K., & Tetsuka T. The existence of a growth-specific DNA binding factor for the promoter region of mouse ST2 gene. *FEBS Lett*. 1994; 354: 311-314.

343. Trub T., Kalousek M.B., Frohli E., & Klemenz R. Growth factor-mediated induction of the delayed early gene T1 depends on a 12-O-tetradecanoylphorbol 13-acetate-responsive element located 3.6 kb upstream of the transcription initiation site. *Proc.Natl.Acad.Sci.U.S.A* 1994; 91: 3896-3900.

344. Kumar S., Tzimas M.N., Griswold D.E., & Young P.R. Expression of ST2, an interleukin-1 receptor homologue, is induced by proinflammatory stimuli. *Biochem.Biophys.Res.Commun*. 1997; 235: 474-478.

345. Brint E.K., Xu D., Liu H., Dunne A., McKenzie A.N., O'Neill L.A., & Liew F.Y. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat.Immunol*. 2004; 5: 373-379.

346. Feterowski C., Novotny A., Kaiser-Moore S., Muhlradt P.F., Rossmann-Bloeck T., Rump M., Holzmann B., & Weighardt H. Attenuated pathogenesis of polymicrobial peritonitis in mice after TLR2 agonist pre-treatment involves ST2 up-regulation. *Int.Immunol*. 2005; 17: 1035-1046.

347. Rossler U., Thomassen E., Hultner L., Baier S., Danescu J., & Werenskiold A.K. Secreted and membrane-bound isoforms of T1, an orphan receptor related to IL-1-binding proteins, are differently expressed in vivo. *Dev.Biol*. 1995; 168: 86-97.

348. Moritz D.R., Rodewald H.R., Gheyselinck J., & Klemenz R. The IL-1 receptor-related T1 antigen is expressed on immature and mature mast

cells and on fetal blood mast cell progenitors. *J Immunol*. 1998; 161: 4866-4874.

349. Werenskiold A.K., Rossler U., Lowel M., Schmidt J., Heermeier K., Spanner M.T., & Strauss P.G. Bone matrix deposition of T1, a homologue of interleukin 1 receptors. *Cell Growth Differ*. 1995; 6: 171-177.

350. Thomassen E., Kothny G., Haas S., Danescu J., Hultner L., Dormer P., & Werenskiold A.K. Role of cell type-specific promoters in the developmental regulation of T1, an interleukin 1 receptor homologue. *Cell Growth Differ*. 1995; 6: 179-184.

351. Kumar S., Minnich M.D., & Young P.R. ST2/T1 protein functionally binds to two secreted proteins from Balb/c 3T3 and human umbilical vein endothelial cells but does not bind interleukin 1. *J Biol.Chem*. 1995; 270: 27905-27913.

352. Moritz D.R., Gheyselinck J., & Klemenz R. Expression analysis of the soluble and membrane-associated forms of the interleukin-1 receptor-related T1 protein in primary mast cells and fibroblasts. *Hybridoma* 1998; 17: 107-116.

353. Oshikawa K., Yanagisawa K., Tominaga S., & Sugiyama Y. ST2 protein induced by inflammatory stimuli can modulate acute lung inflammation. *Biochem.Biophys.Res.Commun*. 2002; 299: 18-24.

354. Tajima S., Bando M., Ohno S., Sugiyama Y., Oshikawa K., Tominaga S., Itoh K., Takada T., Suzuki E., & Gejyo F. ST2 gene induced by type 2 helper T cell (Th2) and proinflammatory cytokine stimuli may modulate lung injury and fibrosis. *Exp.Lung Res*. 2007; 33: 81-97.

355. Rossler U., Andres A.C., Reichmann E., Schmahl W., & Werenskiold A.K. T1, an immunoglobulin superfamily member, is expressed in H-ras-dependent epithelial tumours of mammary cells. *Oncogene* 1993; 8: 609-617.

356. Werenskiold A.K., Prechtel D., Harbeck N., & Hofler H. Tumor-associated overexpression of the soluble T1-S receptor in lymph node-negative breast cancer. *Diagn.Mol.Pathol*. 2000; 9: 26-34.

357. Shimizu M., Matsuda A., Yanagisawa K., Hirota T., Akahoshi M., Inomata N., Ebe K., Tanaka K., Sugiura H., Nakashima K., Tamari M., Takahashi N., Obara K., Enomoto T., Okayama Y., Gao P.S., Huang S.K., Tominaga S., Ikezawa Z., & Shirakawa T. Functional SNPs in the distal promoter of the ST2 gene are associated with atopic dermatitis. *Hum.Mol.Genet*. 2005; 14: 2919-2927.

358. Gachter T., Moritz D.R., Gheyselinck J., & Klemenz R. GATA-Dependent expression of the interleukin-1 receptor-related T1 gene in mast cells. *Mol.Cell Biol*. 1998; 18: 5320-5331.

359. Xu D., Chan W.L., Leung B.P., Huang F., Wheeler R., Piedrafita D., Robinson J.H., & Liew F.Y. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp.Med*. 1998; 187: 787-794.

360. Lohning M., Stroehmann A., Coyle A.J., Grogan J.L., Lin S., Gutierrez-Ramos J.C., Levinson D., Radbruch A., & Kamradt T. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc.Natl.Acad.Sci.U.S.A* 1998; 95: 6930-6935.

361. Yoshida K., Arai T., Yokota T., Komatsu N., Miura Y., Yanagisawa K., Tetsuka T., & Tominaga S. Studies on natural ST2 gene products in the human leukemic cell line UT-7 using monoclonal antihuman ST2 antibodies. *Hybridoma* 1995; 14: 419-427.

362. Chan W.L., Pejnovic N., Lee C.A., & Al-Ali N.A. Human IL-18 receptor and ST2L are stable and selective markers for the respective type 1 and type 2 circulating lymphocytes. *J Immunol.* 2001; 167: 1238-1244.

363. McGuirk P., McCann C., & Mills K.H. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis. *J Exp.Med.* 2002; 195: 221-231.

364. Lecart S., Lecointe N., Subramaniam A., Alkan S., Ni D., Chen R., Boulay V., Pene J., Kuroiwa K., Tominaga S., & Yssel H. Activated, but not resting human Th2 cells, in contrast to Th1 and T regulatory cells, produce soluble ST2 and express low levels of ST2L at the cell surface. *Eur J Immunol.* 2002; 32: 2979-2987.

365. Yanagisawa K., Naito Y., Kuroiwa K., Arai T., Furukawa Y., Tomizuka H., Miura Y., Kasahara T., Tetsuka T., & Tominaga S. The expression of ST2 gene in helper T cells and the binding of ST2 protein to myeloma-derived RPMI8226 cells. *J Biochem.* 1997; 121: 95-103.

366. Saccani S., Polentarutti N., Penton-Rol G., Sims J.E., & Mantovani A. Divergent effects of LPS on expression of IL-1 receptor family members in mononuclear phagocytes in vitro and in vivo. *Cytokine* 1998; 10: 773-780.

367. Gachter T., Werenskiold A.K., & Klemenz R. Transcription of the interleukin-1 receptor-related T1 gene is initiated at different promoters in mast cells and fibroblasts. *J Biol.Chem.* 1996; 271: 124-129.

