The Role of IL-33/ST2 Pathway in Innate Immune Response in Airway Inflammation

Bartosz Stolarski

MSc

A thesis submitted for the Degree of Doctor of Philosophy to the College of Medical, Veterinary and life Sciences, University of Glasgow

September 2010

Containing work carried out in the Institute of Infection, Immunity & Inflammation, University of Glasgow, G12 8QQ

Abstract

Asthma is a common and complex inflammatory disease of the airways characterized by deregulated immune responses that involves activation of multiple cell types including Th2 cells, IgE producing B cells, mast cells, basophils and eosinophils as well as resident lung cells such as epithelial, smooth muscle cells and macrophages. Despite intensive research, there are still unmet needs in the treatment of asthma. Recently, a new cytokine of IL-1 family, named IL-33 emerged as a potentially important factor in the immunopathogenesis of allergy and asthma. It was recently shown in our laboratory that intranasal administration of IL-33 can induce certain physiological features that are characteristic of experimental asthma, such as eosinophilic inflammation, Th2 cytokine and antibody production as well as increased airway hyperresponsiveness.

The effect of IL-33 on the activation and differentiation of allergen specific Th2 cells has been well studied. However, the contribution of IL-33 to the activation of lung resident and inflammatory innate cells remains undefined. In this project I focused on alveolar macrophages and eosinophils as both cell types were reported to express IL-33R, ST2L and are thought to play a crucial role in asthma pathogenesis.

I raised the hypothesis that IL-33 released locally in the lungs may trigger symptoms resembling asthma through the activation of airway alveolar macrophages. Furthermore, I hypothesize that IL-33 may exacerbate and maintain inflammation in the lungs by the direct activation of eosinophils.

In our previous study we showed that IL-33 could switch the quiescent phenotype of alveolar macrophages toward the alternatively activated phenotype (M2, AAM). In the first part of my thesis I looked at the consequences of this phenomenon for airway inflammation. Using clodronate liposomes *in vivo* I was able to eliminate macrophage population from the lungs and demonstrated that resident alveolar macrophages are crucial for the development of IL-33-induced eosinophilic inflammation in the airways. I then examined the contribution of IL-13, a known M2 differentiation factor, to airway inflammation. Using anti-IL-13 neutralizing antibodies I showed that IL-13 is required for the IL-33-triggered differentiation of alveolar macrophages toward M2 phenotype as well as for eosinophilic inflammation.

Next, I looked at how IL-33/ST2 pathway modulates the differentiation and activation of eosinophil. I demonstrated that bone marrow hematopoietic progenitors $CD117^{+}$ express ST2L and that IL-33 is able to differentiate these cells toward eosinophils. By employing deficient mice or neutralizing antibodies I found that this process is ST2 and IL-5 dependent and independent of IL-13.

I then extended my research interests to include mature mouse and human eosinophils. I showed that both human and mouse resting eosinophils express low levels of ST2L which can be markedly increased by IL-33. Moreover, I demonstrated that eosinophils that are recruited to the lungs during experimental allergic airway inflammation express high levels of ST2L. Furthermore, I carried out a study on effector function of eosinophils. I found that IL-33 induces IL-13, IL-6 and increases TARC, TGF-β production by mouse eosinophils. In addition, IL-33 exacerbated IgG-induced human and mouse

eosinophil degranulation, likely by enhancing FcγRII expression. Having shown earlier that IL-13 is requited for the polarization of alveolar macrophages toward AAM by IL-33 *in vitro* and in light of the fact that IL-33-stimulated eosinophils can be a significant source of IL-13; I went on to investigate the interaction between macrophages and eosinophils. Using co-cultures of $ST2^{-/-}$ macrophages with WT eosinophils in Transwell system, I demonstrated that IL-33- but not IL-5-activated eosinophils can support macrophage polarization toward the pro-inflammatory AAM phenotype, partially through the production of IL-13.

Finally, given the role of IL-33/ST2L axis in eosinophil activation *in vitro*, I investigated the contribution of IL-33-activated eosinophils to airway inflammation *in vivo*. Using adoptive transfer protocol I showed that the contribution of IL-33-activated eosinophils to airway inflammation is mediated primarily by the release of cytokines from these cells which, in turn, recruits other inflammatory cells and supports the differentiation of alveolar macrophages towards AAM.

These data show that IL-33/ST2 pathway regulates multiple features of alveolar macrophage and eosinophil biology that can have a significant impact on asthma pathophysiology in the airways. Studies carried out in our laboratory and elsewhere suggest that IL-33 is equally capable of activating other cell types that have been implicated in asthma pathology such as Th2, B1 cells, DCs, mast cells and basophils. Therefore, targeting IL-33/ST2 pathway may potentially offer a promising therapeutic approach to asthma and allergy.

Acknowledgments

My first gratitude goes to my promoters, Professor Eddy Liew and Dr Damo Xu for giving me the opportunity to undertake this research.

I am grateful to Dr Charles McSharry who generously agreed to review my thesis before it was submitted. He had also generously shared his deep knowledge of the field as well as had helped me master some of the most effective *in vivo* techniques that were required for this project.

I would also like to collectively thank Dr Alistair Gracie, Dr Ashley Gilmour, Mrs Helen Arthur and Mr James Reilly for helping me with many of those countless technical problems that I had to deal with throughout this study.

Finally, my special thanks go to my wife Mariola, for her steady love, patience and moral support, all of which helped me overcome moments of discouragement.

Declaration

The work presented in this thesis represents original work carried out by the author. This thesis has not been submitted in any form to any other University. Where reagents, materials or practical support has been provided by others, appropriate acknowledgement has been made in the text.

TABLE OF CONTENTS

List of figures

List of tables

List of publications

Zaiss MM, Kurowska-Stolarska M, Böhm C, Gary R, Scholtysek C, **Stolarski B**, Reilly J, Kerr S, Millar NL, Kamradt T, McInnes IB, Fallon PG, David JP, Liew FY, Schett G. *IL-33 Shifts the Balance from Osteoclast to Alternatively Activated Macrophage Differentiation and Protects from TNF-a-Mediated Bone Loss*. Journal of Immunology. 2011 Apr 22. PMID: 21515798.

Kurowska-Stolarska M, Hueber A, **Stolarski B**, McInnes IB. *Interleukin-33: a novel mediator with a role in distinct disease pathologies*. Journal of Internal Medicine 2011 Jan;269(1):29-35. Review. PMID: 21158975.

Kurowska-Stolarska M, Hueber A, **Stolarski B**, McInnes IB: *IL-33: a novel mediator with a role in distinct disease pathologies*. Journal of Internal Medicine 2011 Jan;269(1):29-35. PMID: 21158975.

Stolarski B, Kurowska-Stolarska M, Kewin P, Xu D, Liew FY: *IL-33 Exacerbates Eosinophil-Mediated Airway Inflammation*. Journal of Immunol., 2010, 185: 3472-80. PMID: 20693421.

Kurowska-Stolarska M, **Stolarski B**, Kewin P, Murphy G, Corrigan CJ, Ying S, Pitman N, Mirchandani A, Rana B, van Rooijen N, McSharry Ch, Shepherd M, McInnes IB, Xu D, Liew FY: IL-33 amplifies the polarization of alternatively-activated macrophages that contribute to airway inflammation. J Immunol., 2009, 183:6469-7. PMID: 19841166.

Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, **Stolarski B**, Garcia CC, Komai-Koma M, Pitman N, Li Y, Niedbala W, McKenzie AN, Teixeira MM, Liew FY, Xu D: IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. J Immunol., 2008, 181:4780-90. PMID: 18802081.

Abbreviations

-/-	gene deficient mice
AAM	alternatively activated macrophages, subset of M2
Ab	antibody (Abs, plural)
AHR	airways hyperresponsiveness
alum	aluminium hydroxide
AMCase	acidic mammalian chitinase
AP-1	activator protein-1
APC	allophycocyanin
BAL	bronchoalveolar lavage
BM	bone marrow
BMM	bone marrow derived macrophages
C/EBP	CCAAT enhancer-binding protein
c-kit	stem cell growth factor receptor, SCFR (CD117)
CCR3	C-C chemokine receptor type 3
CD	clusters of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CMP	common myeloid progenitor
DAMP	damage-associated molecular patterns
DC	dendritic cell
ECM	extracellular matrix
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
ELISA	enzyme linked immunosorbant assay
EoP	eosinophil progenitor

EPO	eosinophil specific peroxidase
ERK	extracellular-signal-regulated kinase
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FIZZ/RELMα	resistin-like secreted protein
FLT3L	FMS-like tyrosine kinase ligand
FOG-1	friend of GATA-1
FSC	fetal calf serum
GATA-1	GATA-binding factor 1
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte/macrophage progenitor
HMGB1	high mobility group box 1
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
HTH	helix-turn-helix
I.N.	intranasal
I.P.	intraperitoneal
IC	immuno-complex
Icsbp	interferon consensus sequence binding protein also known as IFN response factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1RAcP	IL-1 receptor accessory protein

IP-10	interferon-gamma-induced protein (CXCL10)
IRAK	IL1 receptor-associated kinase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KC	keratinocyte chemoattractant (CXCL1)
LPS	lipopolysaccharide
M1	classically activated macrophages
M2	non-classically activated macrophages
M2c	immunosuppressive macrophages, subset of M2
MAPK	mitogen-activated protein kinases
MBP	major basic protein
MCP-1	monocyte chemotactic protein-1 (CCL2)
M-CSF	macrophage colony stimulating factor
MDC	macrophage-derived C-C chemokine (CCL22)
MEP	megakaryocyte-erythroid progenitor
MIG	monokine induced by gamma interferon (CXCL9)
MIP-1α	macrophage inflammatory protein-1 (CCL3)
MIP-2α	chemokine (C-X-C motif) ligand 2 (CXCL2)
MMP	matrix metalloproteinase
MR	mannose receptor (CD206), M2 macrophages marker
MyD88	myeloid differentiation primary response gene (88)
NF-HEV	nuclear factor from high endothelial
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell

OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PB	peripheral blood
PBS	phosphate buffered saline
PD-L1 and L2	ligands for programmed death 1 (PD-1)
PE	phycoerythrin
PGE2	prostaglandin E2
PMA	phorbol 12-myristate 13-acetate
PU.1	transcription factor that binds to the PU-box
RANTES	regulated upon activation, normal T cell expressed and secreted (CCL5)
RT-PCR	reverse transcription polymerase chain reaction
SCF	stem cell factor
SEM	standard error of the mean
SIGIRR	single Ig IL-1-related molecule
SMC	smooth muscle cells
sST2	soluble ST2; soluble receptor for IL-33
ST2L	membrane bound ST2, receptor for IL-33
STAT	signal transducers and activators of transcription
T1/ST2	IL-1 receptor like molecule (IL-1RL1), receptor for IL-33
TARC	thymus- and activation-regulated chemokine (CCL17)
TGF-β	transforming growth factor beta
Th1, Th2	T helper 1; T helper 2
TIR	Toll-IL-1R domain
TLR	toll-like receptor

TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
TRAF	TNF receptor-associated factor
Treg	regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
VEGF	vascular endothelial growth factor
WT	wild type

Chapter 1:

Introduction

1.1. Interleukin-33

A cytokine interleukin-33 (IL-33, IL-1F11) as it is now called, was initially reported as a clone DVS27 gene product, highly upregulated in canine vasospastic cerebral arteries after subarachnoid hemorrhage (2). Then, in 2003, the same sequence was described as a nuclear factor present in endothelial cells of high endothelial venules (HEVs) of secondary lymphoid organs (HEVECs), namely human lymph nodes, tonsils and Peyers's patches and referred to as NF-HEV (3). However, apart from the suggestion by the authors, that NF-HEV can help controlling the specialized HEV phenotype there was a paucity of data on the role of the protein.

In 2005 Schmitz and colleagues used a molecular profile based on the properties of already known IL-1 cytokine family members to search genetic sequence databases (4). They established that NF-HEV molecule was in fact a member of IL-1 cytokine superfamily and shared many molecular properties with several closely related cytokines, such as IL-1α/β (IL-1F1/IL-1F2), IL-1Ra (IL-1F3), and IL-18 (IL-1F4). As a newly identified member of IL-1-like cytokines, NF-HEV was renamed to IL-33 (IL-1F11) .

1.1.1. Structure and molecular properties of IL-33

Schmitz et al. matched IL-33 to its specific receptor, a protein known as orphan receptor T1/ST2 (IL-33Rα) which will be discussed in detail in a separate chapter (4). These authors found the human IL-33 gene to be located on chromosome 9 in 9p24.1 region, while its murine counterpart was mapped to chromosome 19qC1 region. The lengths of IL-33-encoding cDNA sequences are

similar: in humans a full length IL-33 protein (pro-IL33) of 30 kDa molecular mass is constituted by 270 amino-acids while its murine counterpart is 266 amino-acid in length and forms a 29.9 kDa protein. At the amino acid level the two proteins share 55% of the sequence. Moreover, by aligning amino acid sequences of human and murine mature IL-33 with members of the IL-1 family, Schmitz and colleagues demonstrated that both IL-33 proteins share 12 β strands that make up the characteristic β-trefoil fold, a structure first described in IL-1 by Priestle et al (5). Phylogenetic tree analysis revealed that among other known IL-1 family members IL-33 is most closely related to IL-18 (IL-1F4). In contrast to other family members neither of the two cytokines is linked to the IL-1 gene cluster on human chromosome 2.

The structure of IL-33 suggests that it has the capacity to act both as a cytokine and as a nuclear factor. Indeed, unlike most other members of the IL-1 family of cytokines, with one exception for IL-1α, IL-33 is often found in the nucleus rather than cytoplasm (3, 6-7). In humans, it is commonly localized in nuclei of epithelial and endothelial cells (3, 6, 8) while in mouse it has also been shown to have affinity for chromatin of bone marrow-derived mast cells *in vitro* (9). Its nuclear localization is mediated by a nuclear localization signal in its N'-terminus (NLS), while chromatin binding capabilities are facilitated by a homeodomain (helix-turn-helix-like motif) (10-11), which presents close homology to LANA (latency-associated nuclear antigen), a protein coded by Kaposi's sarcoma-associated herpesvirus (11). The other portion of IL-33 protein at the carboxyl terminus is a cytokine domain formed by 12 βstrands as mentioned above, called the IL-1-like domain. Apart from the fact that IL-33

can bind to the acidic residues of a dimeric histone (H2A-H2B) thereby suppressing gene transcription *in vitro*, the role for IL-33 as a nuclear factor remains obscure (11).

1.1.2. IL-33 processing

Similar to other members of IL-1 family, such as IL-18 and IL-1β, IL-33 lacks signal peptide at the N' terminus that is required for a successful assembly via the endoplasmic reticulum and transport through the Golgi apparatus (4). However, despite early predictions IL-33 differs considerably from the two cytokines in the way that it is cleaved by proteases. Although *in vitro* studies showed that IL-33 can be processed by caspase-1 from pro-IL-33 30 kDa down to what was originally assumed to be an active mature form of 20-22kDa, the reaction is rather inefficient, when compared with the cleavage of IL-1β (12-14), and does not occur in the environment devoid of other proteases (14). Even when cells were modified by introducing mutations carrying a potential caspase-1 binding site for the IL-33 to be expressed, the 30kDa pre-IL-33 was still dominant. However, studies by other groups showed that the cleavage could be prevented by using a specific caspase-1 inhibitor (4, 12). It was suggested that the activation of the NLRP3-inflammasome with alum adjuvant *in vivo* activates caspase-1 which, in turn, cleaves pro-IL-33 allowing for its secretion (15). The mature form of IL-33 can also be produced by calpain-mediated cleavage (16). Interestingly, experiments showed that proteases that are activated during cell apoptosis, such as caspase-7 and caspase-3 could do the cleavage (12-13, 17). These apparently conflicting data may be partially explained by the observation that pro-IL-33 lacks a classical

caspase-1 binding site (Derek Gilchrist, unpublished data). Instead, it contains cleavage sites for caspase-7 and caspase-3 are located within its IL-1-like domain (cytokine domain). Therefore it is possible that pro-IL-33 is not a primary target for caspase-1. Rather, the protein is actually processed by other proteases that are controlled by caspase-1. The important point is that the maturation process facilitated by caspases renders the IL-33 biologically inactive.

1.1.3. Secretion of IL-33

There are limited and inconclusive data available about how IL-33 is released. It was shown that IL-33 is released by human acute monocytic leukemia cell line (THP-1) upon stimulation with alum adjuvant, lypopolysaccharide (LPS), LPS with alum, or upon infection with *Listeria moncytogenes* and *Salmonella typhimurium* (14-15). Stimulation with a known cell activator PMA (phorbol 12-myristate 13-acetate) was reported to induce IL-33 secretion from rat cardiac fibroblasts (18). Supporting data was also obtained in mixed murine glial cells cultured in the presence of LPS and ATP (19).

Recently, an alternative mechanism for IL-33 release through cell necrosis has been proposed that takes into account the indirect nature of IL-33 processing by caspases as well as the primarily nuclear localization of a full-length protein *in vivo* (6, 10). This notion stems from the observation that IL-33 harbours structural elements very similar to those found in IL-1α and so-called high mobility group box 1 (HMGB1). The latter is a nuclear factor with transcription-suppressing capabilities commonly found in the nucleus and is

only released through cell necrosis when it contributes locally to the inflammatory processes at sites of tissue damage (20-22). Although HMGB1 is effectively acting as a potential danger signal associated with tissue damage, it is rendered inactive by the caspase-dependent processes associated with apoptosis (23). In this case it contributes to the apoptotic cell-mediated tolerance. The necrosis-associated mechanism of IL-33 release is consistent with its postulated role as a novel 'alarmin'. By definition, alarmins are extra-cellular mediators that serve as an endogenous, 'danger signal', designed to be released immediately upon and as a direct result of tissue damage or pathogens breaching the epithelial barriers. It is possible that IL-33 may be part of such warning system by complementing signals from so-called pathogen-associated molecular pattern receptors (PAMPs), such as TLR family, or NOD-like receptor protein family (nucleotide-binding oligomerization domain (24-25). If so, the release mechanism through cell necrosis may actually be the primary one, while the caspase-dependent mechanism, which renders mature IL-33 biologically inactive (12-13) may be used during steady state tissue turnover where there is no need for triggering inflammation and mounting a defence response.

Taken together, it seems that the truncated form of IL-33 is inactive as a cytokine and released during apoptosis, while the immature (proIL-33) form of the protein is released during cell necrosis and may act as an endogenous 'alarmin'.

1.1.4. Expression pattern of IL-33 in homeostasis and disease

The list of cellular sources of IL-33 seems to be consistent with the proposed role of IL-33 as an alarmin. The cytokine is predominantly expressed in structural lining cells that act as a primary defence mechanism against invading pathogens. IL-33 can be found across the vascular system, particularly in endothelial cells of high endothelial venules (HEV cells), but also in large and small vessel endothelial cells, including liver, colon, small intestine, stomach, kidney, prostate, skin and skeletal muscles (6, 8) where it is commonly mapped to the nucleus. Another common location for IL-33 protein is the epithelial cells of many organs, including stomach, tonsilar crypts, salivary glands, skin, and lung tissue (6, 26). IL-33 protein expression can be markedly elevated compared to steady state at several locations under pathologic conditions which includes lung epithelial cells and smooth muscle cells of asthmatic patients (26-28), skin from atopic dermatitis patients (29), brain of Alzheimer's disease patients, pancreatic myofibroblasts of chronic pancreatatis patients (30), colon of ulcerative colitis patients (31), and synovium of rheumatoid arthritis (RA) patients (6-7, 32). In inflammatory arthritis model, TNF-α and IL-1β are thought to be responsible for IL-33 upregulation (7).

Although full-length, nuclear IL-33 protein was found constitutively expressed in resting blood vessel endothelium, the expression is rapidly down-regulated on the onset of tumor- or wound healing-associated angiogenesis, compared to vessels in healthy tissue. Some *in vitro* experiments strongly suggest that nuclear IL-33 expression can only occur during cell cycle arrest. However, the non-proliferatory status alone was not sufficient to restore the level of nuclear

IL-33 that could be detected by immunohistochemistry. This required an additional cell contact inhibition signal that *in vivo* may come from spatial restrictions in densely packed endothelial cells of healthy vasculature. Similar cell-to-cell signal could be generated in super-confluent endothelial cell monolayers *in vitro,* as shown in HUVEC cultures. Consistently, nuclear IL-33 was scarcely detectable (or not at all) in immature blood vessels of human tumours which are highly angiogenic (8). In contrast, another study concluded that long, immature form of nuclear IL-33 protein is abundant in many human tumours, as well as in normal tissues, making it suitable for use as a general nuclear marker of endothelial cells (6). This apparent discrepancy still awaits to be explained as tumour tissues, owing to their high proliferation status, tend to become hypoxic which, on its own, should enhance nuclear IL-33 expression (Neal Millar, personal communication), unless the inhibition caused by VEGF can counteract the effect of hypoxia. Interestingly, as a cytokine, IL-33 induces both angiogenesis and vaso-permeability, which is similar to what VEGF does. Furthermore, IL-33 increases the endothelial cell proliferation, migration, and tubular network formation comparable with VEGF *in vitro*. Consistently, IL-33 promoted vessel sprouting and neovascularization in mice *in vivo*, as well as the proliferation, migration, and morphologic differentiation of human endothelial cells *in vitro*, apparently by stimulating endothelial NO production (33). Given the distinct roles of nuclear and released forms of other cytokines, such as IL-1, similar effect may occur in endothelial cells expressing IL-33. However, the precise contribution of both forms of IL-33 to the regulation of angiogenesis and homeostatic tissue turnover awaits further clarification.

In terms of IL-33 expression on transcriptional level, IL-33 mRNA is ubiquitously expressed across many tissues and can be detected virtually at any location in human and murine systems. To date, the only major organ in which IL-33 mRNA was not detected is human spleen, although IL-33 message was found in mouse spleen tissue (4), as well as in mouse splenic dendritic cells upon stimulation with LPS.

1.1.5. IL-33 signaling via ST2L and IL-1RAcP complex

In 2005 Schmitz et al. (4) identified that so called "orphan receptor", T1/ST2 (or IL-1R4, now also called IL-33Rα), a molecule known for sixteen years, is a receptor for IL-33 (IL-1F11). This was soon supplemented by the observation that another molecule, IL-1R accessory protein (IL-1RAcP), is required in the formation of a fully functional IL-33 receptor complex (34-35). In fact, IL-1RAcP is widely shared between several other IL-1-related cytokines, namely IL-1α (IL-1F1), IL-1β (IL-1F2), IL-1F6, IL-1F8 and ILF-9, to form heterodimer receptor complexes with the respective alpha chains that assure single-cytokine specificity of the receptor complexes they constitute. The expression of ST2 gene can produce two major splice variants of ST2 mRNA, the membrane bound form, ST2L, and truncated soluble form, sST2. Each splice variant is controlled by a pair of distinct promoters (36). Also, alternate splicing variants (ST2LV and sST2V) were reported in humans and chickens (37-38). The membrane-bound ST2L is required for mediating IL-33 signaling, whereas soluble sST2 acts as a decoy receptor by masking IL-33 biologic activity (39-40) in a similar fashion that soluble IL-1R does to IL-1 signaling. IL-33 binds specifically to ST2L and does not cross-react with any other IL-1R related

receptors (4, 41-42). Both ST2L and IL-1RAcP are necessary for IL-33 action as mice deficient either for IL-1RAcP or ST2 are completely unresponsive to IL-33 administration while wild type animals exhibit a marked inflammatory reaction (35, 43). Consistently, it was demonstrated that soluble IL-1RAcP enhances the ability of soluble ST2 to inhibit IL-33 signaling (42).

Several recent studies demonstrated that IL-33-generated signal can also be regulated through the binding of IL-33 to an alternate receptor complex that consists of ST2L and so called single Ig IL-1-related molecule (SIGIRR), also referred to as Toll IL-1R8 (TIR8) (44). This molecule has been implicated to be a negative regulator of Toll-like receptor (TLR)- and IL-1R-mediated immune responses (45). SIGIRR-deficient dendritic cells exhibit increased responsiveness to the stimulation with TLR ligands as well as IL-1 and IL-18 (46) and SIGIRR-deficient Th2 cells showed a more abundant expression of Th2-associated cytokines in response to IL-33 (44). These data suggest that SIGIRR/ST2L receptor complex may function as a negative regulator of IL-33, similarly to sST2 decoy receptor.

The membrane bound IL-33 receptor, ST2L contains common intercellular domain called Toll-IL-1R (TIR) domain, which is shared among the entire TLR/IL-1R receptor superfamily. It was demonstrated that TIR domain of IL-1RAcP directly interacts with the TIR domain of ST2L/IL-33 and contributes to facilitating the IL-33-mediated signal (35). Downstream of the IL-33/ST2L/IL-1RAcP complex, IL-33 signal is passed on by several adapter molecules, most of which being commonly used by other members of IL-1R family. Schmitz et al. showed that IL-33 binding triggers the recruitment of the

myeloid differentiation primary-response protein 88 (MyD88), and TNF receptor-associated factor (TRAF6) to TIR domain in the cytoplasmic portion of ST2L (and IL-1RAcP) where the molecules are activated (4). The involvement of protein MyD88 adaptor, but not TRIF, was found indispensable for ST2L/IL-33 signaling, both *in vitro* (43, 47) and *in vivo* (48). Downstream of TIR/MyD88/TRAF6 interactions, a cascade of phosphorylation includes IL-1R-associated kinase 1 (IRAK1) and IRAK4, which eventually leads to the activation of all three mitogen-activated protein kinases (MAP kinases) and a transcription factor called nuclear factor-κB (NF-κB) in T cells and mast cells (4, 43). Consistently, Schmitz et al. demonstrated that Erk1/2, p38 and NF-κB activation can be blocked in the presence of anti-ST2 antibody *in vitro* (4). Furthermore, the detailed analysis of IL-33-induced mast cell activation revealed that IL-33 triggers NF-κB phosphorylation by intracellular calcium mobilization mediated by phospholipase D and sphingosine kinase 1 (29).

As mentioned above, IL-33 can also signal entirely independent of Ca^{2+} in which case a phosphorylation cascade of MAP kinases is triggered, including Erk1/2, p38 and JNK, that results in the activation of transcription factor called activator protein 1 (AP-1) (4, 29, 43). Interestingly the IL-33-induced activation of p38, JNK and NF-κB depends on TRAF6 phosphorylation, while ERK can be activated in a TRAF6-independent fashion as demonstrated in mouse embryonic fibroblasts (MEFs) (49). Both pathways (NF-κB and MAP kinases) can be regarded as complementary to one another in a sense that each facilitates some of the responses, but not the others. For example, in T cells IL-33 mediates the production of IL-5 and IL-13 via different mechanisms: whereas

the IL-33-induced IL-5 expression could be suppressed by specific inhibitors of p38, ERK1/2 and JNK1/2 but not the inhibitor of NF-κB, the expression of IL-13 was suppressed by p38, NF-κB and, to a lesser extent, by ERK1/2 and JNK1/2 inhibitors. Also, it was recently shown that IL-33 mediated the production of IL-1β, IL-3, IL-6, TNF-α, MIP-1α (CCL3), MIP-2α (CXCL2), MCP-1 (CCL2), prostaglandin D_2 leukotriene B_4, and triggered degranulation in IgE-primed human and murine mast cells in an NF-κB-dependent manner. In contrast, the production of IL-5, IL-13, Eotaxin-2 (CCL24), RANTES (CCL5) and TARC (CCL17) was mediated by MAPK pathway and was essentially NF-κB-independent (29).

1.1.6. The role of IL-33 in type 1 immune responses

1.1.6.1. Rheumatoid arthritis

Based on the postulated role for IL-33 as a novel alarmin, one can predict that the levels of IL-33 are likely to be elevated at sites of high burden of cell death and that its function may be modulated by local milieu of cellular mediators. Indeed, elevated levels of IL-33 have been reported in patients with Th1/Th17-driven autoimmune diseases (7, 50). IL-33 and its receptor have been specifically mapped to disease-affected tissues - synovial fluid (51) and synovium of RA (7) and in skin lesions of psoriatic patients (52). In vitro analysis points at synovial fibroblasts as a possible source of IL-33 in RA synovium as they markedly up-regulate IL-33 expression upon TNF-α or IL-1β stimulation, both of which are hallmark cytokines in the immunopathogenesis of RA (7). Further evidence for the stromal expression of IL-33 comes from hTNF

transgenic mice that show abundant IL-33 expression in the bone and cartilage-invading synovium (Dr M. Kurowska-Stolarska, personal communication). Studies on ST2 deficient mice revealed that IL-33/ST2 pathway is required for the development of both cellular and humoral autoimmunity as well as chronic articular inflammation in arthritis. ST2 deficient mice developed significantly attenuated collagen induced arthritis manifested by lower incidence and severity score. This was associated with lower levels of collagen-specific antibodies detected in sera and inhibited production of pro-inflammatory cytokines such as IFN-γ, IL-17 and TNF-α by draining lymph node cells and by articular tissue. A study on the mechanism behind this process revealed that IL-33-triggered, mast cell-derived IL-1 and IL-6 are responsible for shifting the balance of T cell response toward pathogenic Th17 and Th1 (7).

1.1.6.2. Atherosclerosis and obesity

Recently, IL-33/ST2 has been identified as a crucial pathway in adipose tissue biology. This is especially interesting in light of a current hypothesis that links adipose tissue immune status to glucose homeostasis. It is believed that adipose tissue infiltration by Th1 cells and pro-inflammatory macrophages (also known as M1) results in obesity and obesity-induced metabolic damage. Conversely, Th2- and Treg-derived cytokines and, most importantly, the alternatively activated macrophages (AAM, also known as M2 macrophages) are protective factors against obesity and type 2 diabetes (53). It was found that IL-33 is expressed in human adipose tissue predominantly in adipocytes and pre-adipocytes (54). Furthermore, IL-33R is expressed by adipose tissue

macrophages and adipocytes themselves (55). Mature mice deficient in ST2 have an enhanced weight gain and more adipose tissue compared to WT mice while on a high fat diet suggesting that the endogenous IL-33/ST2 pathway plays a beneficial role in glucose homeostasis. *Ex vivo* treatment of epididymal stromal vascular fraction (eSVF) containing pre-adipocytes with IL-33 caused the inhibition of lipid accumulation that was associated with reduced expression of genes linked to lipid metabolism and adipogenesis. Most importantly, IL-33 decreased the expression of resistin a mediator that is responsible for development of insulin resistance and type 2 diabetes. Administration of IL-33 has a protective effect on body composition and glucose homeostasis by decreasing body fat, adipocytes size and total serum cholesterol. Obesity is also an independent risk factor for the development of chronic inflammation of arterial wall (atherosclerosis) which can eventually cause myocardial infraction and stroke. The development of atherosclerosis involves a complex interaction between vascular endothelium, serum lipids, inflammatory macrophages, Th1 cells, platelets and vascular smooth muscle cells (56). Recently, it has been found that the IL-33/ST2 pathway, in addition to being protective against adipose tissue inflammation, also protects against atherosclerosis. IL-33 is expressed in normal and atherosclerotic vasculature of mice deficient in ApoE, a protein essential for the transport and metabolism of lipids. Administration of IL-33 to $ApoE^{-/-}$ mice on a high fat diet reduced the size of the atherosclerotic lesion. The underlying mechanism of this protective effect of IL-33 against atherosclerosis and obesity is the ability of IL-33 to trigger type 2 immune responses such as Th2 cytokine production and AAM (M2) macrophage

differentiation as originally reported by Kurowska-Stolarska et al (26) and described in detail in chapter 1.2.2.

1.1.7. The role of IL-33 in type 2 immune responses

1.1.7.1. Asthma and murine airway inflammation models

Asthma is a complex and heterogeneous disease which is characterized by chronic inflammation of the airways associated with variable airflow obstruction arising from various genetic and environmental factors (57-58). The disease stems from an individuals hyper-response to innocuous environmental antigens that with time may lead to marked histlogical changes that can compromise lung function. This disproportional immune response to allergens is mediated by $CD4^+$ T helper 2 cells, eosinophils, mast cells, neutrophils, macrophages, and IgE antibodies. The pathophysiology of asthma includes mucus hyper-secretion, bronchial hyper-responsiveness and smooth muscle hypertrophy and thickening of the epithelial basement membrane (57, 59).

There are several lines of evidence that IL-33 is an important effector cytokine in asthma and allergy. IL-33 is expressed more abundantly in asthma patients compared to healthy individuals, which could be detected systemically in sera from affected individuals (60). However, the primary source tissue is thought to be the lung epithelial cells (26) and smooth muscle cells (27) which, owing to their localization in pulmonary mucosal barrier, may readily respond to a variety of environmental stimuli. Recently, Genome-Wide Association Studies (GWAS) identified several single nucleotide polymorphisms (SNPs) within IL-33 and ST2 coding genes that are more frequent in asthma sufferers compared to healthy

subjects. These findings may represent the first step toward understanding the genetic mechanisms responsible for allergy and asthma (61-63). Moreover, in the ovalbumin (OVA)-induced murine asthma model of airway inflammation the levels of both soluble ST2 and IL-33 mRNA are markedly elevated in sera and lung tissues respectively (41, 43, 64). In addition, the administration of exogenous IL-33 into lungs, leads to asthma-like phenotype (43, 48).

Numerous *in vivo* studies allowed for a fairly detailed investigation into the role of ST2/IL-33 pathway in allergy and asthma pathology. Those which used ST2-deficient animals demonstrate that permanent lack of IL-33 signaling can markedly attenuate key inflammatory responses such as BAL fluid eosinophilia, macrophage count and levels of IL-5, Eotaxins (CL11 and CCL24) and TARC (CCL17), together with pulmonary inflammation, while BAL fluid expression of IL-4 and IL-13 did not change, compared to BALB/c mice (26, 43). Serum IgG_1 and IgE levels were also normal (43). This outcome was reproducibly obtained in so-called acute airway inflammation model with a single OVA/alum sensitization, followed by three challenges on day 8, 9 and 10, with analysis on day 12 (43). However, if a model that involves two sensitizations (12 to 14 days apart) was investigated, most of the responses in ST2-deficient animals were similar to those in BALB/c mice (43, 65-66). These data suggest that, at least in animal models of asthma, the contribution of IL-33/ST2 pathway is indispensable in the acute phase of the antigen-driven responses, whereas in the chronic phase it can be compensated for by different inflammatory pathways.

Several groups assumed a different approach at investigating the role of the ST2/IL-33 pathway in asthma models and employed various techniques to transiently disrupt IL-33 signal, such as the use of anti-ST2 antibody (clone E310) (67-68), anti-IL-33 antibody (69), or soluble ST2-Fc fusion protein (64). Regardless of which particular technique was used to transiently block IL-33 signal the differences include reduced eosinophil count and IL-5 levels detected in BAL fluid (64, 67, 69), reduced IL-4 in BAL fluid (64, 68-69), lower levels of IL-13 detected in BAL fluid, as well as systemically reduced OVA-specific IgE levels (67, 69), reduced airway hyper-responsiveness, mucus secretion and $ST2^+$ cell infiltration in lungs (68), and reduced pulmonary inflammation (69). Thus, these studies confirmed the results obtained from ST2 deficient mice.

While IL-33 acts on many different cell types in experimental asthma, the contribution of ST2/IL-33-activated T cells is among the most extensively studied aspects of the disease. By means of adoptive transfer of Th2 cells it was demonstrated that the response to OVA in recipient animals that had received either anti-ST2 antibody or ST2-IgG fusion protein is diminished, as reflected by reduced eosinophil numbers, reduced IL-4 and IL-5, as well as IL-6, IL-13 levels in BAL fluid (67, 70), while levels of IL-10 and IFN-γ remained unchanged (67). Moreover, it has been demonstrated recently that the resolution of allergic inflammation and airway hyperresponsiveness (AHR) depends on the neutralization of ST2/IL-33 pathway in T cells. This suggests that Il-33 is necessary not only for the development of allergic response but also for its maintenance (68).

In addition, a group from our own laboratory found that in the presence of antigen stimulation (OVA) or anti-CD3 antibody, IL-33 can potently drive a subpopulation of $CD4^+$ cells that produce mainly IL-5 and IL-13 but not IL-4, which makes it distinct from the classical Th2 cells (43). Unlike Th2 cells, this IL-33-differentiated, IL-5-producing sub-population is entirely independent of IL-4 as it has been demonstrated to develop in IL-$4^{-/-}$ mice and, most importantly, could exacerbate OVA-induced airway inflammation both in IL-$4^{-/-}$ and WT background animals on a similar scale. The key role of IL-33 in the activation of those cells is consistent with the fact that IL-33 signaling does not induce GATA-3 or STAT6 factors, that classical Th2 cell population depend on. Furthermore, it was shown that dendritic cells (DCs) respond directly to IL-33 in a ST2-dependent manner and are capable of supporting the development of a IL-5^+IL-4^- Th2 cell subset (71). Therefore, these studies provide a possible mechanism explaining the existence and activation of IL-5-producing T cells in IL-4-deficient mice in different disease models (72-76). In the light of an increasing interest in different pathological phenotypes of asthma, further clinical studies into the contribution of the classical Th2 versus IL-33-triggered IL-4-independent T cells to airway inflammation may provide important insights into the etiology of different phenotypes of asthma, and may offer a novel therapeutic approach (77).

Along with Th2 activation, IL-33 strongly affects the innate arm of type 2 immunity, causing splenomegaly, blood eosinophilia and pulmonary inflammation as demonstrated by the administration of IL-33 to naïve mice (4, 43) and later confirmed in transgenic mice that overexpress IL-33 (78). Furthermore, IL-33 was reported to induce goblet cell hyperplasia and massive

mucus overproduction in the lungs and stomach (4). Several groups demonstrated that those changes could be attributed to the activation of mast cells (29), basophils (79), and epithelial cells (80). For instance, IL-33 is capable of mast cell degranulation, independent of T or B cells although it does require sustained exposure to IgE. Furthermore, IL-33 triggers the release of leukotriens (LTB4) and prostaglandin D2 (PGD2) and stimulates mast cells to secrete an array of cytokines, such as IL-1β, IL-3, IL-6, TNF-α and chemokines, such as MIP-2α (CXCL2), MCP-1 (CCL2) and MIP-1α (CCL3), in an NF-κB dependent manner. It also induces the release of IL-5, IL-13, and chemokines, Eotaxin-2 (CCL24), RANTES (CCL5) and TARC (CCL17), through the MAP kinase pathway and independent of NF-κB (29). Interestingly, IL-33 was found substantially elevated in atopic patients during systemic (anaphylactic) shock or in atopic dermatitis patients but not in subjects without ongoing inflammation. Consistently, in the murine models of systemic anaphylaxis the response to allergen was effectively attenuated by the use of a soluble decoy IL-33 receptor (sST2), anti-IL-33 antibody or mast cell depletion. Thus, IL-33 appears to be an important molecule in the pathogenesis of IgE- and mast cell-associated diseases and may represent a useful target for drugs aimed at preventing an anaphylactic shock (29).

As for the basophils, it was shown recently that IL-33 can act on freshly sorted murine bone marrow basophils without any priming with IL-3 or FcεR cross-linking and it stimulates the release of substantial amounts of histamine, IL-4 and IL-6 from these cells, thereby mimicking the response to GM-CSF, at least in the murine system. Also, IL-33 expands basophil population in the

murine bone marrow *in vivo*, through an indirect mechanism dependent on common β chain that is shared between receptors for IL-3, IL-5 and GM-CSF (79).

Human basophils, on the other hand, do not express IL-33 receptor constitutively but its expression is IL-3-inducible. Yet, they readily respond to IL-33, without prior priming, and the outcome is very similar to that exerted by IL-3, namely, the secretion of IL-4, IL-8 and IL-13. However, IL-33 does not prime human basophils for C5a-triggered LTC4 release (81).

IL-33 was also shown to promote histamine release triggered by antigen-bound (cross-linked) IgE. Moreover, IL-33 can trigger migratory responses of human basophils by inducing CD11b that is thought to regulate their adhesiveness to endothelial cells thereby facilitating basophil transmigration from blood to tissues. Furthermore, it was reported that IL-33 enhanced basophil migration along the gradient of Eotaxin (CCL11), although no apparent change in CCR3 expression was observed (82).

Taken together, there is growing evidence that IL-33 is one of the important factors that are capable of modulating allergic inflammation by skewing the balance of adoptive responses towards type 2 and/or by acting directly on several innate cells that contribute to the pathogenesis of lung inflammatory disease.

1.1.7.2. Helminthic infections

The process of developing an effective immunity against helminth parasites depends upon the host's ability to mount an effective T helper type 2-associated cytokine response as well as the activation of innate immune cells. Recently, IL-33 joined other type 2 cytokines such as IL-4, IL-9, IL-13, IL-25 on a list of essential factors known to facilitate worm expulsion. IL-33 seems to play a particular role in fighting gastrointestinal helminths, such as *Trichuris muris* where it shifts the balance toward Th2 cells producing IL-4, IL-9, and IL-13. Moreover, exogenous IL-33 induced the expression of thymic stromal lymphopoietin (TSLP) from epithelial cells of the infected caecum which itself is important for the generation of Th2-driven parasite immunity. Interestingly, IL-33 can also act independent of T cells, as demonstrated in SCID mice by increasing intestinal epithelium cell proliferation and crypt length, particularly in the initiation phase of the infection (83).

More recently, by employing novel IL-13-eGFP reporter mice Neill et al. identified a distinct subset of Th2-inducible innate effector cells, named nuocytes that may represent the predominant early source of IL-13 during helminth infection with *Nippostrongylus brasiliensis*. Nuocyte leukocytes expand rapidly in response to IL-33 and IL-25 and fail to develop in the combined absence of these two cytokines causing a severe defect in worm expulsion (84).

Taken together, the above data suggest that IL-33 is essential for the development of immunity against helminth parasites by activation Th2 cells,

epithelial cells and nuocytes. A summary of IL-33 biology is shown in **Figure 1.2.**

1.2. Eosinophil

Eosinophils have long been considered merely a type of terminally differentiated effector cells specialized in host defence against parasite helminths. However, there is now growing evidence to support the notion of a much more complex role that eosinophils play in the immune system as they seem to be involved in both adaptive and innate arms of immunity.

1.2.1. Eosinophil development

Eosinophil differentiation process occurs in the bone marrow and is induced and sustained by several cytokines and growth factors. It is now well established that three closely related cytokines, namely IL-3, IL-5 and GM-CSF, play a major role in controlling eosinophilopoiesis. Several reports clearly demonstrated that IL-5 is the most specific and most effective factor that controls both eosinophil differentiation and recruitment *in vivo* (85-88). Produced mainly by activated T cells and mast cells, IL-5 exerts its effect in the bone marrow by stimulating the expansion of a narrow pool of IL-5Rα-expressing eosinophil progenitor (hEoPs) cells that had differentiated under the control of more pluripotent cytokines and growth factors, including IL-3, CM-CSF, stem cell factor (SCF) and FMS-like tyrosine kinase 3 ligand (FLT3-L) (89). IL-5Rα expression is regarded a prerequisite and early lineage-specific event during the course of eosinophilopoiesis (90-91), (**Figure 1.3.**).

In the human system, EoPs differentiate from IL-5Rα^- common myeloid progenitor (CMP) cells (92), that were defined by Mori et al. as IL-5Rα^+CD34$^+$CD38$^+$IL-3Rα^+CD45RA$^-$-expressing cells (91), (**Figure 1.3.**).

In the mouse system, eosinophils originate from the eosinophil lineage-committed progenitors (mEoPs) which arise downstream of granulocyte/macrophage progenitors (GMPs) and are CD117$^+$ (c-kit-positive) cells (**Figure 1.3.**). Thus, unlike in human hematopoiesis, murine eosinophils, neutrophils and monocytes all originate from GMP cells. The precursor cells (mEoPs) that give rise to murine eosinophils were defined as (Lineage$^-$Sca-1$^-$) IL-5Rα^+CD34$^+$CD117^{low}, and are characterized by the activation of GATA-1 transcription factor (90).

Despite the fact that eosinophil development pathways differ considerably between the human and mouse in regard to the precise distribution of immediate precursor populations, a range of transcription factors involved in eosinophilopoiesis appears to be conserved across the two species. In particular, a highly coordinated expression of several transcription factors, including PU.1 (93-96), CCAAT enhancer-binding protein α (C/EBP-α and C/EBP-β) (95, 97-98) and the lineage-instructing GATA-1 (95, 99-101) allows for the commitment to eosinophil lineage and terminal eosinophil differentiation.

Another GATA-1 closely related transcription regulator, called friend of GATA-1 (FOG-1) acts as GATA-1 antagonist and has to be downregulated to allow for the eosinophil lineage commitment (102). Decidedly, GATA-1 transcription

factor plays a central role for eosinophil generation (103). This suggestion was confirmed by a study on the targeted deletion of high-affinity GATA-binding site (so-called double GATA site) which is commonly spread across many eosinophil-related genes, including major basic protein (MBP), CCR3, IL-5Rα chain and in the GATA-1 gene promoter itself. The deletion of a double GATA site abrogates eosinophil lineage development *in vivo* in so-called ΔdblGATA mice (95, 101, 103). Deficiency for C/EBP-α exerts a more broad effect preventing the differentiation of any granulocyte sub-populations, including eosinophils (104). The disruption of PU.1 gene also can significantly impair terminal differentiation of eosinophil. In eosinophil lineage committed cells, PU.1 appears to combine its effect with GATA-1 to produce the characteristics typical of eosinophil leukocyte, whereas in most other cells the two transcription factors mutually antagonize one another, where PU.1 represents the myeloid and GATA-1 the erythroid differentiation choice (95). Interestingly, the level of GATA-1 transcription required for eosinophil lineage commitment has to be just right, and can be characterized as low to moderate, with no FOG-1 expressed. There are data suggesting that moderate GATA-1 expression is both indispensable and sufficient for driving eosinophil development (105). Beyond the commitment of common myeloid progenitors to eosinophil lineage a different member of C/EBP family, more specifically, certain isoforms of C/EBP-ε transcription factor are required for terminal differentiation and functional maturation of human eosinophils while other forms counteract terminal differentiation (106). Moreover, mice deficient for this transcription factor lack both mature eosinophils and neutrophils (104, 107). Finally, loss of C/EBP-ε function caused by a known human SNP leads to the failure of secondary

granule proteins expression (both in the eosinophil and neutrophil lineages) in patients with so called specific granule deficiency (SGD) (108).

In 2008, another transcription factor, Icsbp (interferon consensus sequence binding protein; also known as IFN response factor-8) was reported to be essential for eosinophil development as Icsbp deficient mice have a lower number of eosinophil progenitor cells, compared to wild type as well as exhibit lower eosinophil counts in a steady-state and fail to produce eosinophilia in response to nematode *Nippostrongylus brasiliensis*, despite high amounts of IL-5 expressed upon infection (109). Authors suggest that Icsbp controls GATA-1 expression.

This precisely balanced interplay between GATA-1 FOG-1, PU.1, C/EBPα, Icsbp and C/EBP-ε transcription factors, both in terms of their relative expression levels and timing, is essential for effective eosinophil generation. The end-result is the development of a unique eosinophil lineage-affiliated pattern of gene expression where genes such as eosinophil peroxidase (EPO), and major basic protein (MBP) are exclusive to eosinophil lineage while several other granulocyte-associated genes are permanently shut down in eosinophils, such as basophil-specific histidine decarboxylase (HDC), neutrophil-specific myeloperoxidase (MPO) and mast cell-specific proteinases. This unique gene pattern was used to molecularly engineer a mouse strain specifically devoid of eosinophil, so-called PHIL mice, by linking the expression of bacteria-derived toxin with the expression of EPO, thus preventing eosinophils from undergoing maturation process (110).

1.2.2. Eosinophil recruitment and trafficking

Terminal stages of eosinophil maturation seem to be inherently linked to eosinophil exit from the bone marrow into the circulation. This is largely dependent on the combinatorial action of cytokines, such as IL-5 and other Th2 cytokines, IL-4 and IL-13, chemokines, in particular of eotaxin family and RANTES (111), and several adhesion molecules such as α4, β-1, β-2 and β-7 integrins (112) and surface markers, such as CCR3 (113-115). Again, IL-5 appears to be the central cytokine in eosinophil recruitment and trafficking as IL-5 deficient mice exhibit markedly reduced numbers of both circulating eosinophils and bone marrow resident eosinophil precursors (116). This was further confirmed in the human system in trials of anti-IL-5 therapy (mepolizumab) when decreased eosinophil counts were reported in the bronchial mucosa of atopic asthmatics (117-118). Along with IL-5 also the eotaxins are well recognized as the most lineage-specific factors that control eosinophil mobilization, trafficking and survival in the tissue, e.g. lung infiltration following antigen exposure (119-120). Their chemotactic effects are mediated by the G protein-coupled membrane bound receptor, CCR3 (121-123). It is noteworthy that several CCR3 ligands, particularly eotaxin-1 (CCL11) and eotaxin-2 (CCL24) cooperate with IL-5 in orchestrating eosinophil trafficking and tissue accumulation by expanding the $CCR3^+$ pool and/or priming eosinophils to respond to eotaxins (111). Interestingly, while CCR3 signaling usually results in the activation and prolonged survival of eosinophil, more recent studies reported that CCR3 can also mediate strong negative signal able to arrest key eosinophil responses, particularly when Mig (CXCL9) chemokine acts as a ligand, through a Rac2 dependent pathway (124). In

humans, eosinophil trafficking is also regulated by eotaxin-3 (CCL26), which is only distantly related to the other two mentioned previously and it does not have a functional counterpart in mouse (125).

Eotaxins are able to synergize with IL-5 to induce IL-13 production in the lung. In turn, IL-13 can synergize with IL-4 to potently induce eotaxins in the lung tissue through the STAT6-dependent mechanism which partially explains the causative relationship between Th2 responses and associated lung eosinophilia (111). This finding was further confirmed in IL-4 reporter mice infected with the migrating intestinal helminth, *Nippostrongylus brasiliensis* where eosinophil and Th2 cell recruitment into the lung was shown to be dependent on STAT6 expression by bone marrow-derived tissue resident cells (that were characterized as non-T, non-B cells) (126).

Importantly, in the course of antigen-induced airway inflammation, Th2 cell-derived IL-13 facilitates eosinophil trafficking from peripheral blood into the lungs by controlling the expression of eotaxin and eotaxin-2. Each of those chemokines is responsible for eosinophil trafficking into a different lung compartment. While eotaxin expression appears to be induced in lung pneumocytes, the expression of eotaxin-2 occurs in alveolar macrophages (luminal inflammatory cells). Consistently, mice deficient for eotaxin-2 show markedly reduced BAL eosinophilia upon IL-13 administration (127).

An interesting example of a surface marker involved in eosinophil trafficking is a sialic acid-binding immunoglobulin-like lectin, Siglec-F (and its human functional paralog Siglec-8). This sialoadhesin is markedly upregulated on blood and bone

marrow eosinophils in response to the induction of allergic lung inflammation despite its well established role as part of a negative eosinophil regulation both *in vitro* and *in vivo* (128-129).