368. Hayakawa M., Yanagisawa K., Aoki S., Hayakawa H., Takezako N., & Tominaga S. T-helper type 2 cell-specific expression of the ST2 gene is regulated by transcription factor GATA-3. *Biochim.Biophys.Acta* 2005; 1728: 53-64.

369. Carter R.W., Sweet M.J., Xu D., Klemenz R., Liew F.Y., & Chan W.L. Regulation of ST2L expression on T helper (Th) type 2 cells. *Eur J Immunol.* 2001; 31: 2979-2985.

370. Iwahana H., Yanagisawa K., Ito-Kosaka A., Kuroiwa K., Tago K., Komatsu N., Katashima R., Itakura M., & Tominaga S. Different promoter usage and multiple transcription initiation sites of the interleukin-1 receptor-related human ST2 gene in UT-7 and TM12 cells. *Eur J Biochem.* 1999; 264: 397-406.

371. Abdulahad W.H., van der Geld Y.M., Stegeman C.A., & Kallenberg C.G. Persistent expansion of CD4+ effector memory T cells in Wegener's granulomatosis. *Kidney Int*. 2006; 70: 938-947.

372. Coyle A.J., Lloyd C., Tian J., Nguyen T., Erikkson C., Wang L., Ottoson P., Persson P., Delaney T., Lehar S., Lin S., Poisson L., Meisel C., Kamradt T., Bjerke T., Levinson D., & Gutierrez-Ramos J.C. Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J Exp.Med*. 1999; 190: 895-902.

373. Meisel C., Bonhagen K., Lohning M., Coyle A.J., Gutierrez-Ramos J.C., Radbruch A., & Kamradt T. Regulation and function of T1/ST2 expression on CD4+ T cells: induction of type 2 cytokine production by T1/ST2 cross-linking. *J Immunol*. 2001; 166: 3143-3150.

374. Lohning M., Grogan J.L., Coyle A.J., Yazdanbakhsh M., Meisel C., Gutierrez-Ramos J.C., Radbruch A., & Kamradt T. T1/ST2 expression is enhanced on CD4+ T cells from schistosome egg-induced granulomas: analysis of Th cell cytokine coexpression ex vivo. *J Immunol*. 1999; 162: 3882-3889.

375. Kropf P., Bickle Q., Herath S., Klemenz R., & Muller I. Organ-specific distribution of CD4+ T1/ST2+ Th2 cells in Leishmania major infection. *Eur J Immunol*. 2002; 32: 2450-2459.

376. Kropf P., Schopf L.R., Chung C.L., Xu D., Liew F.Y., Sypek J.P., & Muller I. Expression of Th2 cytokines and the stable Th2 marker ST2L in the absence of IL-4 during Leishmania major infection. *Eur J Immunol*. 1999; 29: 3621-3628.

377. Wiley R., Palmer K., Gajewska B., Stampfli M., Alvarez D., Coyle A., Gutierrez-Ramos J., & Jordana M. Expression of the Th1 chemokine IFN-gamma-inducible protein 10 in the airway alters mucosal allergic sensitization in mice. *J Immunol*. 2001; 166: 2750-2759.

378. Gajewska B.U., Swirski F.K., Alvarez D., Ritz S.A., Goncharova S., Cundall M., Snider D.P., Coyle A.J., Gutierrez-Ramos J.C., Stampfli M.R., & Jordana M. Temporal-spatial analysis of the immune response in a murine model of ovalbumin-induced airways inflammation. *Am.J Respir Cell Mol.Biol*. 2001; 25: 326-334.

379. Kropf P., Herath S., Tewari R., Syed N., Klemenz R., & Muller I. Identification of two distinct subpopulations of Leishmania major-specific T helper 2 cells. *Infect.Immun*. 2002; 70: 5512-5520.

380. Chen C.C., Grimbaldeston M.A., Tsai M., Weissman I.L., & Galli S.J. Identification of mast cell progenitors in adult mice. *Proc.Natl.Acad.Sci.U.S.A* 2005; 102: 11408-11413.

381. Hoshino K., Kashiwamura S., Kuribayashi K., Kodama T., Tsujimura T., Nakanishi K., Matsuyama T., Takeda K., & Akira S. The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. *J Exp.Med*. 1999; 190: 1541-1548.

382. Townsend M.J., Fallon P.G., Matthews D.J., Jolin H.E., & McKenzie A.N. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp.Med.* 2000; 191: 1069-1076.

383. Mangan N.E., Dasvarma A., McKenzie A.N., & Fallon P.G. T1/ST2 expression on Th2 cells negatively regulates allergic pulmonary inflammation. *Eur J Immunol.* 2007; 37: 1302-1312.

384. Sweet M.J., Leung B.P., Kang D., Sogaard M., Schulz K., Trajkovic V., Campbell C.C., Xu D., & Liew F.Y. A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol.* 2001; 166: 6633-6639.

385. Kropf P., Herath S., Klemenz R., & Muller I. Signaling through the T1/ST2 molecule is not necessary for Th2 differentiation but is important for the regulation of type 1 responses in nonhealing Leishmania major infection. *Infect.Immun.* 2003; 71: 1961-1971.

386. Yin H., Huang B.J., Yang H., Huang Y.F., Xiong P., Zheng F., Chen X.P., Chen Y.F., & Gong F.L. Pretreatment with soluble ST2 reduces warm hepatic ischemia/reperfusion injury. *Biochem.Biophys.Res.Commun.* 2006; 351: 940-946.

387. Takezako N., Hayakawa M., Hayakawa H., Aoki S., Yanagisawa K., Endo H., & Tominaga S. ST2 suppresses IL-6 production via the inhibition of IkappaB degradation induced by the LPS signal in THP-1 cells. *Biochem.Biophys.Res.Commun.* 2006; 341: 425-432.

388. Amatucci A., Novobrantseva T., Gilbride K., Brickelmaier M., Hochman P., & Ibraghimov A. Recombinant ST2 boosts hepatic Th2 response in vivo. *J Leukoc.Biol.* 2007; 82: 124-132.

389. Leung B.P., Xu D., Culshaw S., McInnes I.B., & Liew F.Y. A novel therapy of murine collagen-induced arthritis with soluble T1/ST2. *J Immunol.* 2004; 173: 145-150.

390. Fagundes C.T., Amaral F.A., Souza A.L., Vieira A.T., Xu D., Liew F.Y., Souza D.G., & Teixeira M.M. ST2, an IL-1R family member, attenuates inflammation and lethality after intestinal ischemia and reperfusion. *J Leukoc.Biol.* 2007; 81: 492-499.

391. Senn K.A., McCoy K.D., Maloy K.J., Stark G., Frohli E., Rulicke T., & Klemenz R. T1-deficient and T1-Fc-transgenic mice develop a normal protective Th2-type immune response following infection with Nippostrongylus brasiliensis. *Eur J Immunol.* 2000; 30: 1929-1938.

392. Chensue S.W., Warmington K., Ruth J.H., Lukacs N., & Kunkel S.L. Mycobacterial and schistosomal antigen-elicited granuloma formation in IFN-gamma and IL-4 knockout mice: analysis of local and regional cytokine and chemokine networks. *J Immunol.* 1997; 159: 3565-3573.