1.2.3. The effector functions of eosinophils

Current knowledge about this highly specialized yet multifunctional leukocyte is still expanding. At homeostatic baseline, eosinophils are commonly found in the bone marrow where they constitute a small but stable sub-population that consists of both mature eosinophil cells and also eosinophil lineage precursors at various stages of development. As mentioned in previous section, rapid expansion of this population upon diverse stimuli, the most specific of which is IL-5, serves as a major, and probably the only, source of terminally differentiated eosinophil leukocytes that are subsequently recruited to the sites of inflammation mobilized by a range of chemokines, such as eotaxin, together with several adhesion molecules. Outside the bone marrow, eosinophils normally constitute 1-5% of blood nucleated cells and resident populations of tissue eosinophils are commonly found in the gastrointestinal tract (GI), particularly in the transitional area between the small intestine and the colon (cecum). These are also present in thymus, spleen, lymph nodes and endometrial lining of the uterus (endometrial stroma) (130-131) and are required for postnatal mammary gland development (132). Although it is a long-known phenomenon that eosinophils can be recruited in response to tissue damage and tissue repair, the details about the nature of their involvement in those processes are incomplete and poorly understood (133-136). In addition, eosinophil major basic protein (MBP) have been implicated as a pro-angiogenic

factor, based on evidence from *in vitro* and *in vivo* studies which may be of particular relevance to tissue repair and remodelling that contributes to asthma pathology (137).

Eosinophils are widely recognized as the major effector cells in type 2 inflammatory diseases, including helminthic infection, asthma, and allergy (130, 138). The important role of eosinophils in the immunopathogenesis of asthma was shown in studies using eosinophil-deficient mice that develop markedly attenuated responses in experimental airway inflammation models (110, 139).

Two distinct mouse strains devoid of eosinophils have been generated using different molecular approaches. In one case, mice harbor targeted mutations in the gene encoding a transcription factor GATA-1 (ΔdblGATA mice) (139-140). In so-called PHIL mice, on the other hand, eosinophil ablation was achieved through the lineage-specific expression of the diphtheria toxin A chain, under control of the eosinophil peroxidase promoter (110). The latter strain (on a C57BL/6 background), is fully protected from airway hyperresponsiveness (AHR) and exhibits diminished airway mucous metaplasia as well as Th2 cytokine levels in OVA-induced airway inflammation. Similar phenotype characterized by reduced AHR as well as diminished IL-4, IL-5 and IL-13 production was reported in ΔdblGATA mice if bred on C57BL/6 (140). In contrast, ΔdblGATA mice on the BALB/c background develop AHR and mucus production comparable to WT mice in OVA-induced airway inflammation model (139). However, they showed reduced lung remodeling (which was not tested in PHIL mice). The above studies suggest that on the C57BL/6 but not BALB/c background, eosinophils are essential to the development of allergic airway

inflammation. These differences in phenotype between the two ΔdblGATA mouse strains may also provide an insight into why there is no direct correlation between lung eosinophilia and the severity of asthma in a subset of asthma patients treated with IL-5 blocking monoclonal antibody (141).

In asthma, eosinophils mediate the inflammatory process through the release of cytotoxic granules and lipid mediators that induce tissue damage and affect nerves, which, in turn, causes bronchoconstriction (129-130, 138, 142-143). In addition, eosinophils show numerous immune regulatory functions, including the production of a range of cytokines and chemokines that leads to the exacerbation of inflammation, mucus secretion, and lung remodelling (129-130, 138, 143). Recently, it was demonstrated that bone marrow eosinophils are essential for the survival of plasma cells through the release of the proliferation-inducing ligand APRIL and IL-6; eosinophil-deficient mice exhibit much lower numbers of plasma cells both in the resting BM and after immunization (144). Thus, eosinophils support humoral immune response thereby helping to ensure long-term protection against pathogens.

Eosinophils are also one of the primary sources of IL-4 during the initiation phase of type 2 immune responses (126), and they can recruit T cells by releasing TARC (CCL17) locally into the lungs during asthma development (145). There is also growing evidence that eosinophils can serve as antigen presenting cells (146). A summary of the multiple effector functions of eosinophils in the airways is shown in **Figure 1.4.**

1.3. Macrophage

1.3.1. Macrophage subpopulations

Macrophages are specialized hematopoietic cells distributed throughout different tissues of a body where they play central role in homeostasis, tissue remodelling and host defence. One of the key functional characteristics of macrophages is that, depending on micro-environmental factors, they can be specifically polarized so they are able to deal with a particular stimulus in a most adequate manner (147). In addition, the heterogeneity of circulating monocytes may pre-define their polarization fate once they arrive at tissue (148-149).

Polarized macrophages have been broadly classified as either M1 or M2 macrophages. (see **Figure 1.5.**). Classically activated M1 macrophages were described as being responsive to type 1 inflammatory cytokines and microbial products, and by producing high levels of IL-12, whereas M2 macrophages have been generally characterized by their low expression of IL-12. M2 macrophages can be further divided into three groups, based on the activation factors they are responsive to: M2a (alternatively activated macrophages, AAM) are induced by IL-4 or IL-13; M2b can be induced by immune complexes and agonists of TLRs or IL-1 receptors; M2c (immunosupressive) are induced by IL-10 or TGF-β or glucocorticoids (150-151). Furthermore, macrophage subsets differ in their receptor expression, cytokine and chemokine expression and in their effector functions (152). M1 macrophages are well defined as potent effector cells involved in responses against microorganisms and tumor cells as

well as in pathologic type 1 inflammation. Their inflammatory repertoire is characterized by the secretion of IL-12, TNF-α, IL-1β, IL-23, IL-6, IP-10, RANTES and the high expression of iNOS. M1 macrophages express many TLRs, including TLR2, and opsonic receptors such as CD16. In contrast, M2 macrophages ameliorate type 1 inflammatory responses and adaptive immunity, and regulate type 2 immune responses, angiogenesis as well as tissue remodeling. Various subsets of M2 macrophages exhibit these general properties. Promotion of type 2 response in helminthic infection, allergy and asthma are typical of AAM, whereas suppression of both type 1 and 2 inflammation is a predominant feature of M2c cells. The M2 macrophage profile and the AAM in particular can be characterized by abundant expression of non-opsonic receptors such as the mannose receptor (MR; CD206) and CD163. Moreover, Arginase-I is upregulated in AAM and M2c macrophages, which results in generation of polyamines and proline by these cells, thereby contributing to wound healing and pathological fibrosis (150-153).

A growing interest in the role and function of AAM in parasitic infection and asthma led to the identification of more specific markers for AAM. The most relevant signature markers besides Arginase-I, MR and IL-4Rα are chitinase-like lectin Ym1, resistin-like secreted protein FIZZ1 and acidic mammalian chitinase (AMCase) (154). Furthermore, a distinct chemokine profile, including TARC (CCL17), MDC (CCL22) and Eotaxin-2 (CCL24), has been associated with AAM activation (152).

1.3.2. The role of alveolar macrophages in the regulation of immune response

The function of the lungs is to allow the uptake of oxygen and the excretion of carbon dioxide. With its large surface area, the lungs are exposed to many pathogens as well as harmless antigens. Since oedema and inflammation can lead to the thickening of alveolar walls and compromise gas exchange, immune defence of this barrier has to be tightly controlled (155). A cellular component of the immune response in the lungs consists mainly of alveolar macrophages which adhere closely to alveolar epithelial cells. It has been shown that to avoid damage to alveoli, alveolar macrophages are generally kept in a quiescent state which resembles M2c phenotype (immunosuppressive) (156). By producing cytokines such TGF-β1 they actively suppress the induction of adaptive immunity to harmless antigens through their effects on alveolar and interstitial DCs and T cells (156-158). However, in the presence of 'danger signal' which either comes from a pathogen or damaged tissues, alveolar macrophages are believed to be responsible for the initiation of the inflammatory response (159-162).

1.4. Aims of the project

Numerous clinical studies and animal models revealed much of the complexity of the asthma and allergic diseases including a prominent role of adaptive arm of immunity, in particular, Th2 cells but also cells associated with innate immunity such as eosinophils, mast cells, and macrophages. All those cell populations were thought to be driven chiefly by cytokines and other mediators derived from allergen-activated Th2 cells, in particular by IL-5, IL-4 and IL-13. Among those cytokines, IL-5 is widely recognized as one of the key factors that drive asthma pathogenesis owing to its known activity as a selective eosinophilopoietin and chemoattractant, thus required for eosinophil infiltration of the airways that eventually leads to profound histological changes including airway hyperresponsiveness, excessive mucus production and airway tissue remodeling. Indeed, atopic patients often exhibit elevated levels of serum IL-5 which was commonly associated with high eosinophil counts in the sputum and more frequent asthma exacerbation episodes. Furthermore, data obtained from mouse airway inflammation models suggested that disrupting the IL-5 signaling may constitute an effective therapeutic approach through controlling airway eosinophilia.

However, clinical investigations revealed that the use of monoclonal anti-IL-5 antibody in asthma patients is not necessarily the best way to control eosinophil counts and/or eosinophil activation status in the airways (163-165). This may have been partially due to the reported a decrease in the IL-5Rα expression in the airways (166) in conjunction with a possible compensative increase in blood

IL-5 (suggestive of 'endogenous' IL-5 auto regulatory pathway) (167), which could not be counteracted with anti-IL-5 administration. Thus, the strategy aimed at preventing pathological changes in the atopic airways by controlling eosinophil population trough disrupting the signaling of IL-5 has been deemed insufficient.

Recent studies, including those carried out in our laboratory suggest that there are, in fact, other factors that may contribute to asthma pathology. For instance, a recently discovered interleukin-33 was found to be markedly elevated in lung epithelial cells of asthma patients compared to healthy donors (**Figure 1.1.**), which strongly suggested that it may play a role in asthma pathogenesis through interfering with a local microenvironment of the airways. Indeed, we further demonstrated that IL-33 can profoundly alter the activation status of alveolar macrophages which, in turn, contributes to airway inflammation (26). Furthermore, IL-33 has been shown to induce IL-5 and IL-13 in $CD4^+$ cells (43) and serves as a T cell chemoatractant (168) thereby contributing to antigen-driven airway inflammation.

The main aims of this thesis therefore, were to further characterize the role of IL-33 in airway inflammation, by (I) investigating the mechanism in which this cytokine drives the differentiation of alveolar macrophages toward alternatively activated (M2) macrophages and (II) how this affects airway cellular composition. Furthermore, since eosinophilia seems to be a prerequisite for several pathological changes in asthmatic lungs, including airway hyperresponsiveness and airway tissue fibrosis, I sought to investigate (III) how IL-33 contributes to eosinophil biology and (IV) how, in turn,

IL-33-induced eosinophil mediators affect airways homeostasis. To do this I employed a cell specific depletion method or an adoptive cell transfer method followed by the intranasal administration of IL-33 which mimics much of the allergic airway inflammation characteristics (4, 26, 43, 48).

Chapter 3 of this thesis provides information about the role of alveolar macrophages in the IL-33-induced airway inflammation. Chapter 4 investigates the effects of IL-33 on the population of eosinophil progenitor cells in the bone marrow and provides an insight onto the role of IL-33 in eosinophil trafficking through the regulation of the expression of CCR3, a key chemokine receptor involved in this process. Chapter 5 describes my studies of how IL-33 induces eosinophil activation and how some of the key eosinophil effector functions are regulated by IL-33 through ST2 receptor expressed on those cells. Finally, Chapter 6 provides an insight into the role of IL-33-activated eosinophils *in vivo*, as evaluated in IL-33-dependent airway inflammation model.

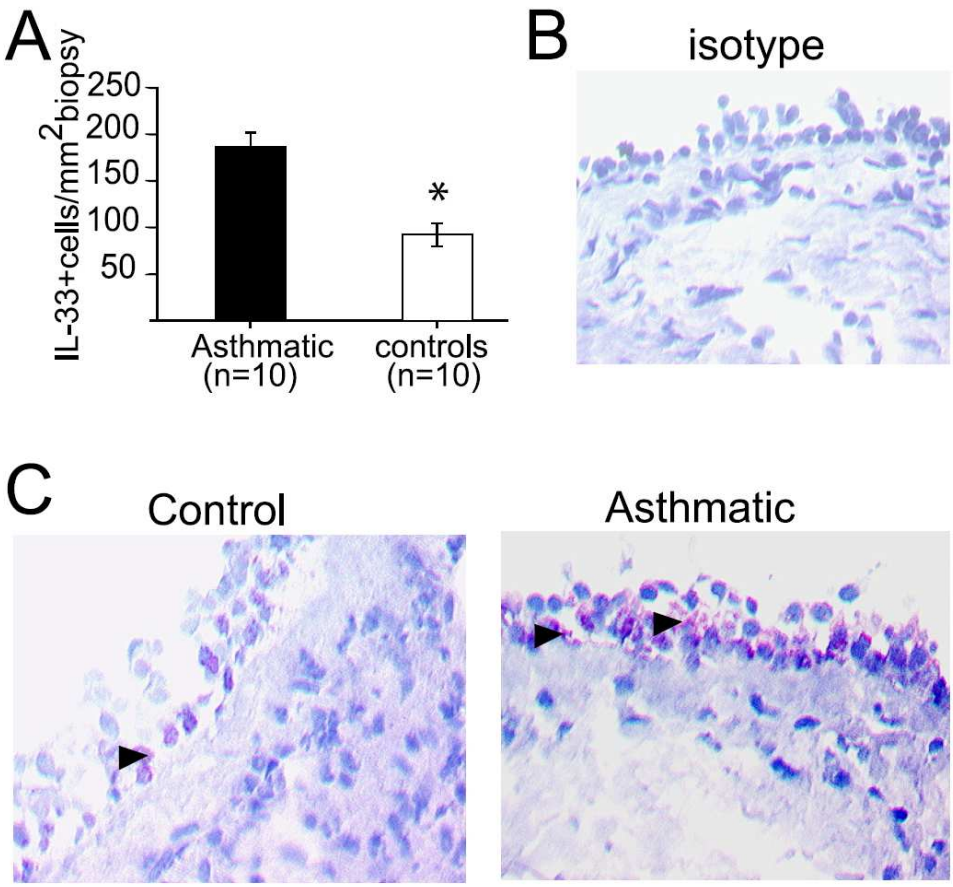
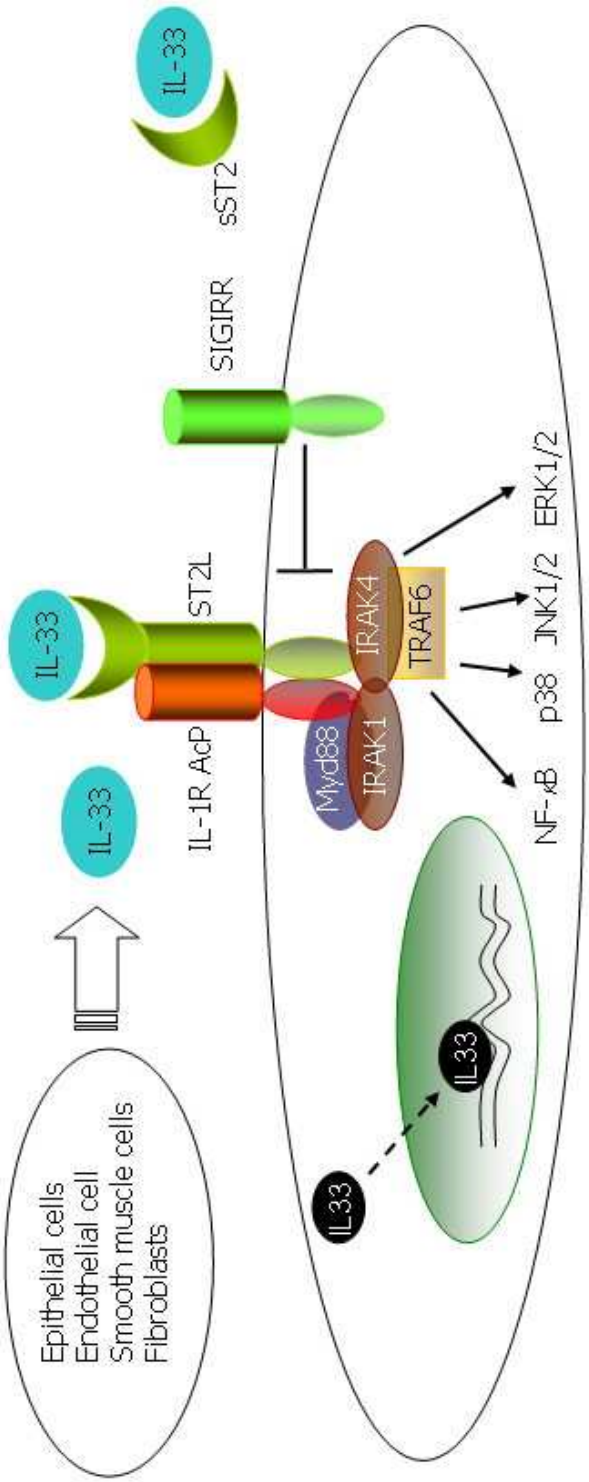


Fig. 1.1. Asthma patients express more IL-33 in the lung epithelial cells compared to healthy subjects. A-C, Frozen sections of lung biopsies of asthmatic patients and healthy donors were stained with anti-IL-33 antibody. Quantitative evalauation (A) and representative staining (B and C) are shown. Positive signal is violet. Data are means ± SEM (n = 10); *, $p < 0.05$, asthmatic versus controls. After Mariola Kurowska-Stolarska et al. (26), PMID: 19841166.



Mast cells	IL-6, IL-1β, MCP-1, MIP-1α, IL-13, MDC, TCA-3; survival, adherence
	CIA, allergy
	allergy/asthma, helminthic infections
T cells (Th2 and IL-5+ T cells)	IL-5, IL-13 production chemoattraction
	protective in atherosclerosis and obesity
	asthma, eosinophilic lung inflammation;
Alternatively Activated Macrophages (AAM)	TARC, Eotaxin-2, IL-10, Arginase I
	protective against obesity
	IL-4, IL-6, IL-8, IL-13 and histamine
Basophils	production; expansion, adhesiveness
	allergy/asthma
Nuocytes	IL-13, IL-5 production
	helminthic infection
Dendritic cells	CD86, MHC II, expansion of IL-5+ T cells
	allergy/asthma, helminthic infection
Neutrophils	CXCR2, neutrophil recruitment
	protective against septic shock (1)

See abbreviations list on page 15.

Figure 1.2. IL-33 biology: target cells and cell-specific effects

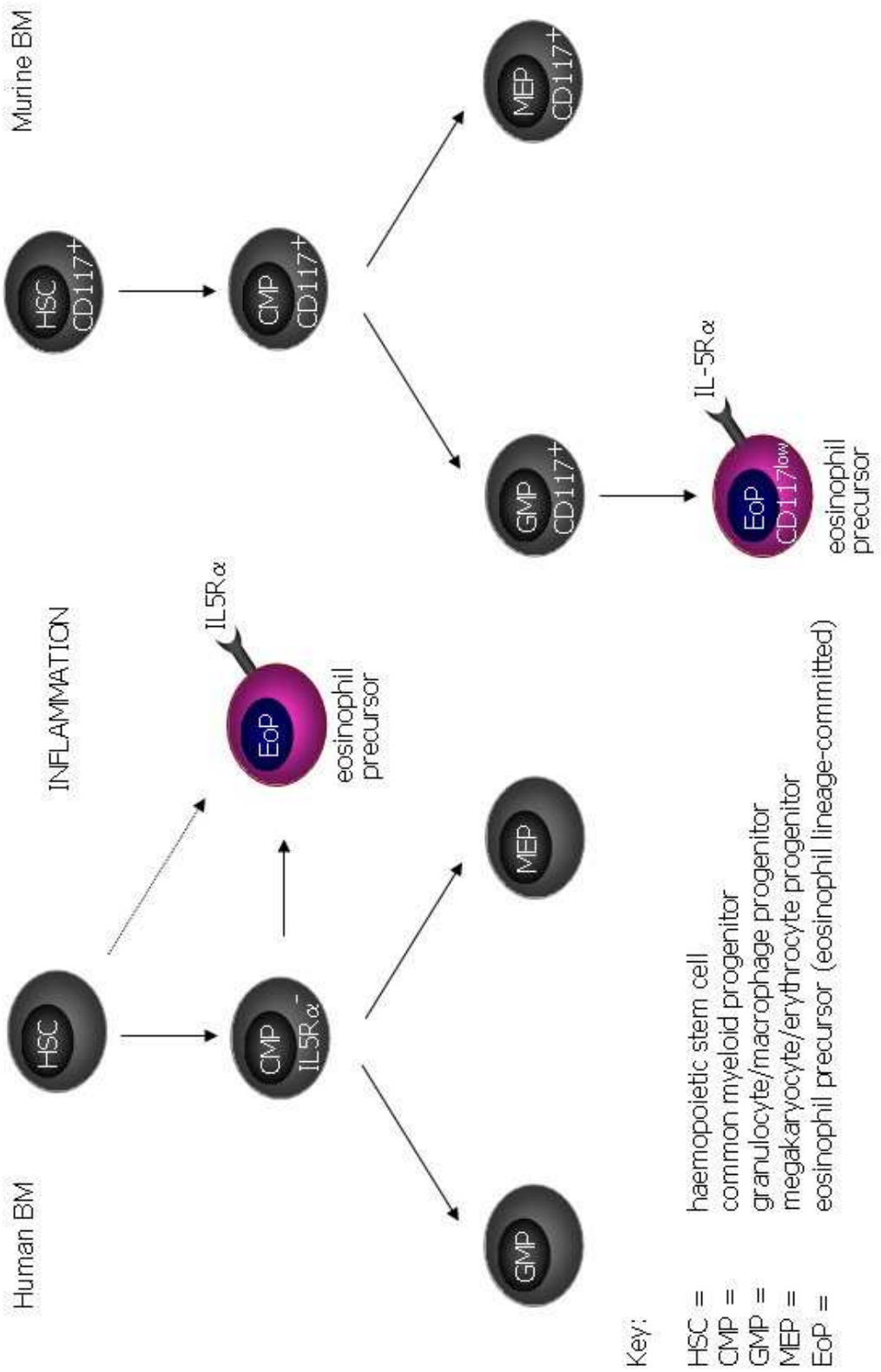


Figure 1.3. Eosinophil development in the human and mouse system

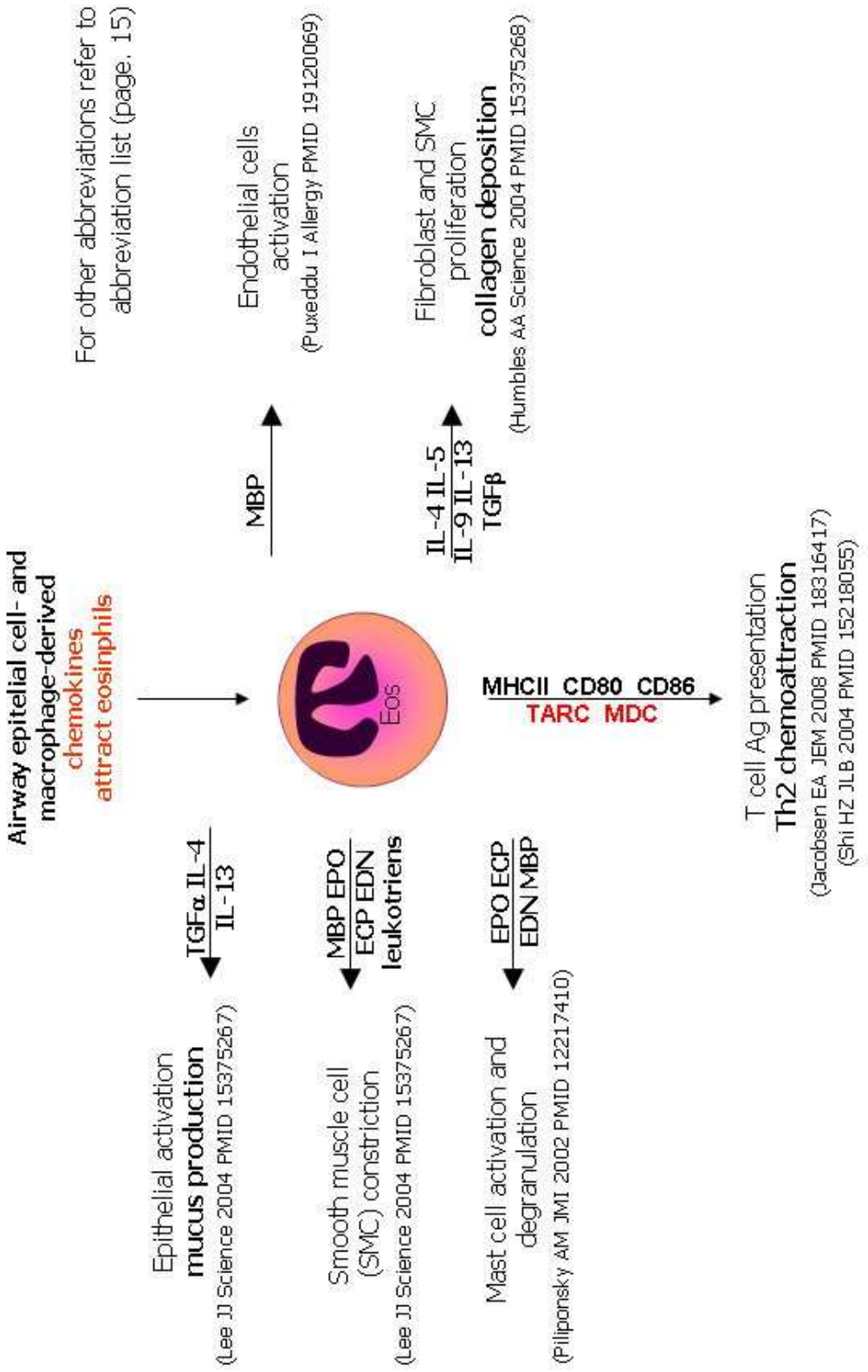


Figure 1.4. Eosinophil effector molecules and their biologic effects in the lungs

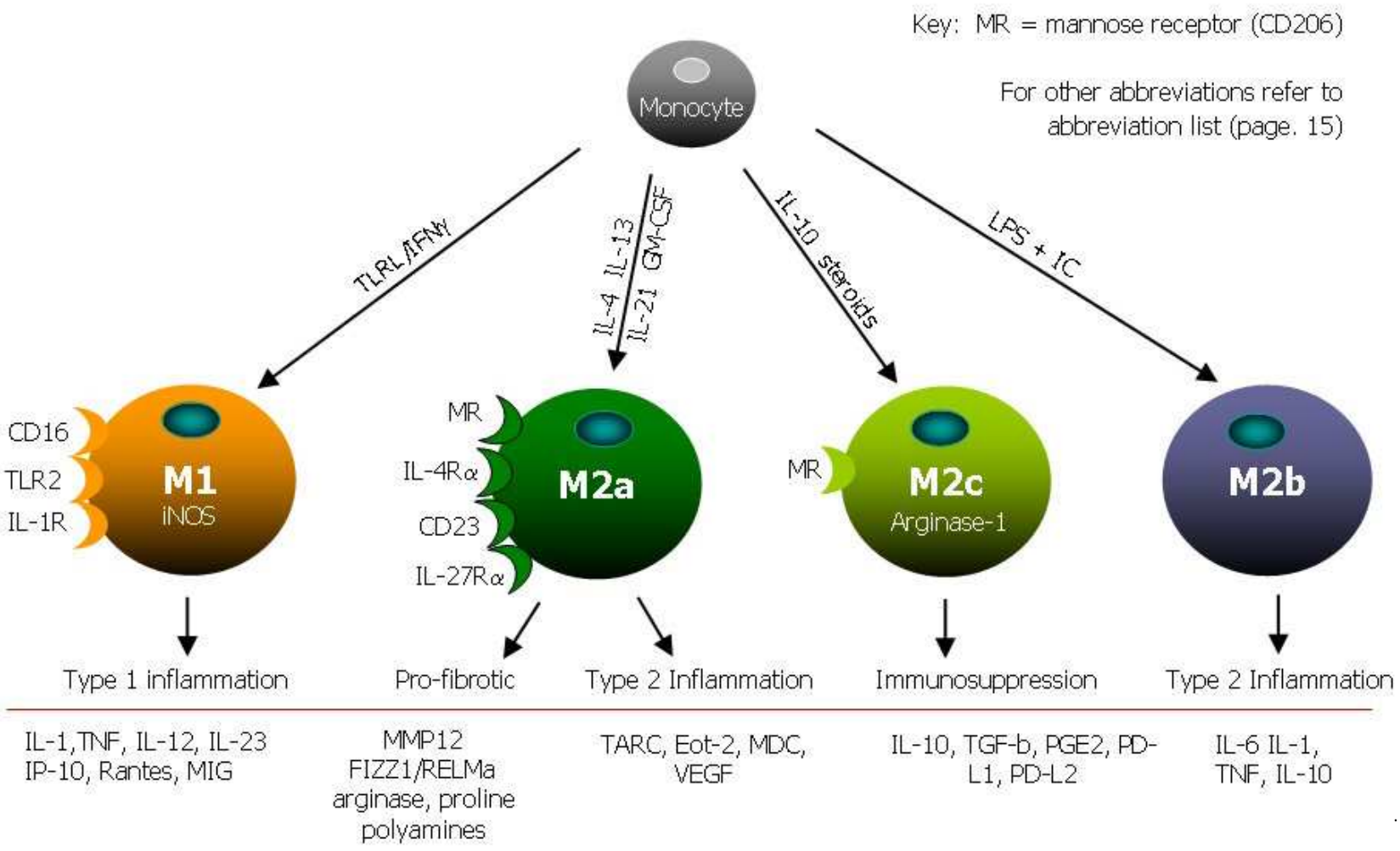


Figure 1.5. Macrophage subpopulations and their effector functions

Adapted from Gordon and Taylor (2005). Nature Reviews Immunology 5(12): 953-964 with modifications

Chapter 2:

Materials and methods

2.1. Animals

Wild type (WT) BALB/c mice were purchased from Harlan Olac, (Bicester, UK). ST2 gene knockout ($ST2^{-/-}$) mice on the BALB/c background that harbor a deletion within the ST2 gene spanning across exon 4 and 5 (169) were obtained previously from Professor Andrew MacKenzie (Laboratory of Molecular Biology, MRC, University of Cambridge) and housed in the Biological Services facilities of the University of Glasgow in accordance with the United Kingdom Home Office regulations under the Animals (Scientific Procedures) Act 1986. All procedures were carried out under Project Licence Number 60/3791, Procedure 3 and Procedure 5.

Bone marrow from C57BL/6, IL-$13^{-/-}$ and IL-5/IL-9/IL-$13^{-/-}$ triple knockout mice on the C57BL/6 background (170-171) were kindly provided by Professor Andrew McKenzie.

2.2. Recombinant IL-33

Human and murine recombinant IL-33 used in all *in vivo* experiments were produced in our laboratory according to published protocols (26, 168). Recombinant IL-33 used for all *in vitro* experiments was purchased from PeproTech (Rocky Hill, NY). Purity and biological activity of the two reagents was similar, as evaluated by its ability to induce IL-5 production from wild type but not $ST2^{-/-}$ cells.

In brief, the IL-33 generation protocol was as follows: mouse cDNA for IL-33 was cloned from IL-1-stimulated fibroblasts, and the cDNA template was

transformed into *Escherichia coli*. IL-33 protein was induced by IPTG and purified by Ni-NTA affinity chromatography. Traces of contaminating bacterial endotoxin were removed by passing the isolate through a polymyxin B column. Typically, the purity of IL-33 was more than 95% and endotoxin levels were less than 0.01 EU/µg of protein as evaluated by the Limulus Amebocyte Lysate QCL-1000 pyrogen test (Bio-Whittaker, Bio-Whittaker).

2.3. Reagents and buffers

All reagents, including buffered saline solutions were sourced from Sigma-Aldrich (Poole, UK). Culture media were purchased from Invitrogen Ltd (Paisley, UK), unless specified otherwise.

Full list of chemicals used throughout the project includes: 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); Complete medium (500 ml RPMI 1640, 50 ml inactivated FBS, 5ml pen / strep, 5 ml L-glutamine); 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); Substrate solution (1.8mM o-phenylenediamine (OPD), 0.4mM H_2O_2 in 0.1M PBS containing 0.1% Triton-X-100, pH 8.0); 4M sulfuric acid; Formalin-acetone buffer (0.75 mM Na_2HPO4, 7.5 mM KH_2PO_4, pH 7.4, 45% v/v acetone, 10% w/v formaldehyde); DAB/H_2O_2 phosphate buffer (0.60 mM Na_2HPO4, 7 mM KH_2PO_4, pH 7.5, 2 mM 3,'3'-diaminobenzidine tetrahydrochloride); 0.01% H_2O_2 (prepared afresh from

30% v/v solution); 8 mM potassium cyanide (KCN); Avertin stock (1:1 w/v solution of 2,2,2-tribromoethanol in *tert*-amyl alcohol).

2.4. Cell isolation and enrichment

2.4.1. Purification of mouse haematopoietic precursor cells

Mice were sacrificed using carbon dioxide inhalation and the femur and tibia were aseptically removed from both hind limbs and stored in complete medium at $4^{O}C$ until the bone marrow was extracted. Under sterile conditions, bones were cut open from both ends and the shaft was flushed with pre-chilled sterile BPS using a needle and a syringe. Single-cell suspension was achieved by passing the cells through a sterile 70μm nylon mesh (Costar). Then, cells were washed in 50ml of RPMI 1640 medium, centrifuged at 300g for 10 min at $4^{O}C$ and resuspended in complete medium prior to counting in a haemocytometer.

For CD117-positive ($Lin^{-}CD117^{+}$) cell enrichment, murine BM cells were separated using MACS Miltenyi magnetic cell sorter. The protocol recommended by the manufacturer was closely followed (MACS Miltenyi Biotec). In brief, BM cells were passed through 30μm nylon mesh (# 130-0141-407, Miltenyi Biotec) to remove the clumps and obtain a single-cell suspension. Next, lineage–committed cells were depleted using the Lineage Cell Depletion Kit, mouse (#130-090-858, Miltenyi Biotec). Lineage-positive cells that express antigens from a panel of so-called 'lineage' antigens (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C) and Ter-119 antibodies) retained on a MACS column, while lineage-negative cells were allowed to pass through the column (separation by negative selection). In the following step, lineage-negative cells were enriched

for CD117 antigen using CD117 MicroBeads, mouse (#130-091-224, Miltenyi Biotec) by positive selection.

2.4.2. Purification of mouse eosinophils

Mouse eosinophils were obtained from bone marrow or peritoneal wash of WT and $ST2^{-/-}$ mice that have been injected with IL-5 (2μg/mouse, i.p.) or with IL-33 (2μg/mouse, i.p.) over 7 days. Eosinophil leukocytes were separated using a flow cytometry based cell sorter (Becton Dickinson, FACSAria One). In brief, cells were gated for Siglec-F^{+} or Siglec-F^{+} and $CCR3^{high}$ or Siglec-F^{+}, $CCR3^{high}$ and Gr-$1^{intermediate}$ and sorted into medium containing GM-CSF (1ng/ml) or IL-5 (2ng/ml), to maintain cell viability. The purity ranged from 96 to 98%. Details are provided in the sections below.

2.4.3. Purification of human eosinophils

Human eosinophils were acquired from peripheral blood of healthy volunteers by density gradient centrifugation (Histopaque, density 1.077 g/ml). In brief, 20ml of cell suspension were carefully laid over 20ml of Histopaque-1077 solution and in a 50 mL conical tube and centrifuged at 600×g for 30 minutes at $20^{O}C$ in a swinging bucket rotor without brake. This was followed by the lysis of erythrocytes from the bottom fraction in ammonium chloride erythrocyte lysis solution (0.8% NH4Cl, 10mM KHCO3 and 0.1mM EDTA) in a fully-filled 50ml conical tube (10minutes, on ice) and centrifugation at 300×g for 8 minutes. Cells were then washed by adding 50 ml of MACS Running Buffer (Miltenyi Biotec), spun down again, re-suspended in appropriate volume of Running Buffer and counted. Next, eosinophils were magnetically separated from other

granulocytes using Eosinophil Isolation Kit, human (# 130-092-010, Miltenyi Biotec) based on a panel of antibodies consisting of CD2, CD14, CD16, CD19, CD56, CD123, and CD235a (glycophorin A) designed for the negative selection of human eosinophils. Instructions provided by the manufacturer were closely followed to achieve efficient separation. Actual purity of eosinophil population was verified by differential cell staining or staining for eosinophil-specific cyanide resistant peroxidase (EPO) activity of cytospin preparations. The purity ranged from 96 to 98%.

2.5. Cell cultures

2.5.1. Eosinophil development from whole bone marrow and $CD117^+$ precursors

Whole bone marrow ($2x10^6$/well/1ml) or purified $CD117^+$ haemopoietic cells ($0.5x10^6$/well/1ml, purity ≥95%) from BALB/c, $ST2^{-/-}$, $IL\text{-}13^{-/-}$ and $IL\text{-}5/IL\text{-}9/IL\text{-}13^{-/-}$ were cultured in RPMI 1640 (supplemented with 10% FCS, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 0.05M 2-mercaptoethanol) and IL-5 (10ng/ml) or IL-33 (2, 10, 50ng/ml) (all cytokines from Peprotech) ± anti-IL-5 neutralizing antibody or isotype control (10μg/ml, eBioscience). After 4-5 or 7-8 days of culture, cells were stained with PE-conjugated anti-Siglec-F (early eosinophil differentiation marker; BD Bioscience), APC-conjugated anti-CCR3 (an eosinophil marker; R&D Systems), FITC-conjugated anti-ST2L (MD Bioscience), PerCP-conjugated anti-Gr-1 (a granulocyte marker; BD Bioscience), PE-conjugated anti-FcεR1 (a basophil marker, BD Bioscience), or with the appropriate isotype control followed by

FACS analysis (**Table 2.1.**). To analyze the effect of IL-33 on CCR3 expression, cells from WT and $ST2^{-/-}$ bone marrow culture driven with IL-5 (10ng/ml) were resuspended, in PBS/2% FCS ($50x10^6$/ml) and 20μl of PE-conjugated rat anti-mouse Siglec-F antibody per $50x10^6$ cells were added. Cells were incubated for 30min on ice followed by two washing steps (10ml of PBS/2% FCS; 10min, 300g). After the last washing step cells were resuspended in PBS ($50x10^6$/ml) and then equal amount of Cell Dissociation Solution (Sigma) was added to prevent forming cell clumps. The cells were then sorted by FACS, based on high Siglec-F expression into complete medium supplemented with IL-5 (2ng/ml) to prevent cell death. Purity of sorted cells was evaluated and was ≥98%. Cells were then stimulated with IL-33 (10ng/ml) in media containing small amount of IL-5 (2ng/ml). After 24h, the cells were stained with APC-conjugated anti-CCR3 antibody (4μl/100μl) for 30 minutes followed by two washing steps (10ml of PBS/2% FCS; 10 minutes, 300g). Cytospins were prepared from some cultures and stained in the presence of potassium cyanide for EPO (specific for eosinophils) or were differentially stained with eosin/hematoxylin.

2.5.2. Eosinophil cytokine responses and surface markers

WT and $ST2^{-/-}$ mice were injected with IL-5 (2μg) i.p. every second day for 7 days. Twenty four hours after last injection cells were harvested from bone marrow. Cells were then resuspended in PBS/2% FCS ($50x10^6$/ml) and 20μl of PE-conjugated rat anti-mouse Siglec-F antibody (0.2mg/ml; BD Bioscience) and 40μl APC-conjugated rat anti-mouse CCR3 (R&D Systems) per 1ml ($50x10^6$ cells) were added. Cells were incubated for 30 minutes on ice followed by two washing steps (10ml of PBS/2% FCS; 10 minutes at 300g). After last washing

step cells were resuspended in PBS ($50x10^6$/ml). Next, equal volume of Cell Dissociation Solution (Sigma) was added to prevent forming cell clumps. The cells were then sorted by FACS, based on high Siglec-F and CCR3 expression into complete medium containing 1ng/ml of GM-CSF or 2ng/ml of IL-5 to prevent cell death. Purified populations of eosinophils (purity ≥97%, $0.5x10^6$/well/ml) were cultured in IL-5-containing control medium (2ng/ml), supplemented with GM-CSF (1, 10ng/ml) or IL-4 (10ng/ml) or IL-33 (10, 50ng/ml) or a combination of these cytokines. After 24 or 48h, cells were stained with FITC-conjugated anti-ST2L and APC-conjugated anti-CCR3 followed by FACS analysis (see **Table 2.1.** for details). To assay cytokine and chemokine production, purified eosinophils were incubated in medium with GM-CSF (1ng/ml, survival and ST2L inducing factor; control) ± IL-33 (10, 50ng/ml). After 48h culture supernatants were collected. Cell viability was evaluated by counting in trypan blue and was ≥94%. Levels of cytokine and chemokine production were evaluated after 72 hours of culture by 20-plex Luminex (Biosource) or ELISA using paired antibodies against IL-13 , TGF-β1, IL-4 and chemokines MIP-1α, Eotaxin-1, Eotaxin-2, TARC (see **Table 2.2.** for details).

2.5.3. Eosinophil degranulation

Mature human and mouse eosinophils were cultured in the presence of GM-CSF (1ng/ml) ± IL-33 (50ng/ml) as before. After 24h, cells were washed with fresh RPMI medium and seeded into a plate coated with anti-human CD32 or anti-mouse CD32/16 (0.3 or 1μg/ml) or $mIgG_1$ or $hIgG_1$ (1μg/ml). After 90 minutes culture supernatants were collected and EPO assay performed. The amount of

EPO in the degranulation assay was calculated as the percentage of total EPO released from eosinophils that underwent 3 freeze-thaw cycles. EPO colorimetric assay was performed on cell supernatants according to previously published protocol (172). In brief, aliquots of sample (50μl) were incubated with equal volume (50μl) of substrate solution containing 1.8mM o-phenylenediamine (OPD) and 0.4mM H_2O_2 in 0.1M phosphate buffer containing 0.1% Triton-X-100 (pH 8.0) for 30min at 37°C followed by acidification with 4M sulfuric acid. Results are shown as the percentage of total EPO. Cell viability was evaluated by differential cell counts using trypan blue.

In some cultures, cells pre-incubated with GM-CSF±IL-33 were stained with PE-conjugated anti-CD32 or PE-conjugated anti-CD16 antibodies followed by FACS analysis (see details in Table 2.1.).

2.5.4. Macrophage differentiation *in vitro*

Bone marrow cells from WT or $ST2^{-/-}$ were cultured in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100units/ml penicillin, 100μg/ml streptomycin, 0.05 M 2-mercaptoethanol on Petri dishes $10x10^6$ per 5ml per dish in the presence of M-CSF (10ng/ml). After 3 days, cells were fed with 5ml of fresh complete medium supplemented with M-CSF (10ng/ml). On day 6 of culture, cells were evaluated by flow cytometry (FACS) for macrophage marker and cell preparations containing ≥96% of $F4/80^+$ macrophages were used for further experiments.

2.5.5. Co-culture of eosinophils and macrophages

Specially designed culture plates with well inserts were used throughout the co-culture experiment series (Transwell system, 12-well plate, Costar). Upper compartment contained murine wild type eosinophils of $3x10^6$/0.5 ml per well. A small pore diameter (0.4μm), permeable membrane was designed to prohibit cell migration between the compartments without restricting the diffusion of soluble mediators. Lower compartment contained $0.5x10^6$/well/1.5ml of $ST2^{-/-}$ macrophages. Cells were incubated with IL-5 (10ng/ml) or IL-33 (10ng/ml) ± neutralizing anti-IL-13 antibodies (R&D Systems) or isotype controls (both 10μg/ml) for 72h. The expression of TLR2 (eBioscience, clone 6C2) and mannose receptor (MR) (Serotec) on macrophages was then analyzed by FACS (**Table 2.1.**).

2.6. Murine model of allergic asthma

WT and $ST2^{-/-}$ mice were given i.p. injections with 100μg of OVA (Sigma) in 2% alum (Aluminium Hydroxide Gel Adjuvant, Brenntag) and then challenged i.n. on days 8, 9 and 10 with 10μg of OVA or PBS as described previously (173). Mice were sacrificed 48-120 h after the last challenge. Bronchoalveolar lavage (BAL) fluid was collected and analyzed.

Mice were terminally anaesthetized by the i.p. injection of 500μl of Avertin (2,2,2-Tribromoethanol, Sigma-Aldrich, 20mg/ml working solution in PBS, filter sterilized through a 0.5 micron filter, Millipore) then killed by exsanguinations after anesthetized sufficiently (no toe pinch/righting reflex, typically after about 5 minutes). Next, the trachea was exposed and a small incision made at the

proximal end to allow cannulation with a 23G needle sheathed with polythene tubing (VWR International). Then, a volume of 0.8ml of PBS was instilled into the lungs for about 15 seconds and the fluid was aspirated. Subsequently, a second aliquot of 0.8ml of fresh PBS was instilled as in the previous step. The two aspirated aliquots were then pooled in a 1.5ml centrifuge tube and stored on ice until the next step was carried out. Live cell counts were performed in a haemocytometer using trypan blue. Cells were spun onto glass slides using a Shandon Cytospin 3 (Shandon Scientific Limited, Cheshire, UK) at 450 rpm for 8 minutes. Then, slides were air dried and cells were fixed by incubation in methanol at room temperature over 10 minutes. Slides were then stained by the Romanovsky method using Rapi-Diff II (BIOS Europe Limited, Lancashire, UK). Coverslips were affixed onto the air-dried slides using DPX (BDH Laboratory Supplies, Poole, UK).

In addition, BAL cells were stained with PE-conjugated anti-Siglec-F and APC-conjugated anti-CCR3 and FITC-conjugated anti-ST2L antibodies (**Table 2.1.**) or matching isotype control.

2.7. IL-33-induced airway inflammation

IL-33 (2μg/mouse) or PBS was administered intranasally for 3 consecutive days. To evaluate the contribution of IL-13 to IL-33-induced inflammation, mice were given neutralizing antibodies or appropriate isotype controls (20μg/mouse) together with IL-33. To evaluate the contribution of alveolar macrophages to IL-33-induced airway inflammation, clodronate or control liposomes (40μl/mouse) were administered i.n. 72 and 24 hours prior to three intranasal IL-33 inoculations over the next three consecutive days (one IL-33 inoculation daily; see **Figure 2.1.** below). Clodronate compound was a gift of Roche Diagnostics GmbH, Mannheim, Germany. All mice were sacrificed on day 6 and, bronchoalveolar lavage fluids were analyzed. Cells from BAL were counted using a hemocytometer as detailed in previous section. Cytospin preparations were made using a Cytospin 3 apparatus and stained with Diff-Quick (Triangle Biomedical Sciences) in a rapid Romanowsky staining method (see section **2.6.** for details). Differential cell counts were carried out using standard morphological criteria.

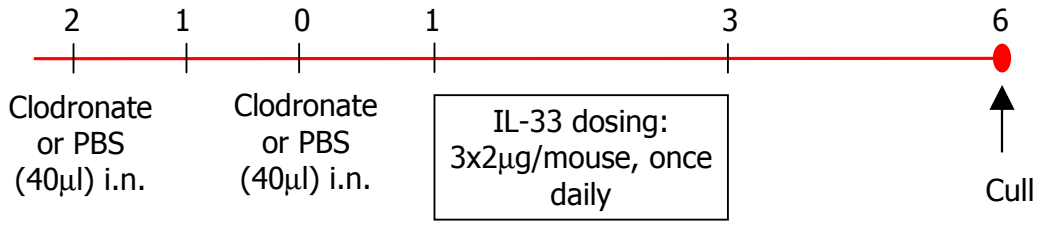


Figure 2.1. Intranasal instillation of clodronate liposomes into alveolar compartment. The depleting effect typically lasts up to 7 days. Two weeks prior to the first clodronate injection and during the experiment mice were kept in filtered cages and given sterile food and water. The 3-dose-6-day protocol for intranasal IL-33 administration was designed and optimised on the basis of the previous dose response and time course experiments that had been carried out by other members of our laboratory, namely Nick Pitman, Peter Kewin and Grace Murphy (unpublished data). These data indicated that the midpoint of eosinophil response occurs three days after the last intranasal IL-33 administration and it is moderate in magnitude, compared to a 7 day model, which was an advantage in terms of animal welfare and corresponded better to the features of clinical asthma.

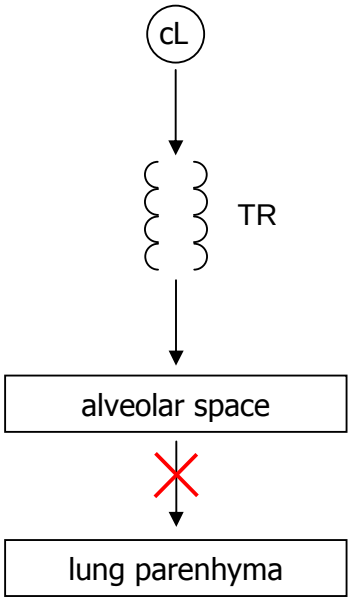


Figure 2.2. Intranasal instillation of clodronate liposomes (adapted from http://www.clodronateliposomes.org/, by Dr. Nico van Rooijen). The method allows for a specific depletion of alveolar macrophages without affecting macrophage populations in any other compartment. Key: **cL** = clodronate liposomes; **TR** = trachea

2.8. Adoptive transfer of eosinophils

BALB/c mice (n=10) were injected with mIL-33 (2μg) intraperitoneally for 7 days. Twenty four hours after last injection cells were harvested from peritoneal wash. Cells were then resuspended in PBS/2% FCS ($50x10^6$/ml) and 20μl of PE-conjugated rat-anti-mouse Siglec-F antibody (0.2mg/ml; BD Bioscience) and 40μl of APC-conjugated rat anti-mouse CCR3 (R&D Systems) per 1ml ($50x10^6$ cells) were added. Cells were incubated for 30 minutes on ice followed by two washing steps (10ml of PBS/2% FCS; 10 minutes, 300g). After last washing steps cells were resuspended in PBS ($50x10^6$/ml) and them equal amount of Cell Dissociation Solution (Sigma) was added to prevent forming cell clumps. The cells were then sorted by FACSA, based on high Siglec-F and CCR3 expression into complete medium containing 1ng/ml of GM-CSF to prevent cell death. Purity of sorted cells was evaluated and was ≥98%. The cells were then incubated 2 days with GM-CSF (1ng/ml), stained with CFSE (2μM) and administered intranasally to $ST2^{-/-}$ recipients ($10x10^6$/mouse). IL-33 (2μg/mouse) or PBS were administered intranasally on day 1, 2, and 3. Control recipient mice received PBS instead of cells followed by IL-33 or PBS inoculations. All mice were sacrificed on day 4 and BAL fluids were analyzed as described previously (see section **2.6.** for details). BAL cells were stained with FITC-conjugated anti-F4/80 antibodies and PE-conjugated anti-TLR2 and APC-conjugated anti-MR or APC-conjugated anti-CD4 or isotype controls (**Table 2.1.**).