393. Mensah-Brown E., Shahin A., Parekh K., Hakim A.A., Shamisi M.A., Hsu D.K., & Lukic M.L. Functional capacity of macrophages determines the induction of type 1 diabetes. *Ann.N.Y.Acad.Sci.* 2006; 1084: 49-57.

394. Hartmann B., Staedtler F., Hartmann N., Meingassner J., & Firat H. Gene expression profiling of skin and draining lymph nodes of rats affected with cutaneous contact hypersensitivity. *Inflamm.Res.* 2006; 55: 322-334.

395. Oshikawa K., Yanagisawa K., Tominaga S., & Sugiyama Y. Expression and function of the ST2 gene in a murine model of allergic airway inflammation. *Clin.Exp.Allergy* 2002; 32: 1520-1526.

396. Walzl G., Matthews S., Kendall S., Gutierrez-Ramos J.C., Coyle A.J., Openshaw P.J., & Hussell T. Inhibition of T1/ST2 during respiratory syncytial virus infection prevents T helper cell type 2 (Th2)- but not Th1-driven immunopathology. *J Exp.Med.* 2001; 193: 785-792.

397. Kuroiwa K., Li H., Tago K., Iwahana H., Yanagisawa K., Komatsu N., Oshikawa K., Sugiyama Y., Arai T., & Tominaga S.I. Construction of ELISA system to quantify human ST2 protein in sera of patients. *Hybridoma* 2000; 19: 151-159.

398. Oshikawa K., Kuroiwa K., Tago K., Iwahana H., Yanagisawa K., Ohno S., Tominaga S.I., & Sugiyama Y. Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *Am.J Respir Crit Care Med.* 2001; 164: 277-281.

399. Kuroiwa K., Arai T., Okazaki H., Minota S., & Tominaga S. Identification of human ST2 protein in the sera of patients with autoimmune diseases. *Biochem.Biophys.Res.Commun.* 2001; 284: 1104-1108.

400. Tajima S., Oshikawa K., Tominaga S., & Sugiyama Y. The increase in serum soluble ST2 protein upon acute exacerbation of idiopathic pulmonary fibrosis. *Chest* 2003; 124: 1206-1214.

401. Brunner M., Krenn C., Roth G., Moser B., Dworschak M., Jensen-Jarolim E., Spittler A., Sautner T., Bonaros N., Wolner E., Boltz-Nitulescu G., & Ankersmit H.J. Increased levels of soluble ST2 protein and IgG1 production in patients with sepsis and trauma. *Intensive Care Med.* 2004; 30: 1468-1473.

402. Untersmayr E., Bakos N., Scholl I., Kundi M., Roth-Walter F., Szalai K., Riemer A.B., Ankersmit H.J., Scheiner O., Boltz-Nitulescu G., & Jensen-Jarolim E. Anti-ulcer drugs promote IgE formation toward dietary antigens in adult patients. *FASEB J* 2005; 19: 656-658.

403. Kanda M., Ohto-Ozaki H., Kuroiwa K., Tominaga S., Watanabe E., & Iwahana H. Elevation of ST2 protein levels in cerebrospinal fluid following subarachnoid hemorrhage. *Acta Neurol.Scand.* 2006; 113: 327-333.

404. Bakos N., Scholl I., Szalai K., Kundi M., Untersmayr E., & Jensen-Jarolim E. Risk assessment in elderly for sensitization to food and respiratory allergens. *Immunol.Lett.* 2006; 107: 15-21.

405. Oshikawa K., Yanagisawa K., Ohno S., Tominaga S., & Sugiyama Y. Expression of ST2 in helper T lymphocytes of malignant pleural effusions. *Am.J Respir Crit Care Med.* 2002; 165: 1005-1009.

406. Weinberg E.O., Shimpo M., De Keulenaer G.W., MacGillivray C., Tominaga S., Solomon S.D., Rouleau J.L., & Lee R.T. Expression and regulation of ST2, an interleukin-1 receptor family member, in cardiomyocytes and myocardial infarction. *Circulation* 2002; 106: 2961-2966.

407. Sanada S., Hakuno D., Higgins L.J., Schreiter E.R., McKenzie A.N., & Lee R.T. IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. *J Clin.Invest* 2007; 117: 1538-1549.

408. Shimpo M., Morrow D.A., Weinberg E.O., Sabatine M.S., Murphy S.A., Antman E.M., & Lee R.T. Serum levels of the interleukin-1 receptor family member ST2 predict mortality and clinical outcome in acute myocardial infarction. *Circulation* 2004; 109: 2186-2190.

409. Brown A.M., Wu A.H., Clopton P., Robey J.L., & Hollander J.E. ST2 in emergency department chest pain patients with potential acute coronary syndromes. *Ann.Emerg.Med.* 2007; 50: 153-8, 158.

410. Szerafin T., Brunner M., Horvath A., Szentgyorgyi L., Moser B., Boltz-Nitulescu G., Peterffy A., Hoetzenecker K., Steinlechner B., Wolner E., & Ankersmit H.J. Soluble ST2 protein in cardiac surgery: a possible negative feedback loop to prevent uncontrolled inflammatory reactions. *Clin.Lab* 2005; 51: 657-663.

411. Weinberg E.O., Shimpo M., Hurwitz S., Tominaga S., Rouleau J.L., & Lee R.T. Identification of serum soluble ST2 receptor as a novel heart failure biomarker. *Circulation* 2003; 107: 721-726.

412. Januzzi J.L., Jr., Peacock W.F., Maisel A.S., Chae C.U., Jesse R.L., Baggish A.L., O'Donoghue M., Sakhuja R., Chen A.A., van Kimmenade R.R., Lewandrowski K.B., Lloyd-Jones D.M., & Wu A.H. Measurement of the interleukin family member ST2 in patients with acute dyspnea: results from the PRIDE (Pro-Brain Natriuretic Peptide Investigation of Dyspnea in the Emergency Department) study. *J Am.Coll.Cardiol.* 2007; 50: 607-613.

413. Danescu J. & Werenskiold A.K. Post-transcriptional inhibition of the interleukin-1 binding protein B15R of vaccinia virus after coexpression of the related T1 protein. *FEBS Lett.* 1995; 367: 89-92.

414. Brint E.K., Fitzgerald K.A., Smith P., Coyle A.J., Gutierrez-Ramos J.C., Fallon P.G., & O'Neill L.A. Characterization of signaling pathways activated by the interleukin 1 (IL-1) receptor homologue T1/ST2. A role for Jun N-terminal kinase in IL-4 induction. *J Biol.Chem.* 2002; 277: 49205-49211.

415. Gayle M.A., Slack J.L., Bonnert T.P., Renshaw B.R., Sonoda G., Taguchi T., Testa J.R., Dower S.K., & Sims J.E. Cloning of a putative ligand for the T1/ST2 receptor. *J Biol.Chem.* 1996; 271: 5784-5789.

416. Mitcham J.L., Parnet P., Bonnert T.P., Garka K.E., Gerhart M.J., Slack J.L., Gayle M.A., Dower S.K., & Sims J.E. T1/ST2 signaling establishes it as a member of an expanding interleukin-1 receptor family. *J Biol.Chem.* 1996; 271: 5777-5783.

417. Baekkevold E.S., Roussigne M., Yamanaka T., Johansen F.E., Jahnsen F.L., Amalric F., Brandtzaeg P., Erard M., Haraldsen G., & Girard J.P. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am.J Pathol.* 2003; 163: 69-79.

418. Carriere V., Roussel L., Ortega N., Lacorre D.A., Americh L., Aguilar L., Bouche G., & Girard J.P. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc.Natl.Acad.Sci.U.S.A* 2007; 104: 282-287.

419. Komai-Koma M., Xu D., Li Y., McKenzie A.N., McInnes I.B., & Liew F.Y. IL-33 is a chemoattractant for human Th2 cells. *Eur J Immunol.* 2007; 37: 2779-2786.

420. Little C.C. & Cloudman A.M. The Occurrence of a Dominant Spotting Mutation in the House Mouse. *Proc.Natl.Acad.Sci U.S.A* 1937; 23: 535-537.

421. Geissler E.N., Ryan M.A., & Housman D.E. The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. *Cell* 1988; 55: 185-192.

422. Kitamura Y., Go S., & Hatanaka K. Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. *Blood* 1978; 52: 447-452.

423. Galli S.J. & Kitamura Y. Genetically mast-cell-deficient W/Wv and Sl/Sld mice. Their value for the analysis of the roles of mast cells in biologic responses in vivo. *Am J Pathol.* 1987; 127: 191-198.

424. Chervenick P.A. & Boggs D.R. Decreased neutrophils and megakaryocytes in anemic mice of genotype W/W. *J Cell Physiol* 1969; 73: 25-30.

425. Lyon M.F. & Glenister P.H. A new allele sash (Wsh) at the W-locus and a spontaneous recessive lethal in mice. *Genet.Res.* 1982; 39: 315-322.

426. Wolters P.J., Mallen-St C.J., Lewis C.C., Villalta S.A., Baluk P., Erle D.J., & Caughey G.H. Tissue-selective mast cell reconstitution and differential lung gene expression in mast cell-deficient Kit(W-sh)/Kit(W-sh) sash mice. *Clin.Exp.Allergy* 2005; 35: 82-88.

427. Grimbaldeston M.A., Chen C.C., Piliponsky A.M., Tsai M., Tam S.Y., & Galli S.J. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am J Pathol.* 2005; 167: 835-848.

428. Tono T., Tsujimura T., Koshimizu U., Kasugai T., Adachi S., Isozaki K., Nishikawa S., Morimoto M., Nishimune Y., Nomura S., & . c-kit Gene was not transcribed in cultured mast cells of mast cell-deficient Wsh/Wsh mice that have a normal number of erythrocytes and a normal c-kit coding region. *Blood* 1992; 80: 1448-1453.

429. McKay A., Leung B.P., McInnes I.B., Thomson N.C., & Liew F.Y. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J Immunol.* 2004; 172: 2903-2908.

430. Patel M., Xu D., Kewin P., Choo-Kang B., McSharry C., Thomson N.C., & Liew F.Y. TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. *J Immunol*. 2005; 174: 7558-7563.

431. Patel M., Xu D., Kewin P., Choo-Kang B., McSharry C., Thomson N.C., & Liew F.Y. Glucocorticoid-induced TNFR family-related protein (GITR) activation exacerbates murine asthma and collagen-induced arthritis. *Eur J Immunol*. 2005; 35: 3581-3590.

432. Henderson W.R., Jr., Lewis D.B., Albert R.K., Zhang Y., Lamm W.J., Chiang G.K., Jones F., Eriksen P., Tien Y.T., Jonas M., & Chi E.Y. The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J Exp.Med* 1996; 184: 1483-1494.

433. Furitsu T., Tsujimura T., Tono T., Ikeda H., Kitayama H., Koshimizu U., Sugahara H., Butterfield J.H., Ashman L.K., Kanayama Y., & . Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin.Invest* 1993; 92: 1736-1744.

434. Butterfield J.H., Weiler D., Dewald G., & Gleich G.J. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk.Res*. 1988; 12: 345-355.

435. Dunn T.B. & Potter M. A transplantable mast-cell neoplasm in the mouse. *J Natl.Cancer Inst*. 1957; 18: 587-601.

436. Rottapel R., Reedijk M., Williams D.E., Lyman S.D., Anderson D.M., Pawson T., & Bernstein A. The Steel/W transduction pathway: kit autophosphorylation and its association with a unique subset of cytoplasmic signaling proteins is induced by the Steel factor. *Mol.Cell Biol*. 1991; 11: 3043-3051.

437. Ra C., Jouvin M.H., Blank U., & Kinet J.P. A macrophage Fc gamma receptor and the mast cell receptor for IgE share an identical subunit. *Nature* 1989; 341: 752-754.

438. Gordon J.R. & Galli S.J. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature* 1990; 346: 274-276.

439. Zhao Y., Leung P.C., Woo K.S., Chen G.G., Wong Y.O., Liu S.X., & van Hasselt C.A. Inhibitory effects of budesonide, desloratadine and dexamethasone on cytokine release from human mast cell line (HMC-1). *Inflamm.Res*. 2004; 53: 664-669.

440. Wright H.V., Bailey D., Kashyap M., Kepley C.L., Drutskaya M.S., Nedospasov S.A., & Ryan J.J. IL-3-mediated TNF production is necessary for mast cell development. *J Immunol*. 2006; 176: 2114-2121.

441. Nakahata T., Kobayashi T., Ishiguro A., Tsuji K., Naganuma K., Ando O., Yagi Y., Tadokoro K., & Akabane T. Extensive proliferation of mature connective-tissue type mast cells in vitro. *Nature* 1986; 324: 65-67.

442. Reuter S., Heinz A., Sieren M., Wiewrodt R., Gelfand E.W., Stassen M., Buhl R., & Taube C. Mast cell-derived tumour necrosis factor is essential for allergic airway disease. *Eur Respir J* 2008; 31: 773-782.

443. Moulin D., Donze O., Talabot-Ayer D., Mezin F., Palmer G., & Gabay C. Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. *Cytokine* 2007; 40: 216-225.

444. Hallgren J. & Gurish M.F. Pathways of murine mast cell development and trafficking: tracking the roots and routes of the mast cell. *Immunol.Rev.* 2007; 217: 8-18.

445. Ho L.H., Ohno T., Oboki K., Kajiwara N., Suto H., Iikura M., Okayama Y., Akira S., Saito H., Galli S.J., & Nakae S. IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcepsilonRI signals. *J Leukoc.Biol.* 2007; 82: 1481-1490.

446. Iikura M., Suto H., Kajiwara N., Oboki K., Ohno T., Okayama Y., Saito H., Galli S.J., & Nakae S. IL-33 can promote survival, adhesion and cytokine production in human mast cells. *Lab Invest* 2007; 87: 971-978.

447. Kondo Y., Yoshimoto T., Yasuda K., Futatsugi-Yumikura S., Morimoto M., Hayashi N., Hoshino T., Fujimoto J., & Nakanishi K. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int.Immunol.* 2008; 20: 791-800.