2.9. Flow cytometry (FACS)

Cells from each sample were washed with FACS Staining Buffer (2% FCS in PBS, 0.09M NaN3), resuspended in 100μl of FACS Staining buffer and incubated with FcBlocker (anti mouse CD32/16 2μl/tube, BD Pharmingen). After 10 minutes specific fluorochrome-conjugated anitibodies or isotype controls were added for further 30 minute incubation, followed by two washing steps in staining buffer (1ml/tube). After washing, the cells were resuspended in 400μl of staining buffer and analyzed by flow cytometry (FACSCalibur). To enable dead cell exclusion, 2μl of 7-Amino-actinomycin-D (7-AAD, Via-probe, BD Biosciences) was added to each FACS tube immediately before acquiring and analysis on FACSCalibur flow cytometer (BD Biosciences). All data analysis was carried out using the FlowJo software (Tree Star Inc., Oregon, USA) or CellQuest (BD Biosciences). Details concerning antibodies used for FACS staining are summarized in **Table 2.1.**

Antigen	Label	Isotype	Stock	Volume/100 µl	Company
mST2L hST2L	FITC	rat IgG_1 mouse IgG_1	1mg/ml	2µl	MD Bioscience
mCCR3	APC	rat IgG_{2a}	25µg/ml	4µl	R&D Systems
mSiglec-F	PE	rat IgG_{2a}	0.2mg/ml	2µl	BD Biosciences
mGr-1	PerCP	rat IgG_{2b}	0.2mg/ml	2µl	BD Biosciences
hCD69	APC	mouse IgG_1	0.2mg/ml	5µl	BD Biosciences
hCD32	PE	mouse IgG_1	100 tests	2µl	BD Biosciences
hCD16	PE	mouse IgG_1	100 tests	2µl	BD Biosciences
mFcεR1	PE	Rat IgG	0.2mg/ml	2µl	BD Biosciences
mF4/80	APC	rat IgG_{2b}	0.2mg/ml	2µl	AbD Serotec
mMR	Alexa647	rat IgG_{2a}	50µg/ml	10µl	AbD Serotec
mCD11c	FITC	Hamster IgG_1	0.2mg/ml	2µl	BD Biosciences
mTLR2	FITC	rat IgG_{2b}	0.2mg/ml	2µl	eBioscience

Table 2.1. Monoclonal antibodies used for flow cytometry.

Key: m = mouse; h = human

2.10. Cytokine immunoassays

Cytokine and chemokine contents in culture supernatants was assessed by means of enzyme linked immunosorbent assay (ELISA) kits, ELISA paired antibodies and multiplexed immunoassay. The latter will be described in a next paragraph while this section provides details about standard ELISA protocols.

In regard to antibody pairs from BD Biosciences, all reagents and samples were applied at a volume of 50μl/well. Flat-bottomed ELISA plates (Immulon 4 HBX, Thermo Labsystems, Franklin, USA) were coated with capture antibody in BD ELISA coating buffer (0.1M NaHCO3 pH 8.4) and incubated overnight at 4^{O}C. Plates were washed three times with ELISA wash buffer (0.05% Tween-20 in PBS pH 7.4) and incubated 1 hour at 37^{O}C with 300μl/well of assay buffer (1% BSA in PBS). Following the blocking step, plates were washed three times and samples and standards were added. A 7 point doubling dilution standard curve in duplicates was used. Assay buffer served as blank (also in duplicates) and for the dilution of the standards. Samples and standards were incubated for 2 hours at room temperature after which plates were washed five times and biotinylated detection antibody diluted in assay buffer was added to all wells and incubated for 2 hours at room temperature. Subsequently, plates were washed three times and Streptavidin-HRP conjugate (Extravidin, Sigma) was added in 1:1000 dilution. After 30 minutes of incubation at room temperature plates were washed three times and 100μl/well of TMB substrate was added (KPL, Gaithersburg, USA). Plates were developed in the dark for a maximum of 30 minutes. Some assays were terminated earlier if blue colour had become clearly visible. Optical density was measured using an MRX II microplate reader

(Dynex Technologies, Worthing, UK) at 630nm wavelength. Sample values were calculated from the standard curves using Revelation software package (Dynex Technologies).

Similar protocol was followed for R&D antibodies except for the different ELISA assay buffer that was used (see **Table 2.2.**). Also, the supplied Eotaxin-2 detection antibody required dilution in the R&D ELISA assay buffer containing 2% normal goat serum. The latter had to be heat-inactivated and cooled down to room temperature prior to antibody dilution.

All antibody pairs used are detailed in **Table 2.2.**

Analyte	Capture Ab (μg/ml)	Detection Ab (μg/ml)	Sensitivity (pg/ml)	Company
mIl-4	2	2	20-20x10^3	BD Biosciences
mIL-5	4	4	10-10x10^3	BD Biosciences
mIL-6	1	1	10-10x10^3	BD Biosciences
mIFN-γ	1	1	40-40x10^3	BD Biosciences
mIL-13	kit	kit	5-5x10^3	R&D Systems
mMIP-1α	kit	kit	5-5x10^3	R&D Systems
mEotaxin-1	0.8	0.4	5-0.5x10^3	R&D Systems
mEotaxin-2	2	0.08	4-4x10^3	R&D Systems
mTARC	2	0.2	5-1x10^3	R&D Systems
mTGF-β1	4	0.2	15-1.5x10^3	R&D Systems

Table 2.2. Monoclonal antibody pairs used for ELISA.

Key: m = murine

2.11. Multiplexed immunoassay (Luminex)

This type of an immunoassay utilizes specific antibodies for the cytokines and chemokines of choice that have been coated on the designated beads that had been coded with a characteristic combination of fluorescent dyes. Such modification to classical ELISA allows for the simultaneous identification of multiple ligands in a single reaction volume.

All culture supernatants were collected and stored at -20^{O}C until they were assayed using LMC006 (Cytokine Mouse 20-Plex). Full list of cytokines present on the Luminex plate included IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, IL-1β, IL-6, IL-8, TNF-α, VEGF, IL-17, FGF, IL-1α and chemokines: KC, MCP-1, MIG, MIP-1α and IP-10. The assay was used according to the manufacturer's instructions. All reagents and solutions required were purchased from the original kits. A 96-well Luminex filter plate was pre-washed with Wash solution using vacuum apparatus. Next, 50μl of fluorescent bead mixture was added into each well and washed twice with Wash solution. Subsequently, the following reagents were added per well: 50μl of Incubation Buffer, 100μl of standard or 50μl of Assay Diluent plus 50μl of sample. Mixtures of fluorescent beads with samples or standards were then incubated at room temperature for 2 hours with agitation in the dark. Subsequently, the wells were washed twice and 100μl per well of biotinylated detection antibody was added and incubated for 1 hour as previously. Then, the plate wells were washed twice and 100μl per well of Streptavidin-HRP was added and incubated for another 30 minutes as before. Next, the wells were washed three times before resuspending the beads in 100μl per well of Wash solution. The fluorescence bound to the beads was

analysed using a Luminex 100™ analyse (Luminex Corporation, Texas, USA). Data was then analysed by means of Luminex software (Luminex Corporation).

2.12. Staining for cyanide-resistant eosinophil peroxidase

Cytospin preparations of 1-5x10^4 cells were prepared using a Cytospin 3 apparatus (Shandon Scientific Limited, Cheshire, UK). Slides were fixed for 0.5min in ice-cold (4^{O}C) formalin-acetone buffer (see section **2.3.** for details). Next, slides were washed with room temperature tap water and air dried. Subsequently, cells were stained for eosinophil-specific, cyanide-resistant peroxidase activity using a previously described method (174-175); the validity of this method was also verified in human cells (176-177). Briefly, cytospin preparations were incubated in DAB/H_2O_2-containing phosphate buffer supplemented with low concentration of KCN for 10 minutes (KCN was used instead of NaCN as the source of cyanide ion). Subsequently, slides were washed by shortly immersing in fresh tap water, then briefly under running tap water, and air dried. Finally, cells were counterstained with hematoxylin and cover slips were affixed onto the air-dried slides using DPX (BDH Laboratory Supplies, Poole, UK).

Precautions were taken to avoid contact with the poison, and a cyanide antidote was also kept available. At the conclusion of these experiments, all cyanide-containing solutions were disposed of in accordance with relevant safety regulations by technical staff.

2.13. Extraction of RNA

Freshly isolated human peripheral blood neutrophils and eosinophils were washed twice with PBS, gently centrifuged, and PBS residues were completely removed by aspiration. Cells were lysed in RLT solution (Qiagen), and stored at -20^{O}C until they were processed. The RNA isolation was carried out using the RNeasy Micro Kit (Qiagen) according to the protocol provided by the manufacturer. Unwanted genomic DNA was removed by carrying out an optional step with the RNAse-free DNase set (Qiagen) as recommend in the original manufacturer's protocol. The RNA contents and its quality was measured using a NanoDrop 2000c instrument (Thermo Fisher Scientific). Samples were then stored at -80^{O}C until processed.

2.14. Reverse transcription polymerase chain reaction (RT-PCR)

RNA samples were thawed and kept on ice until used as template in a reverse transcription using Superscript II Reverse Transcriptase set (Invitrogen) according to the manufacturer's protocol. The reaction mixture comprised of 300ng of RNA template, 1μg Oligo(dT) (500μg/ml), 1μl of dNTP mix (25mM each) and nuclease-free water (Qiagen). A total volume was 12μl per reaction, placed in a nuclease-free microcentrifuge tube (ABgene, Surrey, UK). The mixture was incubated in 65^{O}C for 5 minutes then rapidly cooled to 4^{O}C. Next,

4μl volume of 5X First-Strand Buffer, 2μl of 0.1M DTT and 1μl of RNase inhibitor (40 units/ml) were added and incubated at 42^{O}C for two minutes. Subsequently, 1μl (200 units) of Superscript II RT was then added and incubated at 42^{O}C for 50 minutes. The reaction was then terminated by incubation at 70^{O}C for 15 minutes. Tubes containing PCR mixture with no Superscript II served as negative controls. cDNA was then stored at -80^{O}C.

2.15. ST2L- and sST2-specific cDNA amplification (PCR)

AmpliTaq Gold 360 DNA Polymerase (Invitrogen) was used to amplify cDNA transcripts of interest. The original manufacturer's protocol was followed. In short, reaction mixture (total of 25μl) consisted of 12.4μl nuclease-free water (Qiagen), 2.5μl AmpliTaq Gold 360 Buffer (10x), 2μl Magnesium Chloride (25mM), 2μl dNTP mix (10-mM, 2.5mM each; final concentration 200μm each), 2μl forward primer (final concentration 1.0μM), 2μl reverse primer (final concentration 1.0μM), 0.1μl (1U), AmpliTag Gold 360 DNA Polymerase, and 2μl cDNA template. Each polymerase chain reaction was performed using standard cycle conditions suggested by the polymerase manufacturer (holding, 10min/95^{O}C for AmpliTaq Gold activation; cycling: 35x denature, 30sec/95^{O}C anneal, 30sec/58^{O}C, extend, 30sec/72^{O}C; final extension, 7min/72^{O}C; final hold/4^{O}C). Amplifications were carried out using a Veriti 96-well Thermal Cycler (Applied Biosystems). Samples were normalized by reference to a housekeeping gene, β-actin, as a reporter gene. Primer pairs for human ST2L, sST2 and β-actin were validated and kindly provided by Mark Moore.

The amplified templates were then analyzed on a 2% agarose (Gibco) gel containing 0.8μg/ml ethidium bromide (Sigma) in 0.5 times TBE buffer. Gels were run on at 80V, powered by a constant power supply (Pharmacia). Gel was then visualised under ultra-violet (UV).

2.16. Statistical analysis

Analysis of variance (ANOVA) followed by Tukey's test or Student's t-test was applied to the data. All data are expressed as mean ± SEM. $p < 0.05$ was considered statistically significant.

Chapter 3:

The role of alveolar macrophages in IL-33-induced airway inflammation

3.1. Alveolar macrophages are required for IL-33-induced airway inflammation

It has been previously reported that IL-33 is abundantly expressed in the lungs of asthma patients. Consistently, IL-33 overexpression or administration of exogenous IL-33 into the lungs leads to the development of symptoms that resemble key asthma features, such as lung eosinophilia, type 2 cytokine and chemokine production as well as the activation of epithelial cells (48, 178). However, it is still unclear which cell type is responsible for an initiation of this response. Using deficient mice or depletion antibodies Kondo et al excluded the contribution of T and B cells, NK cells, basophils and mast cells.

Alveolar macrophages are predominant immune effector cell type resident in the alveolar spaces of the airways and it is believed that they are responsible for the initiation of inflammatory responses. We previously showed that IL-33 can target macrophages and is a potent amplifier of bone marrow-derived and alveolar macrophage differentiation toward alternatively activated macrophage (AAM, M2) phenotype (26). Those cells are characterized by high expression of pro-fibrotic arginase-I and production of chemokines such as TARC (CCL17) and eotaxin-2 (CCL24) (26). Thus, I hypothesized that alveolar macrophages are the cell type that acts as the first line of response upon IL-33 release and, in turn, modulates subsequent immunological events. Naïve mice were administered with IL-33 (2μg i.n.) or PBS for 3 consecutive days and the mice were culled on day 6. Mice receiving IL-33 showed lung inflammation manifested by an increase in total BAL cell count, including eosinophil, macrophage and neutrophil cell counts (**Figure 3.1.**). In order to investigate

the contribution of alveolar macrophages to IL-33-induced airway inflammation, clodronate or control liposomes (40 μl/mouse) were administered intranasally 72 and 24 h before three consecutive days of intranasal IL-33 inoculations as described by Thepen et al. (157). This route of liposome administration affects alveolar but not lung parenchyma macrophage (157) (**Figure 2.2.**). The intranasal administration of clodronate liposomes was able to deplete around 80% of alveolar macrophages compared to the control group. Interestingly, IL-33 induced significantly less severe eosinophilia in alveolar macrophage-depleted mice compared to control mice (**Fig. 3.2.**) indicating that IL-33-driven AAM activation of alveolar macrophages is likely responsible for IL-33-induced airway eosinophilia.

3.2. IL-33-induced airway inflammation and alternatively activated macrophage polarization are IL-13 dependent

In our previous studies *in vitro* we were able to demonstrate that IL-13/IL-4Rα signaling is essential for IL-33-driven AAM activation by inducing the expression of ST2L (26). To confirm the contribution of IL-13 to IL-33-induced airway inflammation and alveolar macrophage differentiation *in vivo*, mice were given IL-13 neutralizing antibodies or appropriate isotype control (20 μg/mouse) for 5 consecutive days. IL-33 was administered over the first three days. All mice were sacrificed on day 6. Neutralization of IL-13 significantly decreased total cell and eosinophil counts in the BAL by 45 and 55% respectively (**Fig. 3.3.**). Importantly, anti-IL-13 neutralizing antibodies markedly decreased the percentage of mannose receptor-positive (MR^+) alveolar macrophages compared to the control group (54 ± 8% and 82 ± 2% of all macrophage,

respectively; $p < 0.05$). The percentage of TLR2-positive macrophages remained unchanged **(Fig. 3.4.).** These data demonstrate that IL-13 is required, at least partially, for IL-33-induced airway inflammation and differentiation of alveolar macrophages toward AAM.

Summary of Chapter 3

Together, these data indicate that IL-33/ST2 signaling plays a significant role in the polarization of alveolar macrophages toward AAM by acting via IL-13 dependent mechanism. Most importantly, alveolar macrophages are likely the first line of response to IL-33 signal and as such are major factor that contributes to the initiation and maintenance of IL-33-induced airway inflammation.

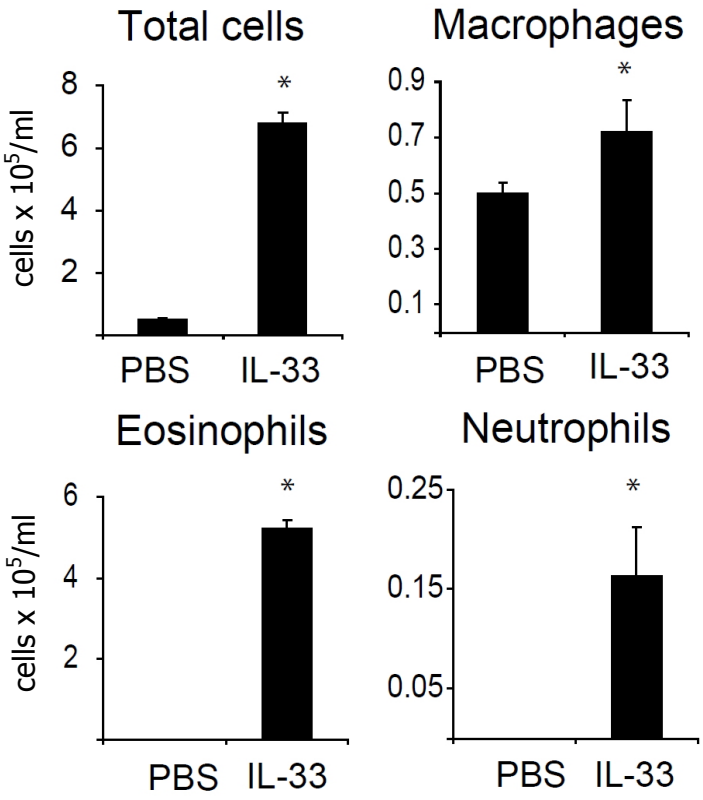


Fig. 3.1. IL-33 induces airway inflammation. BALB/c mice were treated i.n. with IL-33 (2 μg/mouse) or PBS for 3 consecutive days (all groups $n = 5$). Mice were sacrificed on day 6. BAL differential cell counts are shown. Data are means ± SEM. *, $p < 0.05$ PBS versus IL-33-treated mice.

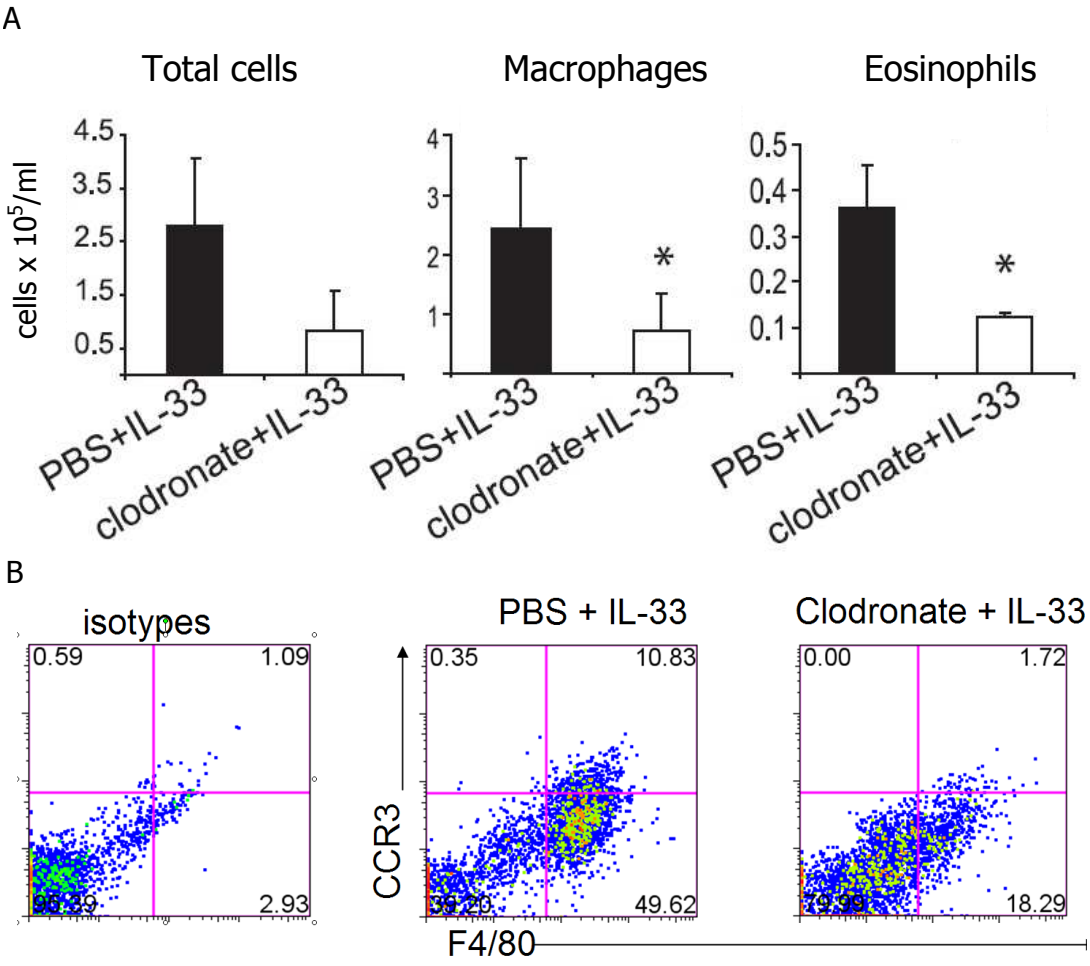


Fig. 3.2. A-B Macrophage depletion results in diminished eosinophilia and lower total cell counts in BAL. Clodronate ($n = 5$) or control ($n = 5$) liposomes (both 40μl/mouse) were administered i.n. 72 and 24h before i.n. IL-33 inoculation (3 consecutive days, 2μg/mouse/day). All mice were sacrificed on day 6 and BAL cells were analyzed. BAL differential cell counts are shown. A, Data are means ± SEM. *, $p < 0.05$, clodronate versus control liposomes. B, Representative data from FACS analysis of BAL cells is shown.

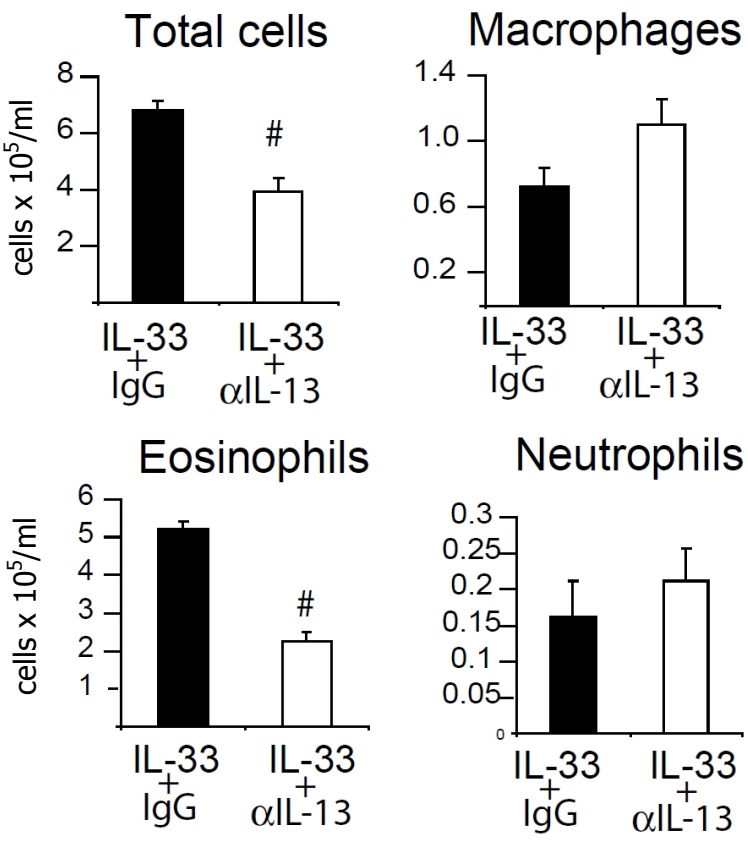


Fig. 3.3. The contribution of IL-13 to IL-33-induced airway inflammation. BALB/c mice were treated i.n. with IL-33 (2μg/mouse) or PBS for 3 consecutive days. Some mice also received anti-IL -13-neutralizing Abs (20μg/mouse) for 5 consecutive days. The control group received isotype-matched rat IgG (all groups $n = 5$). Mice were sacrificed on day 6. BAL differential cell counts are shown. Data are means ± SEM. $^{\#}$, $p < 0.05$ IL-13 neutralizing Abs versus IgG-treated mice.

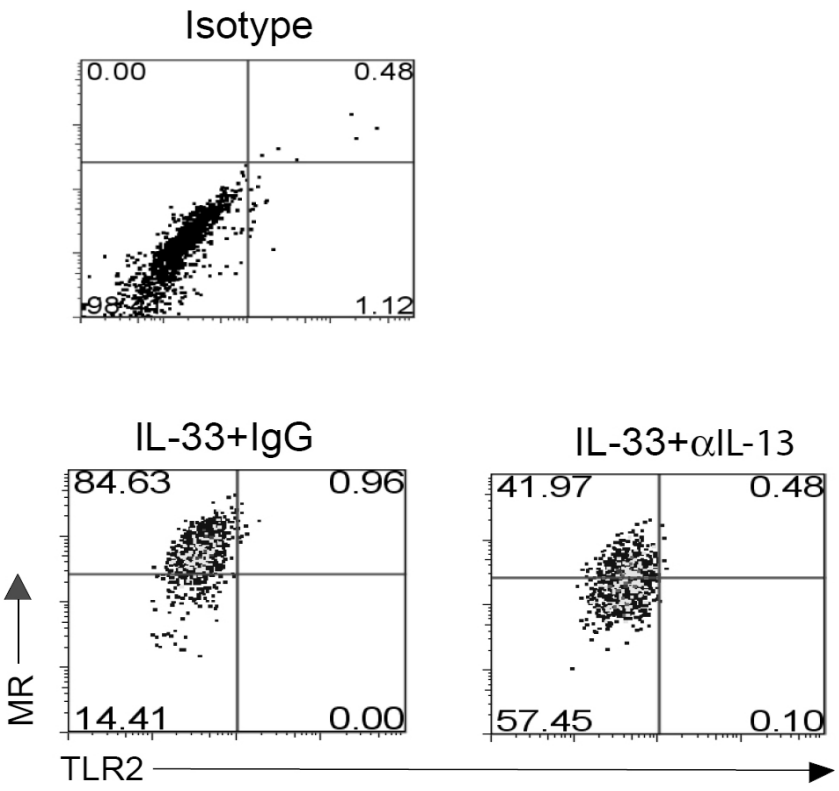


Fig. 3.4. IL-33-induced differentiation of alveolar macrophages toward AAM is diminished by IL-13 neutralization. BALB/c mice were treated i.n. with IL-33 (2μg/mouse) or PBS for 3 consecutive days. Some mice also received anti-IL-13-neutralizing Abs (20μg/mouse) for 5 consecutive days. The control group received isotype-matched rat IgG (all groups $n = 5$). Mice were sacrificed on day 6. Representative staining for MR and TLR2 of alveolar macrophages from anti-IL-13 Abs and IgG-treated mice is shown.

Chapter 4:

The role of IL-33 in eosinophil development

4.1. IL-33 promotes eosinophil development in bone marrow

IL-33 can induce eosinophilia in mice (4, 48, 80). I showed in Chapter 3 that IL-33-activated alveolar macrophages are responsible for attracting eosinophils from the periphery into lungs. However, it is still not clear whether IL-33 can directly affect eosinophil differentiation and activation. In this chapter I addressed the first part of this question by using several different methods and various eosinophil markers (89). Initially, I incubated whole bone marrow cells from WT or $ST2^{-/-}$ mice with IL-33 or IL-5 for 5 or 8 days. IL-5 and IL-33 induced eosinophil differentiation from bone marrow precursors of WT mice as determined by the presence of eosinophil peroxidase (EPO, eosinophil granule-associated marker) (**Fig. 4.1.**). By contrast, IL-5 but not IL-33 induced eosinophil differentiation in $ST2^{-/-}$ mice. There were no EPO^{+} cells detected in the culture with medium alone in WT or $ST2^{-/-}$ cultures. These data indicate that IL-33 can induce eosinophil development from bone marrow cells in a ST2-dependent manner.

4.2. IL-33 directly drives eosinophils from haemopoietic precursor cells ($Lin^{-}/CD117^{+}$)

To determine whether IL-33 can act directly on hematopoietic progenitor cells, $CD117^{+}$ ($c\text{-}kit^{+}$) cells were purified from bone marrow of WT or $ST2^{-/-}$ mice after depletion of committed or mature lineage-positive cells and cultured as above. Freshly isolated BALB/c $Lin^{-}CD117^{+}$ progenitors expressed ST2L (**Fig. 4.2.**). The expression of IL-5Rα on these cells was below the detection level of flow

cytometry (**Fig. 4.3.**). However, the expression of IL-5Rα was markedly increased in the cultures stimulated with IL-33 for 4 days (**Fig. 4.4.**). Eosinophil differentiation was analyzed by FACS using surface eosinophil-specific marker, Siglec-F. IL-5 could induce comparable numbers of Siglec-F^+ cells that stained positively with eosin both in WT and $ST2^{-/-}$ $CD117^+$ cell cultures (10 ± 2.5% and 9.2 ± 2.5% respectively), whereas IL-33 could induce Siglec-F^+-eosin-positive eosinophils only in WT but not in $ST2^{-/-}$ $CD117^+$ cell cultures (10.5 ± 0.5% and 1.2 ± 1% respectively) after 5 days of culture (**Fig. 4.5.**, FACS; **Fig. 4.7.**, quantitative diagram). The morphology of Siglec-F^+ cells from IL-5- and IL-33-driven cultures matched typical eosinophil cell characteristics with pink-orange eosin-stained granules in the cytoplasm as shown in **Fig. 4.6.** Similar data were obtained for 8-day cultures; there were no live cells, including Siglec-F^+, detected in 8-day cultures with medium alone (data not included). In addition, IL-33-driven Siglec-F^+ eosinophils expressed ST2L (**Fig. 4.8.**).

These results therefore indicate that IL-33 is able to act directly on hematopoietic progenitor cells and stimulate their differentiation to mature eosinophils.

4.3. IL-33 driven eosinophil development is partially mediated by IL-5

Given that IL-33 stimulates the production of cytokines associated with type 2 immunity and eosinophil development (4, 43, 130), I examined the contribution of IL-5, IL-9 and IL-13 to the IL-33-induced development of eosinophils from

bone marrow precursors. Bone marrow cells from WT, IL-13$^{-/-}$ and IL-5/IL-9/IL-13$^{-/-}$ triple knockout mice were cultured with IL-33 (5 or 20ng/ml) or medium alone over 5 or 10 days and mature eosinophil population characterized by the expression of CCR3 was evaluated by FACS (CCR3^{+}GR-1^{intermediate}) and eosin-positive staining. IL-33 could induce comparable numbers of CCR3^{+}Gr-1^{+} eosinophils from the precursors of IL-13$^{-/-}$ and WT mice (9.5 ± 2% and 9 ± 1.9% respectively), but not from the precursor cells of triple knockout mice (2.5 ± 1.5%) (**Fig. 4.9.1-2.**). These results suggest that IL-5 and/or IL-9 but not IL-13 are required for the IL-33-induced eosinophil differentiation from precursor cells. Since IL-5 is known as a major eosinophil differentiation factor (179), I cultured CD117^{+} hematopoietic progenitor cells with IL-33 in the presence of an IL-5-neutralizing antibody. The treatment with anti-IL-5 monoclonal antibody markedly reduced the IL-33-induced differentiation of eosinophils (SiglecF^{+}CCR3^{+}) from CD117^{+} cells (**Fig. 4.10.**). Consistent with this finding, WT CD117^{+} cells stimulated with IL-33 produced substantial amounts of IL-5 compared to un-stimulated cultures or IL-33-treated ST2$^{-/-}$ CD117^{+} cells (**Fig. 4.11.**). Furthermore, an increase in FcεRI-positive cells, likely basophils, were detected in cultures stimulated with IL-33 as reported previously by Schneider et al. (79), (**Fig. 4.12.**).

Together, these data suggest that IL-33-driven eosinophils development from progenitor cells is ST2 and IL-5 but not IL-13 dependent.

4.4. IL-33/ST2 signaling is required for optimal expression of CCR3 on eosinophils

CCR3 is a major chemokine receptor responsible for the mobilization of mature eosinophils from the bone marrow and their trafficking to sites of inflammation (130). To investigate whether IL-33 is involved in the regulation of CCR3 expression during eosinophil differentiation, FACS-sorted Siglec-F^+ eosinophils from IL-5 driven bone marrow cultures (7 days) were checked for characteristic eosinophil morphology (**Fig. 4.13.**) and incubated with IL-5 (2ng/ml) ± IL-33 for 24h and CCR3 expression determined by FACS. As expected, the majority of IL-5-driven Siglec-F^+ eosinophils readily expressed CCR3 (**Fig. 4.14.,** histogram), however, IL-33 was able to further increase surface density of CCR3 on these eosinophils (**Fig. 4.15.**, diagram). Next, I investigated whether there was any difference in the basal expression of CCR3 between IL-5-driven eosinophils from WT and $ST2^{-/-}$ mice. Eosinophils from $ST2^{-/-}$ mice expressed significantly less CCR3 than WT eosinophils (**Fig. 4.16.** and **Fig. 4.17.**). Thus, these results clearly indicate that the endogenous IL-33/ST2 signaling pathway enhances the expression of CCR3 on eosinophils.

Summary of Chapter 4

Together, this part of my study suggests that IL-33 is able to act locally in the bone marrow compartment and directly stimulates eosinophil differentiation from hematopoietic progenitor cells in an IL-5-dependent manner.

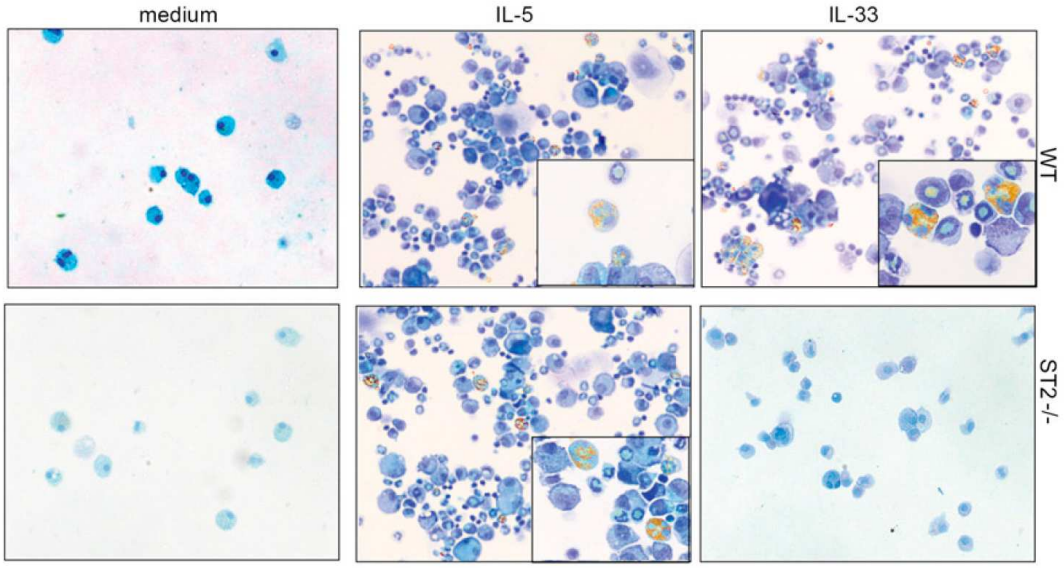


Fig. 4.1. IL-33 induces eosinophil differentiation from bone marrow cells. Bone marrow (2×10^6/ml) from WT or $ST2^{-/-}$ mice were cultured with IL-5 (10ng/ml) or IL-33 (10ng/ml) for 5 days. The cell number and dose of cytokines used were optimized in preliminary experiments (data not shown). EPO staining of cultured bone marrow cells is shown (original magnification $\times$ 10; insets $\times$ 40). Data are representative of three independent experiments.

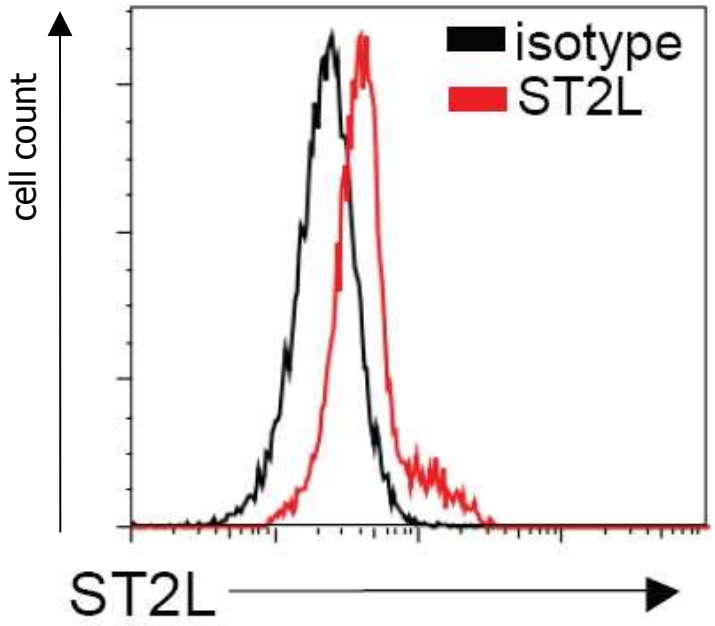


Fig. 4.2. Freshly isolated $Lin^{-}CD117^{+}$ haematopoietic cells from WT mice readily express ST2L. Data are representative of three independent experiments.

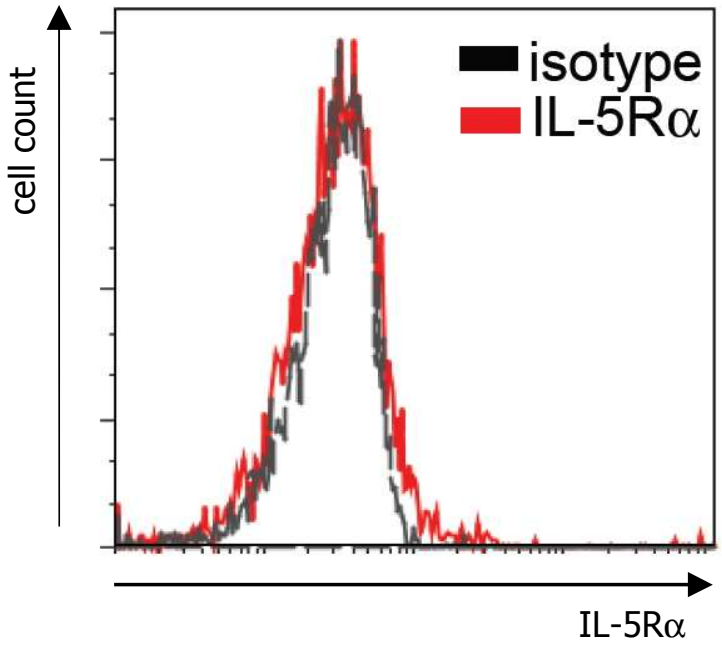


Fig. 4.3. Freshly isolated $Lin^{-}CD117^{+}$ haematopoietic cells from WT mice do not express IL-5Rα at the level detectable by FACS. Data are representative of three independent experiments.

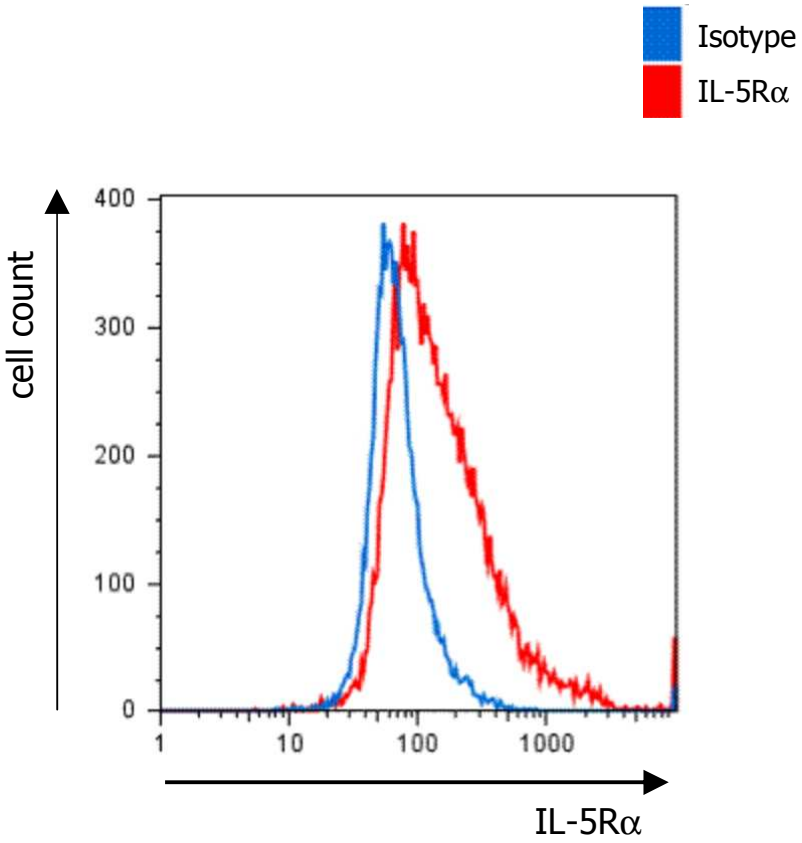


Fig. 4.4. The expression of IL-5 receptor in $CD117^+$ cells can be upregulated by IL-33 stimulation. Representative FACS analysis of $CD117^+$ cultures with IL-33 on day 4 is shown. Data are representative of three independent experiments.

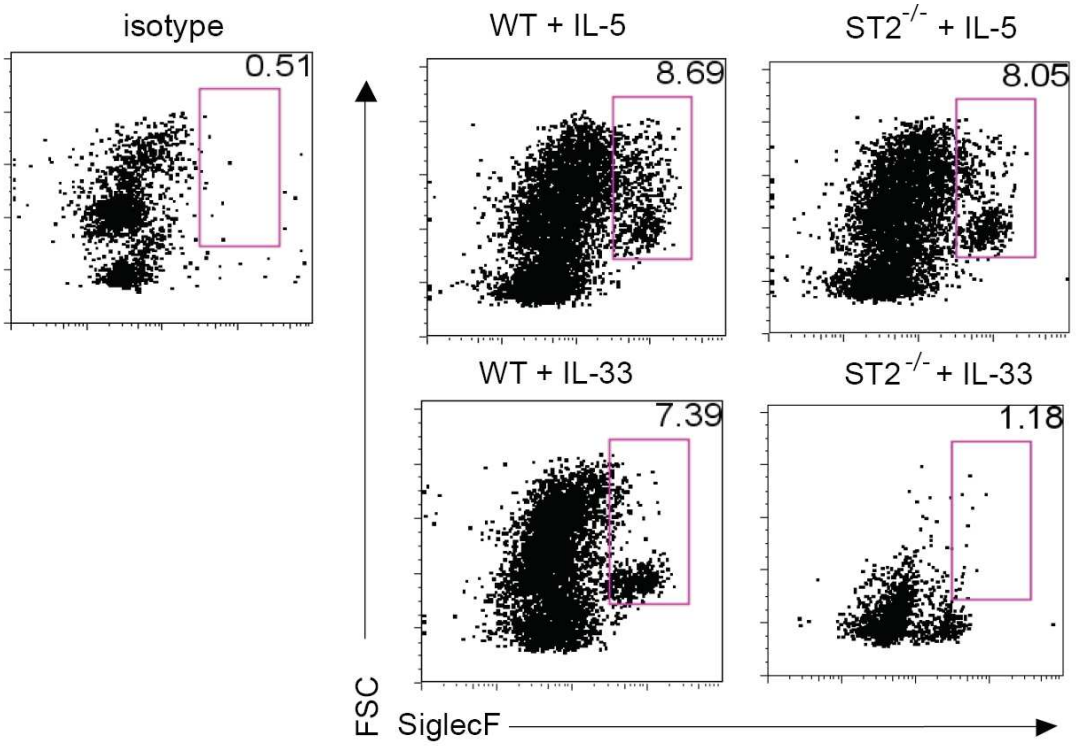


Fig. 4.5. IL-33 induces eosinophil differentiation from haematopoietic progenitor cells (FACS). $Lin^{-}CD117^{+}$ haematopoietic cells (0.5×10^{6}/ml) from BALB/c or $ST2^{-/-}$ mice were cultured with IL-5 (10ng/ml) or IL-33 (10ng/ml) for 5 days. Representative FACS analysis of $CD117^{+}$ cultures is shown. Data are representative of three independent experiments.

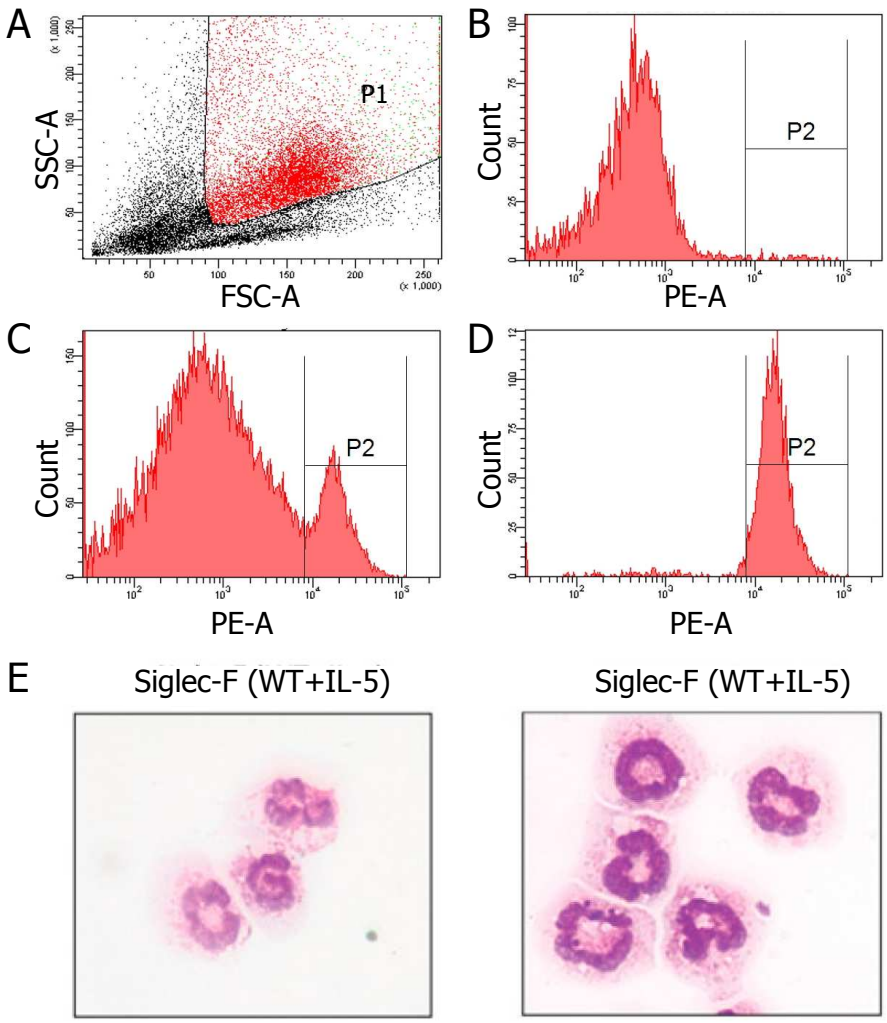


Fig. 4.6. Sorting eosinophils from $Lin^{-}CD117^{+}$ cells cultured in with IL-33. A-D, gating used for sorting Siglec-F^{+} cells; A, Cells gated for big, granule-containing cells in P1; B, cells from P1 stained with IgG-PE before sorting (isotype control); C, cells from P1 stained with Siglec-F-PE before sorting where gate P2 was used to sort for eosinophils; D, Siglec-F^{+} cells in gate P2 after sorting (returned sample); E, Eosin-stained cytoplasm and donut shaped nuclei of Siglec-F-sorted eosinophils obtained from cultures of WT $CD117^{+}$ cells with IL-5 or IL-33; a representative image of Siglec-F^{+} cells shown (original magnification $\times$ 40).

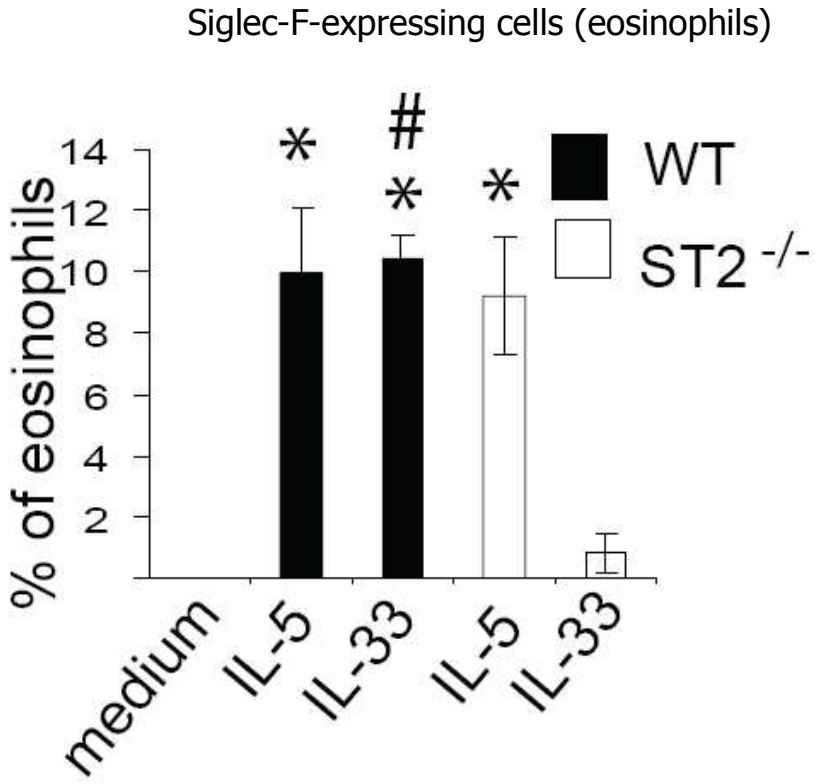


Fig. 4.7. IL-33 induces eosinophil differentiation from haematopoietic progenitor cells. Quantitative evaluation of the eosinophil marker Siglec-F on $CD117^+$ cells. $CD117^+$ haematopoietic cells (0.5×10^6/ml) from BALB/c or $ST2^{-/-}$ mice were cultured with IL-5 (10ng/ml) or IL-33 (10ng/ml) for 5 days. Data are means ± SEM of three independent experiments. *$p < 0.05$, IL-33- or IL-5-treated versus medium; #$p < 0.05$, WT versus $ST2^{-/-}$ sample.