448. Allakhverdi Z., Smith D.E., Comeau M.R., & Delespesse G. Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J Immunol.* 2007; 179: 2051-2054.

449. Pushparaj P.N., Tay H.K., H'ng S.C., Pitman N., Xu D., McKenzie A., Liew F.Y., & Melendez A.J. The cytokine interleukin-33 mediates anaphylactic shock. *Proc.Natl.Acad.Sci U.S.A* 2009; 106: 9773-9778.

450. Ali S., Huber M., Kollewe C., Bischoff S.C., Falk W., & Martin M.U. IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc.Natl.Acad.Sci.U.S.A* 2007; 104: 18660-18665.

451. Kishimoto T., Akira S., Narazaki M., & Taga T. Interleukin-6 family of cytokines and gp130. *Blood* 1995; 86: 1243-1254.

452. Yanagida M., Fukamachi H., Ohgami K., Kuwaki T., Ishii H., Uzumaki H., Amano K., Tokiwa T., Mitsui H., Saito H., Iikura Y., Ishizaka T., & Nakahata T. Effects of T-helper 2-type cytokines, interleukin-3 (IL-3), IL-4, IL-5, and IL-6 on the survival of cultured human mast cells. *Blood* 1995; 86: 3705-3714.

453. Rincon M., Anguita J., Nakamura T., Fikrig E., & Flavell R.A. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp.Med.* 1997; 185: 461-469.

454. Vercelli D., Jabara H.H., Arai K., Yokota T., & Geha R.S. Endogenous interleukin 6 plays an obligatory role in interleukin 4-dependent human IgE synthesis. *Eur J Immunol*. 1989; 19: 1419-1424.

455. Jones S.A. Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol*. 2005; 175: 3463-3468.

456. Kaplanski G., Marin V., Montero-Julian F., Mantovani A., & Farnarier C. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends Immunol*. 2003; 24: 25-29.

457. Ishihara K. & Hirano T. IL-6 in autoimmune disease and chronic inflammatory proliferative disease. *Cytokine Growth Factor Rev*. 2002; 13: 357-368.

458. Ward P.A., Remold H.G., & David J.R. The production by antigen-stimulated lymphocytes of a leukotactic factor distinct from migration inhibitory factor. *Cell Immunol*. 1970; 1: 162-174.

459. Yoshimura T., Yuhki N., Moore S.K., Appella E., Lerman M.I., & Leonard E.J. Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett*. 1989; 244: 487-493.

460. Wolpe S.D., Davatelis G., Sherry B., Beutler B., Hesse D.G., Nguyen H.T., Moldawer L.L., Nathan C.F., Lowry S.F., & Cerami A. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J Exp.Med*. 1988; 167: 570-581.

461. Palmqvist C., Wardlaw A.J., & Bradding P. Chemokines and their receptors as potential targets for the treatment of asthma. *Br.J Pharmacol*. 2007; 151: 725-736.

462. Rot A., Krieger M., Brunner T., Bischoff S.C., Schall T.J., & Dahinden C.A. RANTES and macrophage inflammatory protein 1 alpha induce the migration and activation of normal human eosinophil granulocytes. *J Exp.Med*. 1992; 176: 1489-1495.

463. Taub D.D., Conlon K., Lloyd A.R., Oppenheim J.J., & Kelvin D.J. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. *Science* 1993; 260: 355-358.

464. Menten P., Wuyts A., & Van D.J. Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev*. 2002; 13: 455-481.

465. Chensue S.W., Warmington K.S., Ruth J.H., Sanghi P.S., Lincoln P., & Kunkel S.L. Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (mycobacterial) and Th2 (schistosomal) antigen-induced granuloma formation: relationship to local inflammation, Th cell expression, and IL-12 production. *J Immunol*. 1996; 157: 4602-4608.

466. Karpus W.J., Lukacs N.W., Kennedy K.J., Smith W.S., Hurst S.D., & Barrett T.A. Differential CC chemokine-induced enhancement of T helper cell cytokine production. *J Immunol*. 1997; 158: 4129-4136.

467. Luther S.A. & Cyster J.G. Chemokines as regulators of T cell differentiation. *Nat.Immunol.* 2001; 2: 102-107.

468. Gu L., Tseng S., Horner R.M., Tam C., Loda M., & Rollins B.J. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 2000; 404: 407-411.

469. Marini M., Vittori E., Hollemborg J., & Mattoli S. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J Allergy Clin.Immunol.* 1992; 89: 1001-1009.

470. Yokoyama A., Kohno N., Fujino S., Hamada H., Inoue Y., Fujioka S., Ishida S., & Hiwada K. Circulating interleukin-6 levels in patients with bronchial asthma. *Am J Respir Crit Care Med* 1995; 151: 1354-1358.

471. Yokoyama A., Kohno N., Sakai K., Kondo K., Hirasawa Y., & Hiwada K. Circulating levels of soluble interleukin-6 receptor in patients with bronchial asthma. *Am J Respir Crit Care Med* 1997; 156: 1688-1691.

472. Doganci A., Eigenbrod T., Krug N., De Sanctis G.T., Hausding M., Erpenbeck V.J., Haddad e., Lehr H.A., Schmitt E., Bopp T., Kallen K.J., Herz U., Schmitt S., Luft C., Hecht O., Hohlfeld J.M., Ito H., Nishimoto N., Yoshizaki K., Kishimoto T., Rose-John S., Renz H., Neurath M.F., Galle P.R., & Finotto S. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *J Clin.Invest* 2005; 115: 313-325.

473. Kuhn C., III, Homer R.J., Zhu Z., Ward N., Flavell R.A., Geba G.P., & Elias J.A. Airway hyperresponsiveness and airway obstruction in transgenic mice. Morphologic correlates in mice overexpressing interleukin (IL)-11 and IL-6 in the lung. *Am.J Respir Cell Mol.Biol.* 2000; 22: 289-295.

474. DiCosmo B.F., Geba G.P., Picarella D., Elias J.A., Rankin J.A., Stripp B.R., Whitsett J.A., & Flavell R.A. Airway epithelial cell expression of interleukin-6 in transgenic mice. Uncoupling of airway inflammation and bronchial hyperreactivity. *J Clin.Invest* 1994; 94: 2028-2035.

475. Bradding P., Feather I.H., Wilson S., Bardin P.G., Heusser C.H., Holgate S.T., & Howarth P.H. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol.* 1993; 151: 3853-3865.

476. Sousa A.R., Lane S.J., Nakhosteen J.A., Yoshimura T., Lee T.H., & Poston R.N. Increased expression of the monocyte chemoattractant protein-1 in bronchial tissue from asthmatic subjects. *Am.J Respir Cell Mol.Biol.* 1994; 10: 142-147.

477. Holgate S.T., Bodey K.S., Janezic A., Frew A.J., Kaplan A.P., & Teran L.M. Release of RANTES, MIP-1 alpha, and MCP-1 into asthmatic airways following endobronchial allergen challenge. *Am.J Respir Crit Care Med.* 1997; 156: 1377-1383.

478. Lukacs N.W., Strieter R.M., Warmington K., Lincoln P., Chensue S.W., & Kunkel S.L. Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J Immunol*. 1997; 158: 4398-4404.

479. Campbell E.M., Charo I.F., Kunkel S.L., Strieter R.M., Boring L., Gosling J., & Lukacs N.W. Monocyte chemoattractant protein-1 mediates cockroach allergen-induced bronchial hyperreactivity in normal but not CCR2-/- mice: the role of mast cells. *J Immunol*. 1999; 163: 2160-2167.

480. Lukacs N.W., Strieter R.M., Shaklee C.L., Chensue S.W., & Kunkel S.L. Macrophage inflammatory protein-1 alpha influences eosinophil recruitment in antigen-specific airway inflammation. *Eur J Immunol*. 1995; 25: 245-251.

481. Fulkerson P.C., Zimmermann N., Hassman L.M., Finkelman F.D., & Rothenberg M.E. Pulmonary chemokine expression is coordinately regulated by STAT1, STAT6, and IFN-gamma. *J Immunol*. 2004; 173: 7565-7574.

482. McConville J.T., Williams R.O., III, Carvalho T.C., Iberg A.N., Johnston K.P., Talbert R.L., Burgess D., & Peters J.I. Design and evaluation of a restraint-free small animal inhalation dosing chamber. *Drug Dev.Ind.Pharm*. 2005; 31: 35-42.

483. Cheng Y.S. Aerosol Deposition in the Extrathoracic Region. *Aerosol Sci Technol*. 2003; 37: 659-671.

484. Miller F.J., Mercer R.R., & Crapo J.D. Lower Respiratory Tract Structure of Laboratory Animals and Humans: Dosimetry Implications. *Aerosol Science and Technology* 1993; 18: 257-271.

485. Southam D.S., Dolovich M., O'Byrne P.M., & Inman M.D. Distribution of intranasal instillations in mice: effects of volume, time, body position, and anesthesia. *Am J Physiol Lung Cell Mol.Physiol* 2002; 282: L833-L839.

486. Wang Y. & McCusker C.T. Interleukin-13-dependent bronchial hyper-responsiveness following isolated upper-airway allergen challenge in a murine model of allergic rhinitis and asthma. *Clin.Exp.Allergy* 2005; 35: 1104-1111.

487. Leishman A.J., Garside P., & Mowat A.M. Immunological consequences of intervention in established immune responses by feeding protein antigens. *Cell Immunol*. 1998; 183: 137-148.

488. Mitzner W. & Tankersley C. Interpreting Penh in mice. *J Appl.Physiol* 2003; 94: 828-831.

489. Bates J., Irvin C., Brusasco V., Drazen J., Fredberg J., Loring S., Eidelman D., Ludwig M., Macklem P., Martin J., Milic-Emili J., Hantos Z., Hyatt R., Lai-Fook S., Leff A., Solway J., Lutchen K., Suki B., Mitzner W., Pare P., Pride N., & Sly P. The use and misuse of Penh in animal models of lung disease. *Am J Respir Cell Mol.Biol*. 2004; 31: 373-374.

490. Lundblad L.K., Irvin C.G., Adler A., & Bates J.H. A reevaluation of the validity of unrestrained plethysmography in mice. *J Appl.Physiol* 2002; 93: 1198-1207.

491. Leigh R., Ellis R., Wattie J., Southam D.S., De H.M., Gauldie J., O'Byrne P.M., & Inman M.D. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol.Biol*. 2002; 27: 526-535.

492. Kearley J., Buckland K.F., Mathie S.A., & Lloyd C.M. Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am J Respir Crit Care Med* 2009; 179: 772-781.

493. Ito W., Tanimoto M., Ono K., Mizuno S., Yoshida A., Koga H., Fuchimoto Y., Kondo N., Tanimoto Y., Kiura K., Matsumoto K., Kataoka M., Nakamura T., Gelfand E.W., & Kanehiro A. Growth factors temporally associate with airway responsiveness and inflammation in allergen-exposed mice. *Int.Arch.Allergy Immunol*. 2008; 145: 324-339.

494. Brown J.R., Kleimberg J., Marini M., Sun G., Bellini A., & Mattoli S. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. *Clin.Exp.Immunol*. 1998; 114: 137-146.

495. Ravensberg A.J., Ricciardolo F.L., van S.A., Rabe K.F., Sterk P.J., Hiemstra P.S., & Mauad T. Eotaxin-2 and eotaxin-3 expression is associated with persistent eosinophilic bronchial inflammation in patients with asthma after allergen challenge. *J Allergy Clin.Immunol*. 2005; 115: 779-785.

496. Collins P.D., Marleau S., Griffiths-Johnson D.A., Jose P.J., & Williams T.J. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp.Med* 1995; 182: 1169-1174.

497. Lamkhioued B., Abdelilah S.G., Hamid Q., Mansour N., Delespesse G., & Renzi P.M. The CCR3 receptor is involved in eosinophil differentiation and is up-regulated by Th2 cytokines in CD34+ progenitor cells. *J Immunol*. 2003; 170: 537-547.

498. Watanabe K., Jose P.J., & Rankin S.M. Eotaxin-2 generation is differentially regulated by lipopolysaccharide and IL-4 in monocytes and macrophages. *J Immunol*. 2002; 168: 1911-1918.

499. Kool M., Soullie T., van N.M., Willart M.A., Muskens F., Jung S., Hoogsteden H.C., Hammad H., & Lambrecht B.N. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp.Med* 2008; 205: 869-882.

500. Martinon F., Burns K., & Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol.Cell* 2002; 10: 417-426.

501. Mariathasan S., Weiss D.S., Newton K., McBride J., O'Rourke K., Roose-Girma M., Lee W.P., Weinrauch Y., Monack D.M., & Dixit V.M. Cryopyrin

activates the inflammasome in response to toxins and ATP. *Nature* 2006; 440: 228-232.

502. Eisenbarth S.C., Colegio O.R., O'Connor W., Sutterwala F.S., & Flavell R.A. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 2008; 453: 1122-1126.

503. Li H., Willingham S.B., Ting J.P., & Re F. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. *J Immunol*. 2008; 181: 17-21.

504. Franchi L. & Nunez G. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. *Eur J Immunol*. 2008; 38: 2085-2089.

505. Kool M., Petrilli V., De S.T., Rolaz A., Hammad H., van N.M., Bergen I.M., Castillo R., Lambrecht B.N., & Tschopp J. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J Immunol*. 2008; 181: 3755-3759.

506. Mariathasan S. & Monack D.M. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat.Rev.Immunol*. 2007; 7: 31-40.

507. Marrack P., McKee A.S., & Munks M.W. Towards an understanding of the adjuvant action of aluminium. *Nat.Rev.Immunol*. 2009; 9: 287-293.

508. Schmitz N., Kurrer M., & Kopf M. The IL-1 receptor 1 is critical for Th2 cell type airway immune responses in a mild but not in a more severe asthma model. *Eur J Immunol*. 2003; 33: 991-1000.

509. Nakae S., Komiyama Y., Yokoyama H., Nambu A., Umeda M., Iwase M., Homma I., Sudo K., Horai R., Asano M., & Iwakura Y. IL-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response. *Int.Immunol*. 2003; 15: 483-490.