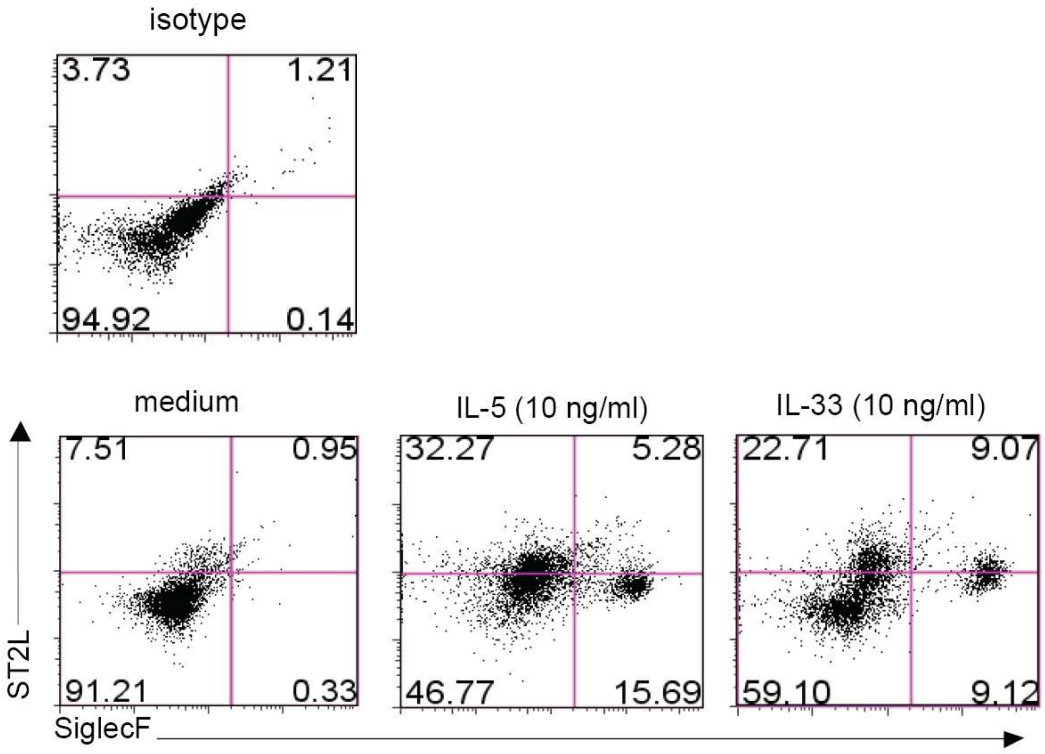


Fig. 4.8. Siglec-F$^+$ eosinophils that differentiate from Lin$^-$CD117$^+$ cells express ST2L. Lin$^-$CD117$^+$ hematopoietic cells (0.5×10^6/ml) from BALB/c mice were cultured with IL-5 (10ng/ml) or IL-33 (10ng/ml) for 5 days. Representative FACS analysis of CD117$^+$ cultures is shown. Data are representative of three independent experiments.

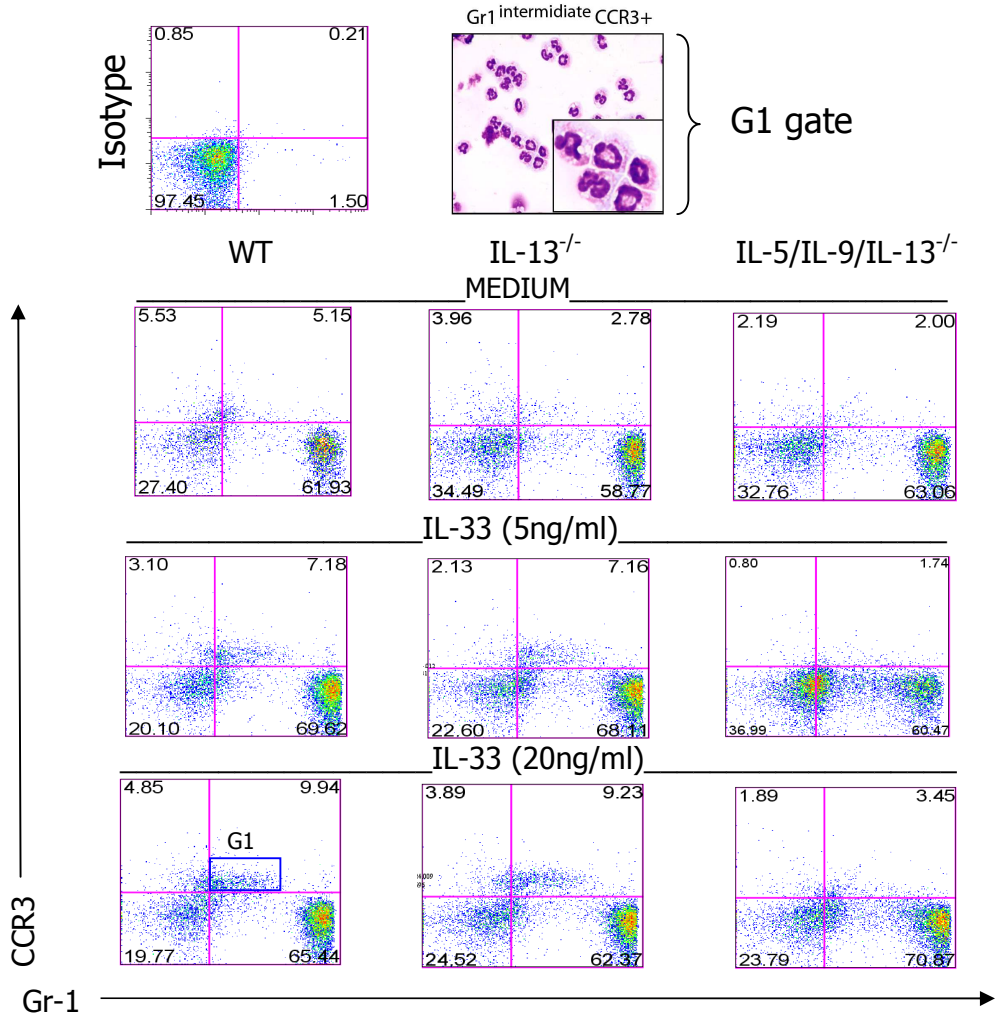


Fig. 4.9.1. IL-33-induced eosinophil differentiation from bone marrow cells is markedly inhibited in IL-5/IL-9/IL-13$^{-/-}$ triple knockout mice (5 days). Bone marrow cells (2×10^6/ml) from WT (C57BL/6 background), IL-13$^{-/-}$ (C57BL/6), or IL-5/IL-9/IL-13$^{-/-}$ triple knockout mice (C57BL/6) were cultured with IL-33 (5 or 20ng/ml) or medium alone for 5 days. Representative data on the expression of eosinophil markers CCR3^{high} and GR-1^{intermediate} of a single experiment are shown. Technical duplicates were used to generate FACS data for each IL-33 dose used. Eosin-stained CCR3^{high} and GR-1^{intermediate}-sorted cells (gate G1). Original magnification $\times$20; inset $\times$100.

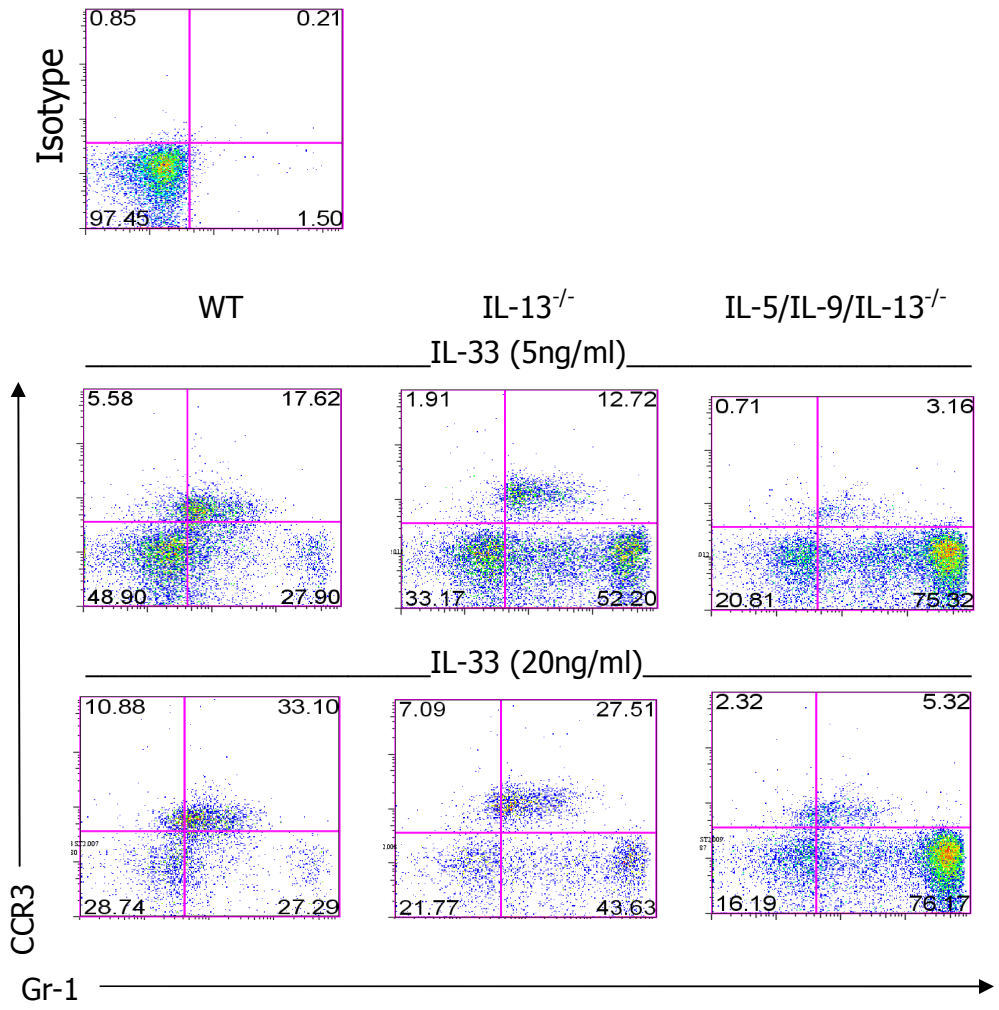


Fig. 4.9.2. IL-33-induced eosinophil differentiation from bone marrow cells is markedly inhibited in IL-5/IL-9/IL-13$^{-/-}$ triple knockout mice (10 days). Bone marrow cells (2×10^6/ml) from WT (C57BL/6 background), IL-13$^{-/-}$ (C57BL/6), or IL-5/IL-9/IL-13$^{-/-}$ triple knockout mice (C57BL/6) were cultured with IL-33 (5 or 20ng/ml) or medium alone for 10 days. Representative data on the expression of eosinophil markers CCR3^{high} and GR-1^{intermediate} of a single experiment are shown. Technical duplicates were used to generate FACS data for each IL-33 dose used.

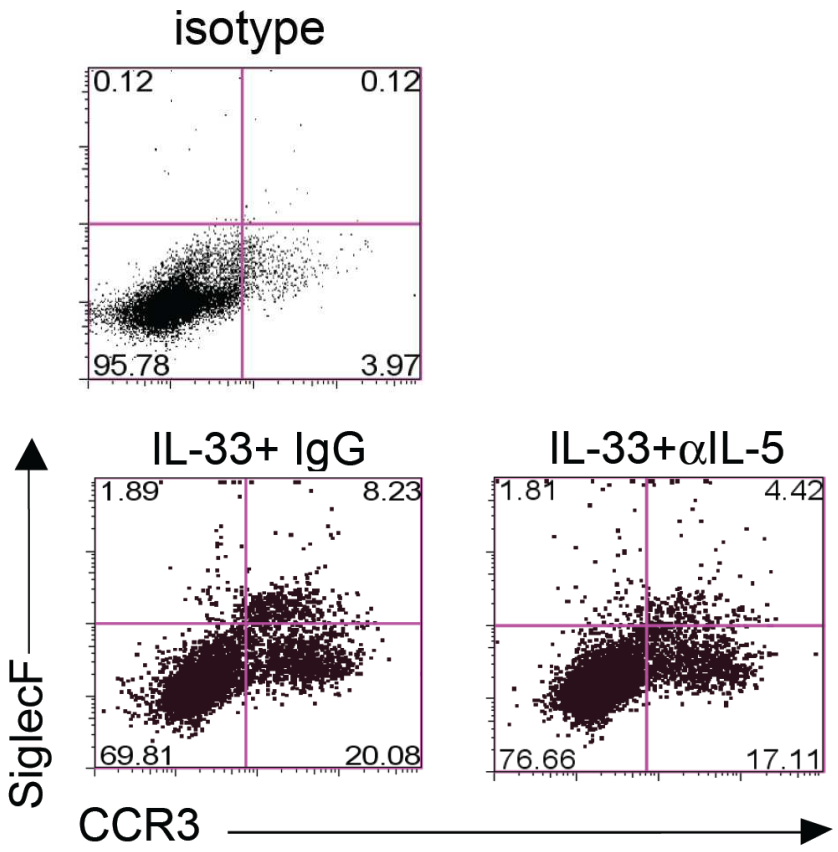


Fig. 4.10. IL-33–induced eosinophil differentiation from haematopoietic progenitor cells is IL-5 dependent. $CD117^{+}$ hematopoietic cells (0.5×10^{6}/ml) from BALB/c were cultured with IL-33 (10ng/ml) with or without IL-5-neutralizing Ab (or isotype control, 10μg/ml) for 5 days. Representative FACS data of mature eosinophil; Siglec-F and CCR3 markers are shown. Data are representative of three independent experiments.

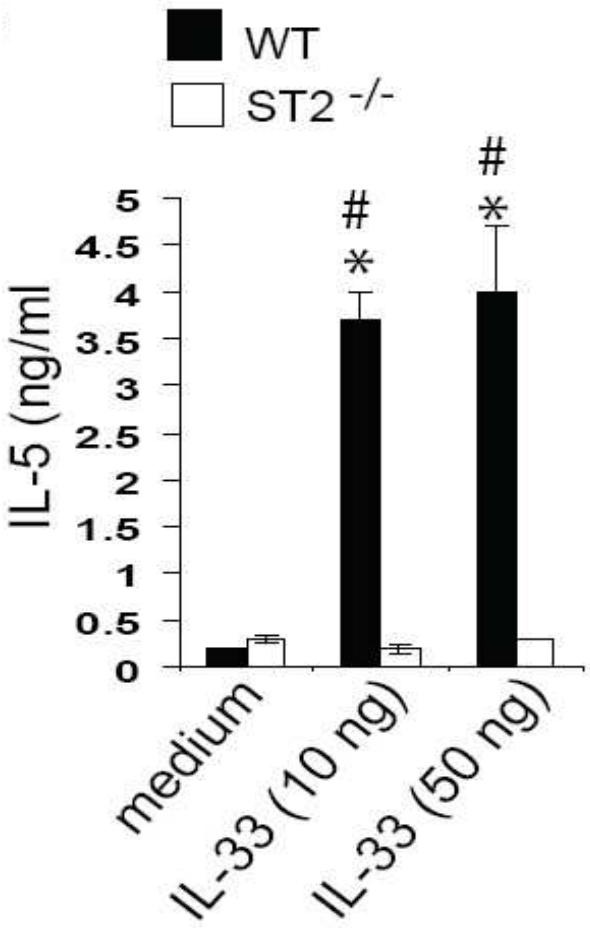


Fig. 4.11. IL-33 induces IL-5 production by $CD117^+$ cells. $CD117^+$ haematopoietic cells (0.5×10^6/ml) from WT or $ST2^{-/-}$ (BALB/c background) mice were cultured with IL-33 (10 or 50ng/ml) for 5 days. IL-5 levels in culture supernatants were determined by ELISA. Data are means ± SEM of three independent experiments, *$p < 0.05$, IL-33 versus medium; $^{\#}p < 0.05$, WT versus $ST2^{-/-}$.

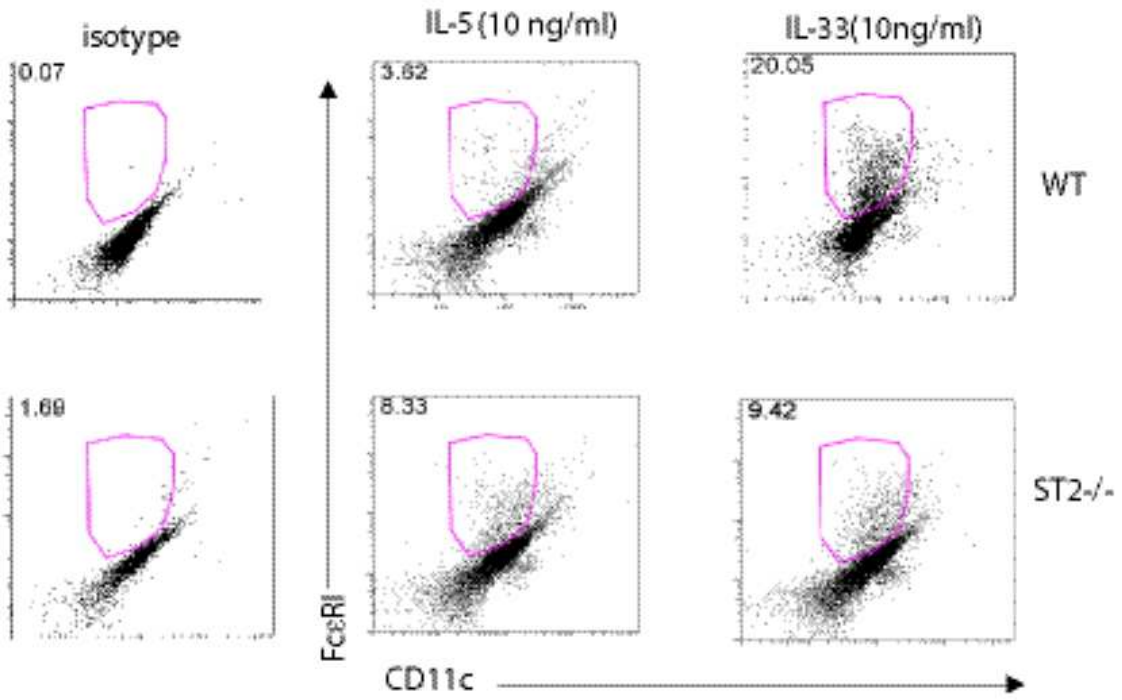


Fig. 4.12. The effect of IL-33 on the development of basophils. A population of FcεRI-positive cells expands in cultures of WT but not $ST2^{-/-}$ cells when stimulated with IL-33. $CD117^{+}$ cells were purified from bone marrow of WT and $ST2^{-/-}$ mice after the depletion of committed and mature lineage-positive cells and culture in the presence of IL-5 (10ng/ml) or IL-33 (10ng/ml) for 4 days. Cell phenotype was analysed by FACS using FITC-anti-CD-11c, PE-anti-FcεRI antibodies and relevant isotype controls. Data from a single experiment are shown. Similar results supporting IL-33-driven basophil differentiation were reported by Schneider et al. (79).

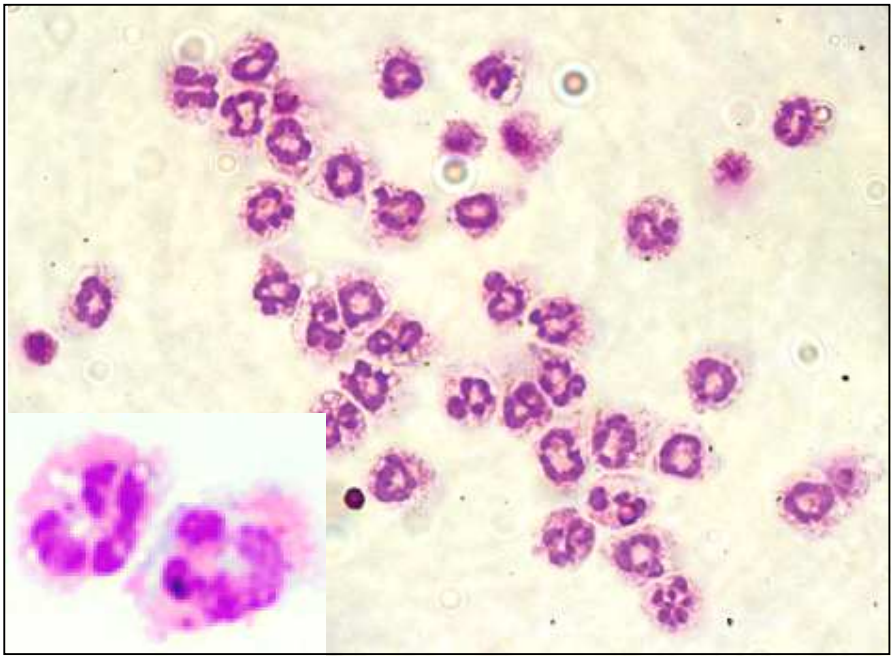


Fig. 4.13. The purity of FACS-sorted eosinophils was confirmed by the presence of eosin (pink cytoplasm) as shown in this microphotography. WT bone marrow cells were cultured for 7 days with IL-5 (10ng/ml) followed by FACS-sorting for Siglec-F^+ cells. Original magnification $\times$20; inset $\times$100).

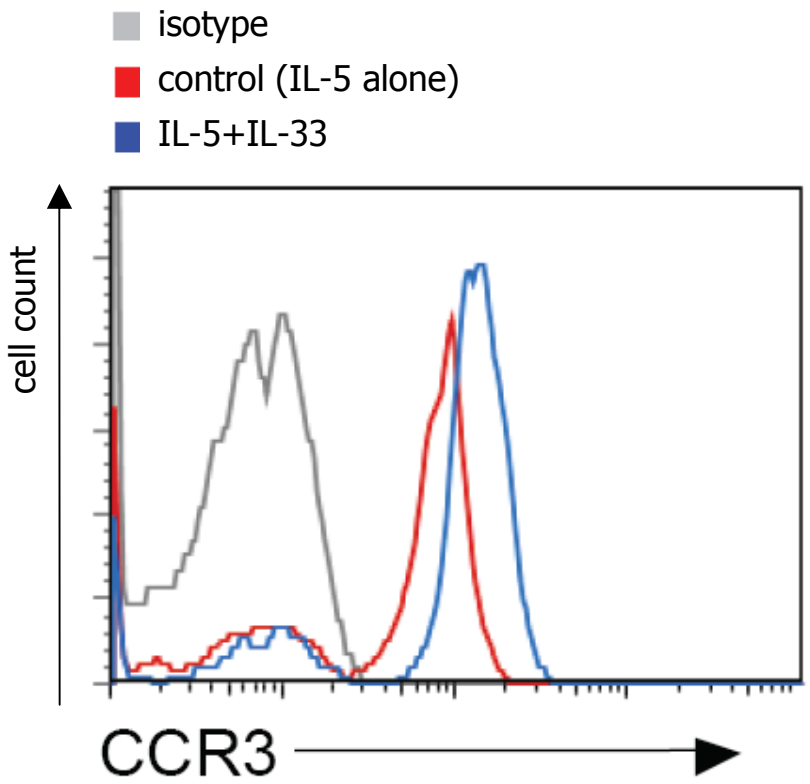


Fig. 4.14. IL-33 increases eosinophil CCR3 expression (i). FACS-sorted Siglec-F^+ eosinophils from IL-5-driven (10ng/ml) WT bone marrow cultures (7 days with IL-5) were incubated with IL-5 (2ng/ml) with or without IL-33 (10ng/ml) for 24h, followed by FACS analysis for CCR3 expression. Expression of CCR3 by FACS is shown. Data are representative of three independent experiments. Control = IL-5 alone (2ng/ml, red line); IL-33 variant = IL-5+IL-33 (2 and 10ng/ml respectively, blue line); Isotype = grey line.

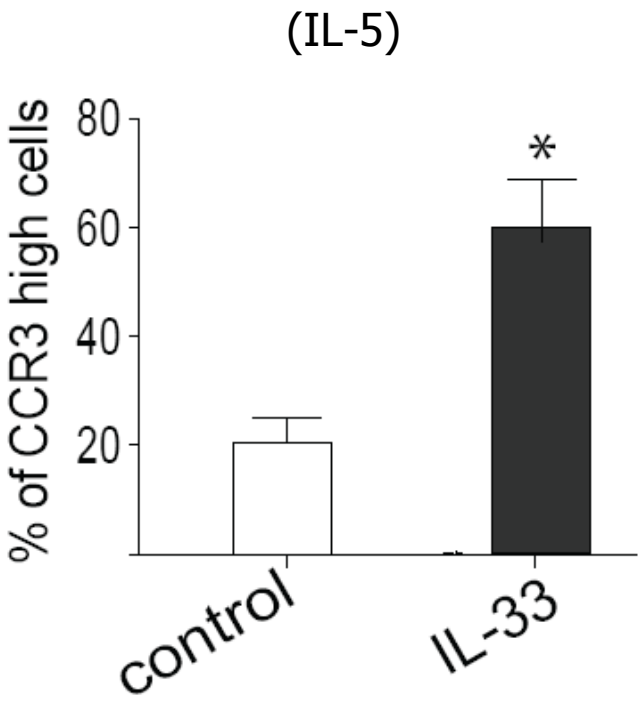


Fig. 4.15. IL-33 increases eosinophil CCR3 expression (ii). FACS-sorted Siglec-F^+ eosinophils from IL-5-driven (10ng/ml) BALB/c bone marrow cultures (7 days with IL-5) were incubated with IL-5 (2ng/ml) with or without IL-33 (10ng/ml) for 24h, followed by FACS analysis of CCR3 expression. Data are means $\pm$ SEM ($n = 3$),*$p < 0.05$, IL-5 versus IL-5 + IL-33 (white and black, respectively).