510. Heinzel F.P., Sadick M.D., Holaday B.J., Coffman R.L., & Locksley R.M. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J Exp.Med* 1989; 169: 59-72.

511. Howard J.G., Hale C., & Liew F.Y. Immunological regulation of experimental cutaneous leishmaniasis. IV. Prophylactic effect of sublethal irradiation as a result of abrogation of suppressor T cell generation in mice genetically susceptible to Leishmania tropica. *J Exp.Med* 1981; 153: 557-568.

512. Noguchi J., Kuroda E., & Yamashita U. Strain difference of murine bone marrow-derived mast cell functions. *J Leukoc.Biol*. 2005; 78: 605-611.

513. Iwama T., Nagai H., Suda H., Tsuruoka N., & Koda A. Effect of murine recombinant interleukin-5 on the cell population in guinea-pig airways. *Br.J Pharmacol*. 1992; 105: 19-22.

514. Griffiths-Johnson D.A., Collins P.D., Rossi A.G., Jose P.J., & Williams T.J. The chemokine, eotaxin, activates guinea-pig eosinophils in vitro and

causes their accumulation into the lung in vivo. *Biochem.Biophys.Res.Commun.* 1993; 197: 1167-1172.

515. Garcia-Zepeda E.A., Rothenberg M.E., Ownbey R.T., Celestin J., Leder P., & Luster A.D. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat.Med* 1996; 2: 449-456.

516. Schwarze J., Hamelmann E., & Gelfand E.W. Barometric whole body plethysmography in mice. *J Appl.Physiol* 2005; 98: 1955-1957.

517. Adler A., Cieslewicz G., & Irvin C.G. Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. *J Appl.Physiol* 2004; 97: 286-292.

518. Kearley J., Burwell T.J., Benjamin E., Kozhich A.T., Donacki N., Kiener P.A., Molfino N.A., Kolbeck R., Coyle A.J., & Humbles A.A. IL-33 Induces Airway Hyperresponsiveness and Alternative Macrophage Activation Via an IL-13 Dependent Mechanism. *American Journal of Respiratory and Critical Care Medicine* 2009; 179: A2254.

519. Kuchler A.M., Pollheimer J., Balogh J., Sponheim J., Manley L., Sorensen D.R., De Angelis P.M., Scott H., & Haraldsen G. Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation. *Am J Pathol.* 2008; 173: 1229-1242.

520. Miller A.M., Xu D., Asquith D.L., Denby L., Li Y., Sattar N., Baker A.H., McInnes I.B., & Liew F.Y. IL-33 reduces the development of atherosclerosis. *J Exp.Med.* 2008; 205: 339-346.

521. Moussion C., Ortega N., & Girard J.P. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS.One.* 2008; 3: e3331.

522. Bartunek J., Delrue L., Van D.F., Muller O., Casselman F., De W.B., Croes R., Verstreken S., Goethals M., de R.H., Sarma J., Joseph L., Vanderheyden M., & Weinberg E.O. Nonmyocardial production of ST2 protein in human hypertrophy and failure is related to diastolic load. *J Am Coll.Cardiol.* 2008; 52: 2166-2174.

523. Palmer G., Talabot-Ayer D., Lamacchia C., Toy D., Seemayer C.A., Viatte S., Finckh A., Smith D.E., & Gabay C. Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis. *Arthritis Rheum.* 2009; 60: 738-749.

524. Thornberry N.A., Bull H.G., Calaycay J.R., Chapman K.T., Howard A.D., Kostura M.J., Miller D.K., Molineaux S.M., Weidner J.R., Aunins J., & . A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 1992; 356: 768-774.

525. Cayrol C. & Girard J.P. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc.Natl.Acad.Sci U.S.A* 2009; 106: 9021-9026.

526. Talabot-Ayer D., Lamacchia C., Gabay C., & Palmer G. Interleukin-33 is biologically active independently of caspase-1 cleavage. *J Biol.Chem.* 2009; 284: 19420-19426.

527. Luthi A.U., Cullen S.P., McNeela E.A., Duriez P.J., Afonina I.S., Sheridan C., Brumatti G., Taylor R.C., Kersse K., Vandenabeele P., Lavelle E.C., & Martin S.J. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity.* 2009; 31: 84-98.

528. Lamkanfi M., Kanneganti T.D., Van D.P., Vanden B.T., Vanoverberghe I., Vandekerckhove J., Vandenabeele P., Gevaert K., & Nunez G. Targeted peptidecentric proteomics reveals caspase-7 as a substrate of the caspase-1 inflammasomes. *Mol.Cell Proteomics.* 2008; 7: 2350-2363.

529. Hayakawa M., Hayakawa H., Matsuyama Y., Tamemoto H., Okazaki H., & Tominaga S. Mature interleukin-33 is produced by calpain-mediated cleavage in vivo. *Biochem.Biophys.Res.Commun.* 2009; 387: 218-222.

530. Hori O., Brett J., Slattery T., Cao R., Zhang J., Chen J.X., Nagashima M., Lundh E.R., Vijay S., Nitecki D., & . The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol.Chem.* 1995; 270: 25752-25761.

531. Park J.S., Svetkauskaite D., He Q., Kim J.Y., Strassheim D., Ishizaka A., & Abraham E. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol.Chem.* 2004; 279: 7370-7377.

532. Klune J.R., Dhupar R., Cardinal J., Billiar T.R., & Tsung A. HMGB1: endogenous danger signaling. *Mol.Med* 2008; 14: 476-484.

533. Kazama H., Ricci J.E., Herndon J.M., Hoppe G., Green D.R., & Ferguson T.A. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity.* 2008; 29: 21-32.

534. Wang H., Bloom O., Zhang M., Vishnubhakat J.M., Ombrellino M., Che J., Frazier A., Yang H., Ivanova S., Borovikova L., Manogue K.R., Faist E., Abraham E., Andersson J., Andersson U., Molina P.E., Abumrad N.N., Sama A., & Tracey K.J. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285: 248-251.

535. Gardella S., Andrei C., Ferrera D., Lotti L.V., Torrisi M.R., Bianchi M.E., & Rubartelli A. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep.* 2002; 3: 995-1001.

536. Scaffidi P., Misteli T., & Bianchi M.E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; 418: 191-195.

537. Wessendorf J.H., Garfinkel S., Zhan X., Brown S., & Maciag T. Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor. *J Biol.Chem.* 1993; 268: 22100-22104.

538. Singer I.I., Scott S., Chin J., Bayne E.K., Limjuco G., Weidner J., Miller D.K., Chapman K., & Kostura M.J. The interleukin-1 beta-converting enzyme (ICE) is localized on the external cell surface membranes and in the cytoplasmic ground substance of human monocytes by immuno-electron microscopy. *J Exp.Med* 1995; 182: 1447-1459.

539. Mandinova A., Soldi R., Graziani I., Bagala C., Bellum S., Landriscina M., Tarantini F., Prudovsky I., & Maciag T. S100A13 mediates the copper-dependent stress-induced release of IL-1alpha from both human U937 and murine NIH 3T3 cells. *J Cell Sci* 2003; 116: 2687-2696.

540. Eigenbrod T., Park J.H., Harder J., Iwakura Y., & Nunez G. Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *J Immunol*. 2008; 181: 8194-8198.

541. Sakurai T., He G., Matsuzawa A., Yu G.Y., Maeda S., Hardiman G., & Karin M. Hepatocyte necrosis induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis. *Cancer Cell* 2008; 14: 156-165.

542. Dinarello C.A. Immunological and inflammatory functions of the interleukin-1 family. *Annu.Rev.Immunol*. 2009; 27: 519-550.

543. Dinarello C.A. Biologic basis for interleukin-1 in disease. *Blood* 1996; 87: 2095-2147.

544. Oppenheim J.J. & Yang D. Alarmins: chemotactic activators of immune responses. *Curr.Opin.Immunol*. 2005; 17: 359-365.

545. Bianchi M.E. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc.Biol*. 2007; 81: 1-5.

546. Hudson C.A., Christophi G.P., Gruber R.C., Wilmore J.R., Lawrence D.A., & Massa P.T. Induction of IL-33 expression and activity in central nervous system glia. *J Leukoc.Biol*. 2008; 84: 631-643.

547. Li J.Y., Burwell T.J., Coyle A.J., Humbles A.A., & Kolbeck R. The Pro-Inflammatory Cytokines, IL-1beta, TNFalpha and IFNgamma, Synergistically Induce IL-33 Secretion from Human Airway Epithelial Cells. *American Journal of Respiratory and Critical Care Medicine* 2009; 179: A5090.

548. Xu D., Jiang H.R., Kewin P., Li Y., Mu R., Fraser A.R., Pitman N., Kurowska-Stolarska M., McKenzie A.N., McInnes I.B., & Liew F.Y. IL-33 exacerbates antigen-induced arthritis by activating mast cells. *Proc.Natl.Acad.Sci.U.S.A* 2008; 105: 10913-10918.

549. Suzukawa M., Iikura M., Koketsu R., Nagase H., Tamura C., Komiya A., Nakae S., Matsushima K., Ohta K., Yamamoto K., & Yamaguchi M. An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J Immunol*. 2008; 181: 5981-5989.

550. Pecaric-Petkovic T., Didichenko S.A., Kaempfer S., Spiegl N., & Dahinden C.A. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 2009; 113: 1526-1534.

551. Kroeger K.M., Sullivan B.M., & Locksley R.M. IL-18 and IL-33 elicit Th2 cytokines from basophils via a MyD88- and p38{alpha}-dependent pathway. *J Leukoc.Biol*. 2009.

552. Smithgall M.D., Comeau M.R., Yoon B.R., Kaufman D., Armitage R., & Smith D.E. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int.Immunol*. 2008; 20: 1019-1030.

553. Kurowska-Stolarska M., Kewin P., Murphy G., Russo R.C., Stolarski B., Garcia C.C., Komai-Koma M., Pitman N., Li Y., Niedbala W., McKenzie A.N., Teixeira M.M., Liew F.Y., & Xu D. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol*. 2008; 181: 4780-4790.

554. Bourgeois E., Van L.P., Samson M., Diem S., Barra A., Roga S., Gombert J.M., Schneider E., Dy M., Gourdy P., Girard J.P., & Herbelin A. The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *Eur J Immunol*. 2009; 39: 1046-1055.

555. Cherry W.B., Yoon J., Bartemes K.R., Iijima K., & Kita H. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *J Allergy Clin.Immunol*. 2008; 121: 1484-1490.

556. Suzukawa M., Koketsu R., Iikura M., Nakae S., Matsumoto K., Nagase H., Saito H., Matsushima K., Ohta K., Yamamoto K., & Yamaguchi M. Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Lab Invest* 2008; 88: 1245-1253.

557. Espinassous Q., Garcia-de-Paco E., Garcia-Verdugo I., Synguelakis M., von A.S., Sallenave J.M., McKenzie A.N., & Kanellopoulos J. IL-33 enhances lipopolysaccharide-induced inflammatory cytokine production from mouse macrophages by regulating lipopolysaccharide receptor complex. *J Immunol*. 2009; 183: 1446-1455.

558. Rank M.A., Kobayashi T., Kozaki H., Bartemes K.R., Squillace D.L., & Kita H. IL-33-activated dendritic cells induce an atypical TH2-type response. *J Allergy Clin.Immunol*. 2009; 123: 1047-1054.

559. McLachlan J.B., Hart J.P., Pizzo S.V., Shelburne C.P., Staats H.F., Gunn M.D., & Abraham S.N. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. *Nat.Immunol*. 2003; 4: 1199-1205.

560. McLachlan J.B., Shelburne C.P., Hart J.P., Pizzo S.V., Goyal R., Brooking-Dixon R., Staats H.F., & Abraham S.N. Mast cell activators: a new class of highly effective vaccine adjuvants. *Nat.Med* 2008; 14: 536-541.

561. Kurowska-Stolarska M., Stolarski B., Kewin P., Murphy G., Corrigan C., Ying S., Pitman N., Mirchandani A., Rana B., van Rooijen N., Shepherd M., McSharry C., McInnes I., & Xu D. IL-33 amplifies the polarization of

alternatively-activated macrophages that contribute to airway inflammation. *J Immunol*. 2009; in press.

562. Kurowska-Stolarska M., Stolarski B., Kewin P., Murphy G., Corrigan C., Ying S., Pitman N., Mirchandani A., Rana B., van Rooijen N., Shepherd M., McSharry C., McInnes I., & Xu D. IL-33 amplifies the polarization of alternatively-activated macrophages that contribute to airway inflammation. *J Immunol*. 2009; in press.

563. Liu X., Li M., Wu Y., Zhou Y., Zeng L., & Huang T. Anti-IL-33 antibody treatment inhibits airway inflammation in a murine model of allergic asthma. *Biochem.Biophys.Res.Commun*. 2009; 386: 181-185.

564. King C., Brennan S., Thompson P.J., & Stewart G.A. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol*. 1998; 161: 3645-3651.

565. Patel J.A., Kunimoto M., Sim T.C., Garofalo R., Eliott T., Baron S., Ruuskanen O., Chonmaitree T., Ogra P.L., & Schmalstieg F. Interleukin-1 alpha mediates the enhanced expression of intercellular adhesion molecule-1 in pulmonary epithelial cells infected with respiratory syncytial virus. *Am J Respir Cell Mol.Biol*. 1995; 13: 602-609.

566. Chang C.H., Huang Y., Issekutz A.C., Griffith M., Lin K.H., & Anderson R. Interleukin-1alpha released from epithelial cells after adenovirus type 37 infection activates intercellular adhesion molecule 1 expression on human vascular endothelial cells. *J Virol*. 2002; 76: 427-431.

567. Chang C.H., Huang Y., & Anderson R. Activation of vascular endothelial cells by IL-1alpha released by epithelial cells infected with respiratory syncytial virus. *Cell Immunol*. 2003; 221: 37-41.

568. Kurowska-Stolarska M., Stolarski B., Kewin P., Murphy G., Corrigan C., Ying S., Pitman N., Mirchandani A., Rana B., van Rooijen N., Shepherd M., McSharry C., McInnes I., & Xu D. IL-33 amplifies the polarization of alternatively-activated macrophages that contribute to airway inflammation. *J Immunol*. 2009; in press.