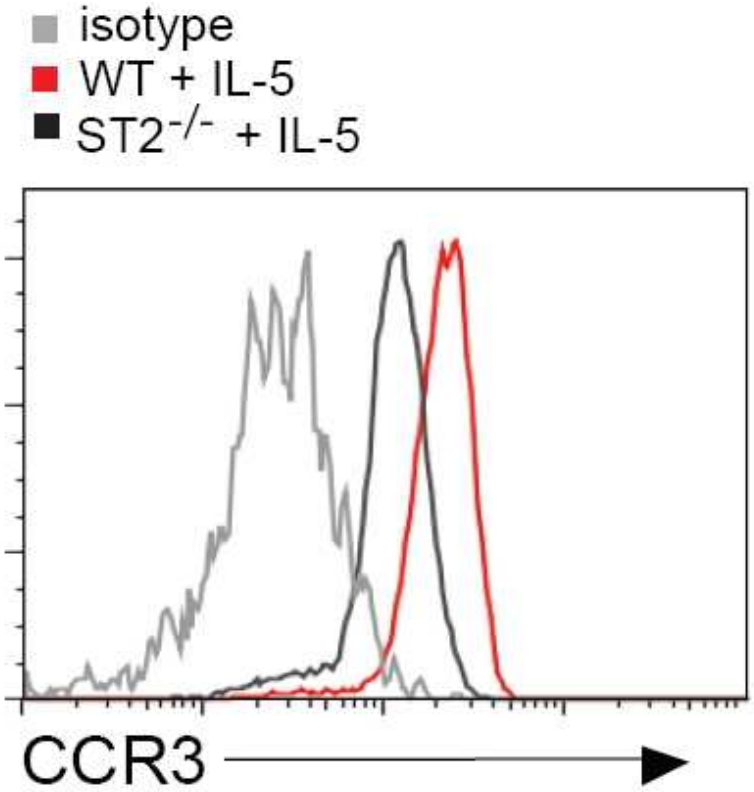


Fig. 4.16. IL-33 increases eosinophil CCR3 expression (iii). FACS-sorted Siglec-F^{+} eosinophils from IL-5-driven (10ng/ml) BALB/c and $ST2^{-/-}$ bone marrow cultures were FACS stained and checked for CCR3 expression. Data are representative of three independent experiments.

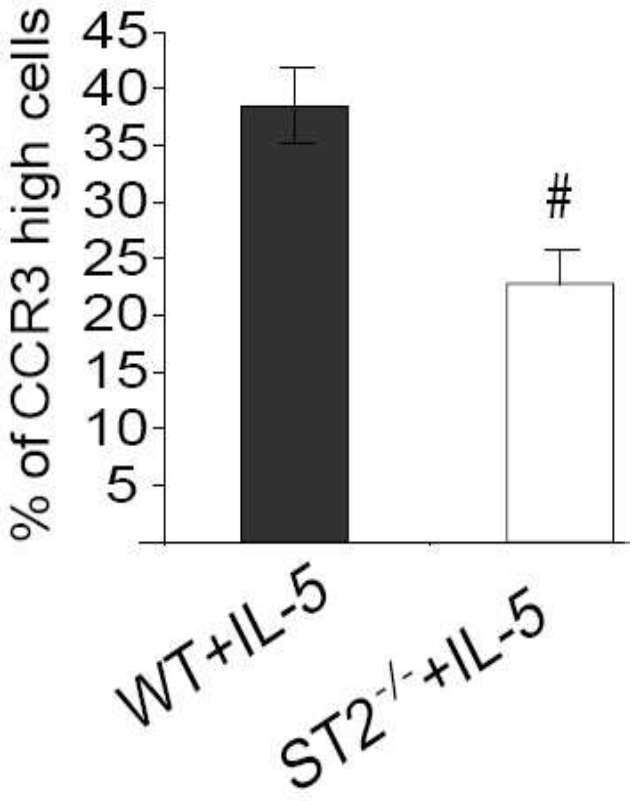


Fig. 4.17. $ST2^{-/-}$ eosinophils expressed reduced levels of CCR3. FACS-sorted Siglec-F^{+} eosinophils from IL-5-driven (10ng/ml) WT and $ST2^{-/-}$ bone marrow cultures were checked for CCR3 expression. The percentage of $CCR3^{high}$ cells is shown. Data are means $\pm$ SEM ($n = 3$), $^{\#}p < 0.05$, WT versus $ST2^{-/-}$ of three independent experiments.

Chapter 5:

The role of IL-33 in modulation of effector functions of eosinophil

5.1. The regulation of ST2L expression on mature human and murine eosinophils

Next, I looked at the regulation of ST2 expression on mature human and murine eosinophils. Human peripheral blood (PB) eosinophils were isolated by negative selection (Miltenyi Biotec). The purity of eosinophils was evaluated by EPO and/or hematoxilin/eosin staining and showed ≥ 96% of cells positive for EPO and or eosin stained granules (**Figure 5.1.**). In contrast to PB neutrophils, eosinophil leukocytes easily expressed mRNA for ST2L and sST2 (**Figure 5.2.**). Subsequently, I evaluated surface expression of ST2L on human PB eosinophils isolated from 6 volunteers. PB eosinophils expressed ST2L protein only marginally (less than 2% of cells) in majority of donors (4 out of 6). However, in remaining 2 donors, between 2 and 15% eosinophils expressed ST2L (**Figure 5.3.**, **Figure 5.4.**). Not surprisingly, the expression of ST2L on human eosinophils could be potently increased by the ST2L ligand itself. A small increase was also observed in the medium alone, suggesting that ST2L expression may be regulated by certain component(s) of fetal calf serum (FCS). Interestingly, IL-5 markedly decreased the medium-induced ST2L expression but this effect could be still overcome by the stimulating action of IL-33 (**Figure 5.3.**, **Figure 5.4.**). Similarly, murine mature eosinophils differentiated from bone marrow precursors in the presence of IL-5 and sorted by FACS for $CCR3^{high}$ Siglec-F^{+} cells (> 98% pure, **Figure 5.5.**), did not express detectable amounts of ST2L (**Figure 5.6.**). To investigate whether cytokines that are abundantly released during type 2 immune response can affect ST2L expression on murine eosinophils, I used IL-5-driven mature eosinophils from WT and $ST2^{-/-}$ mice and incubated those cells in medium containing IL-5 (2ng/ml)

supplemented with IL-33 or GM-CSF or IL-4 (all 10ng/ml) or a combination of these cytokines for 48h. Expression of ST2L on eosinophils incubated in medium with IL-5 alone was either below the sensitivity of FACS (**Figure 5.6.**) or was very low (**Figure 4.8.**). However, IL-33 (**Figure 5.6.**) and GM-CSF (**Figure 5.7.)** but not IL-4 (**Figure 5.8.**) increased ST2L expression on WT eosinophils. An additive effect of GM-CSF and IL-33 on ST2L expression was also observed in WT (**Figure 5.7.**) but not $ST2^{-/-}$ mice (**Figure 5.9.**). Taken together, these results demonstrate that mature human and mouse resting eosinophils express low levels of ST2L which can be markedly increased by cytokines including IL-33 and GM-CSF.

5.2. BAL eosinophils express ST2L during OVA-induced airway inflammation

To determine whether eosinophils that accumulate in the lungs during the course of inflammation actually express IL-33R I evaluated ST2L expression on BAL eosinophils during the course of mouse OVA-induced airway inflammation (see Materials and Methods). BAL cells were isolated 48-120 h after the last antigen challenge and the expression of ST2L on $CCR3^{+}$ eosinophils was evaluated by FACS. Based on the previous data (26, 43) I expected eosinophils ($CCR3^{+}$cells) to start accumulating in BAL as early as 24 h with a maximum 120 h after the last challenge. BAL eosinophils expressed ST2L which was at its peak 48 h after the last challenge (80% of eosinophils) (**Figure 5.10.**) and gradually declined afterwards, down to nominal 2.5% on day 5. ST2L was not

detected in control eosinophils from $ST2^{-/-}$ mice. Thus, the kinetics of ST2L expression on eosinophils suggests that cytokines derived from OVA-activated T cells are likely responsible for the increase of ST2L expression on eosinophils that accumulated in the lungs.

Altogether, these data suggest that in the course of type 2 immune response eosinophils that are mobilized to the site of inflammation may acquire ST2L on the surface likely due to cytokines produced by antigen-specific Th2 cells.

5.3. The role of IL-33 in eosinophil activation

5.3.1. IL-33 induces production of IL-13, IL-6, TGF-1β and TARC

Since IL-33 is abundantly expressed in the lungs of asthma patients and mice with OVA-induced allergic inflammation (26-28, 43) and eosinophils at sites of inflammation express high levels of ST2L, (**Figure 5.10.**) I thus investigated the contribution of IL-33 to mature eosinophil function in more detail. Eosinophils are a source of pleiotropic mediators, including cytokines, chemokines and tissue damaging granule proteins (130). To investigate whether IL-33 can modulate the production of these mediators, FACS-sorted $CCR3^{+}Siglec\text{-}F^{+}$ WT and $ST2^{-/-}$ eosinophils were incubated in medium containing suboptimal amounts of GM-CSF (1ng/ml) in the presence or absence of IL-33 (10, 50ng/ml) over 48h. GM-CSF was used because of its ability to act as eosinophil survival factor; it can also upregulate the expression of ST2L.

Evaluation of cytokine contents in culture supernatants (ELISA and Luminex assay) revealed that IL-33 triggered the production of IL-13 (**Figure 5.11.**), IL-6 (**Figure 5.12.**) and strongly increased the production of TARC and TGF-β (**Figure 5.13**, **Figure 5.14.** respectively) from WT but not $ST2^{-/-}$ eosinophils in a dose-dependent manner. IL-33 had little to no effect on the production of eotaxin, eotaxin-2, IL-4, MCP-1, IP-10 and MIP-1α (data not shown). Other cytokines (IL-17, KC, MIG, FGF, IL-1α, IL-2, IL-5, IL-10, IL-12, IFN-γ, IL-1β, TNF-α, VEGF) remained below the detection level of Luminex assay used (data not shown). These data indicate that IL-33-activated eosinophils may be an important source of IL-13, TARC, IL-6 and TGF-β.

5.3.2. IL-33 exacerbates IgG-induced eosinophil degranulation

It is well documented that eosinophils are source of tissue damaging granule proteins (130, 138, 143, 180-181). In order to evaluate the contribution of IL-33 to the process of eosinophil degranulation, mature murine eosinophils were cultured in the presence of GM-CSF (1ng/ml) ± IL-33 (50ng/ml) as before. After 24 h, cells were washed with fresh RPMI medium and seeded into a plate coated with anti-CD32/16 (0.3 or 1μg/ml) or $mIgG_1$ (1μg/ml). After 90 minutes culture supernatants were collected and EPO assay performed. The amount of EPO in the degranulation assay was calculated as % of total EPO released from eosinophils that underwent 3 freeze-thaw cycles. In addition, the viability of cells was evaluated by cell counts using trypan blue. Both plate-bound anti-CD32/16- and IgG-triggered EPO release that was markedly increased in

the IL-33 pre-treated cells. However, trypan blue-staining showed that, in both instances, an increase in the EPO release from IL-33 pre-treated cells coincided with a higher cell death rate (**Figure 5.15.**, **Figure 5.16**). Thus, the engagement of FcγII receptor on mouse eosinophils *in vitro* can lead to an increase in eosinophil cell death. However, a close comparison of the proportion of the amount of EPO released with the proportion of viable cells that remained in IL-33 pre-treated cultures suggest that there is some contribution of the degranulation-derived EPO. Also, I performed similar experiments in human eosinophils purified from peripheral blood. Again, pre-treatment of cells with hIL-33 increased EPO release by human eosinophils in anti-CD32 Ab-induced culture compared to non-treated cells (**Figure 5.17.**). In contrast to the murine system, there was no difference between the two pre-treatment conditions in anti-CD32 Abs induced cell death. Thus it appears that in human eosinophils, the contribution of apoptosis-derived EPO release is modest compared to IL-33-potentiated degranulation.

These data suggest that IL-33 is, indeed able to significantly increase the IgG-triggered degranulation. In order to investigate the mechanism behind this process, I looked at the expression of FcγRs on human esoinophils. FACS analysis showed that the pre-treatment of human eosinophils with IL-33 increased the expression of FcγRII receptor (CD32) but not FcγRIII (CD16) (**Figure 5.18.**) likely rendering these cells more responsive to IgG binding and thus promoting degranulation.

5.3.3. IL-33-activated eosinophils polarize macrophages into AAM in an IL-13 dependent manner

In the course of IL-33-triggered airway inflammation as well as in OVA-induced asthma model alveolar and lung macrophages differentiate toward alternatively activated macrophages (M2, AAM) (26). This sub-population of macrophages is largely responsible for the production of chemokines and fibrotic factors that in turn perpetuate lung inflammation and fibrosis. Given the abundance of eosinophils in the lungs during asthma models I asked whether IL-33 stimulated eosinophils contribute to the activation of macrophages. To test this hypothesis I co-cultured $ST2^{-/-}$ BM derived macrophages with WT eosinophils *in vitro* in the presence or absence of IL-33 or IL-5. Eosinophils alone modestly elevated the percentage of MR^{+} macrophages which was markedly increased by the presence of IL-33 but not IL-5 (**Figure 5.19**, **Figure 5.20.**). Furthermore, because IL-13 is a well-defined M2-differentiation factor (150) and was found to be produced by eosinophils upon IL-33 stimulation (**Figure 5.13.**), I therefore investigated whether IL-13 produced by IL-33-activated eosinophils may be responsible for the enhanced M2 (AAM) polarization. The presence of anti-IL-13 neutralizing antibodies partially but significantly reduced the number of MR^{+} macrophages polarized by IL-33-activated eosinophils (**Figure 5.21**. and **Figure 5.22.**; assessed by ANOVA followed by Tukey's test), indicating that IL-33- but not IL-5-activated eosinophils polarized macrophage to the pro-inflammatory AAM phenotype, at least in part, via IL-13.

5.3.4. IL-33 induces expression of activation marker CD69 on human peripheral blood eosinophils

Also, I have preliminarily looked at the role of IL-33 in human PB eosinophils activation. I decided to evaluate the expression of CD69 as it was reported in the literature to be an early activation marker of human eosinophils (182). Its expression is elevated in response to several cytokines including IL-13 (183), GM-CSF, IL-3, IL-5 *in vitro* and *in vivo* in BAL fluid of asthma patients (184). PB eosinophils were stimulated with hIL-5 (10ng/ml) or hIL-33 (10 and 50ng/ml) for 48h. Eosinophils cultured in medium alone expressed low levels of CD69 on the surface. IL-5 strongly increased the expression of CD69. Similarly, IL-33 significantly increased the expression of CD69, primarily on ST2L positive eosinophils. These data therefore indicate that indeed IL-33 affects the activation of human eosinophils (**Figure 5.23.**, **Figure 5.24**).

Summary of Chapter 5

Together, this part of my study demonstrates that IL-33 enhances multiple features of eosinophil functions including cytokine/chemokine production, interaction with other cell types as well as degranulation and therefore an IL-33-rich environment in asthma and allergy may contribute to the pathogenic activities of eosinophils.

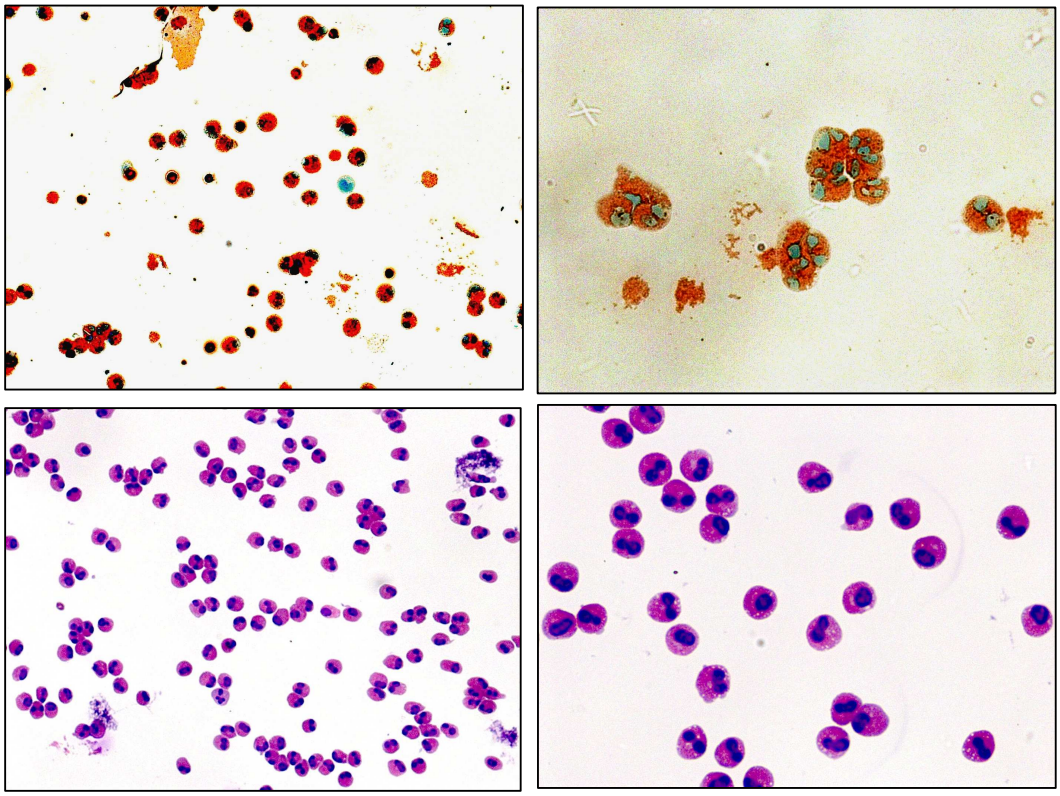


Fig. 5.1. The evaluation of the purity of human eosinophils isolated from peripheral blood. Specific staining for eosinophil peroxidase (EPO) is shown in the upper panel; eosinophil EPO-containing granules stain in red-gold. Bottom panel shows a classical differential eosin/hematoxylin staining. Left panel, original magnification x 10; right panel, original magnification x20.

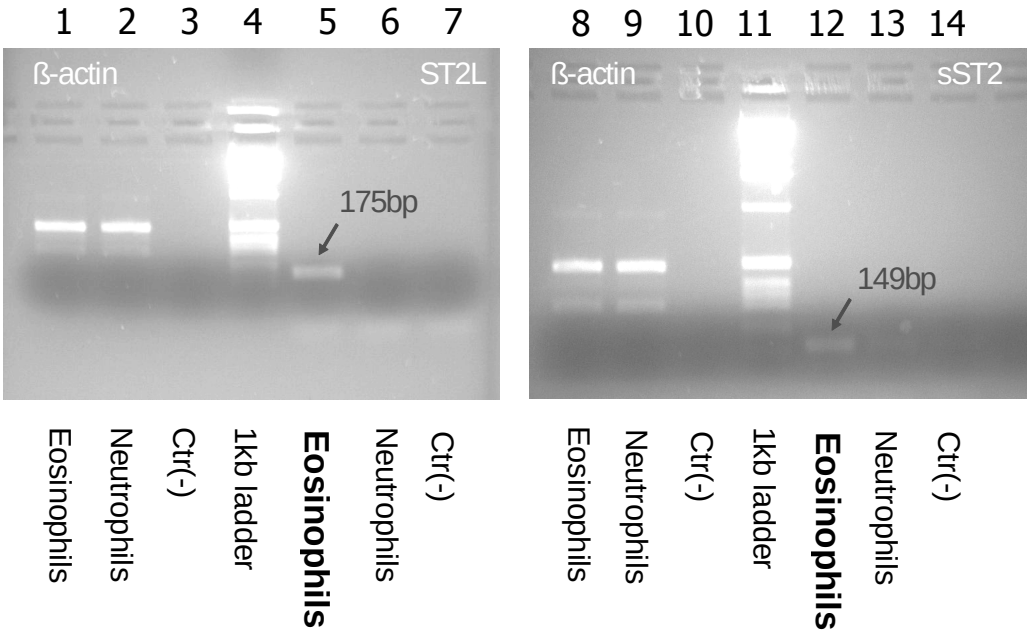


Fig. 5.2. The expression of ST2L and sST2 mRNA can be detected in freshly isolated eosinophils but not neutrophils. Specific band of 175 bp (line 5) represents PCR product of ST2L. Similarly, the band of 149 bp (line 12) represents PCR product of sST2. Lines 4, 11 contain DNA marker (1kb DNA ladder). Lines 1, 2, 8, 9 contain positive control (housekeeping gene, β-actin). Negative control (lines 3, 10 contain no template). The representative pictures of two independent experiments are shown. Primer pairs were kindly provided by Mark Moore.

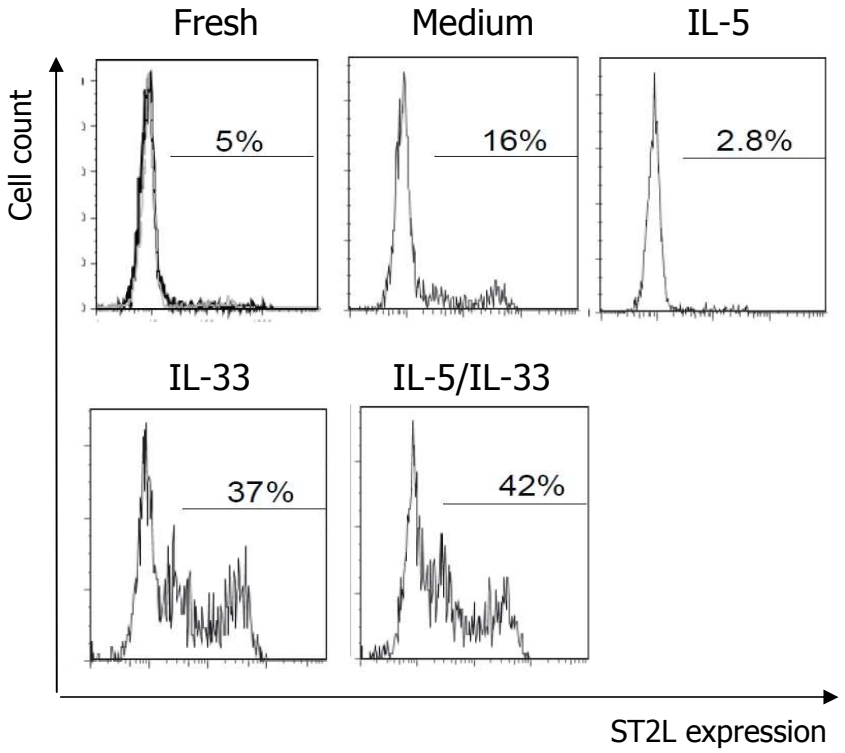


Fig. 5.3. The expression of ST2L by freshly isolated and cultured human eosinophils (representative data). Peripheral blood human eosinophils were acquired as described in Materials and Methods section and FACS analyzed *ex-vivo* or cultured in-vitro over 2 days prior the evaluation of ST2L expression. FACS staining for ST2L was done with FITC-conjugated monoclonal antibody (MD Bioscience). Dead cell were excluded by staining with ViaProbe. Medium alone mildly increased ST2L expression while IL-5 downregulated medium-induced ST2L. IL-33 was able to induce ST2L and to reverse the IL-5-mediated negative regulatory effect. Representative data of six independent experiments is shown.

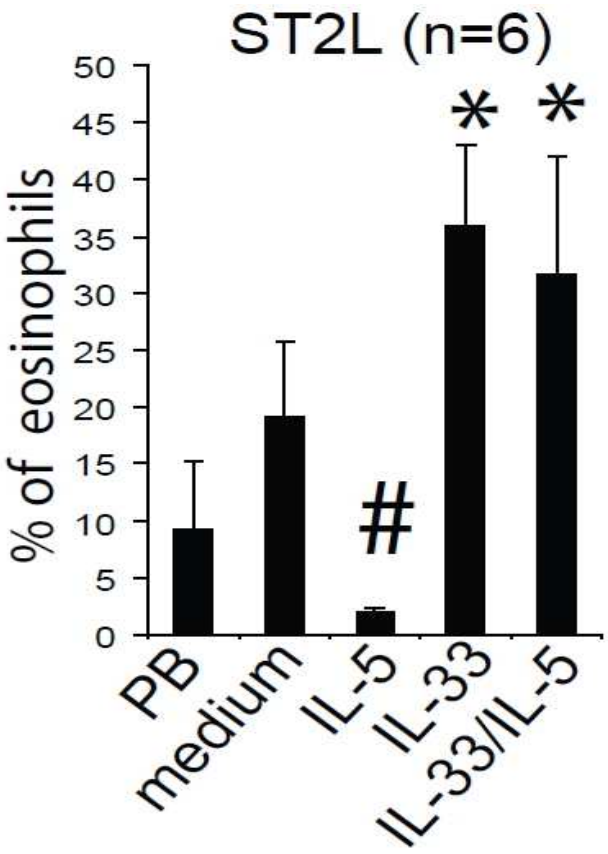


Fig. 5.4. The percentage of ST2L-expressing cells in fresh or cultured human eosinophils (quantitative data). Peripheral blood human eosinophils were acquired as described in Materials and Methods section and FACS analyzed or cultured in-vitro over 2 days prior ST2L expression evaluation. FACS staining for ST2L was done with FITC-conjugated monoclonal antibody (MD Bioscience). Data are means ± SEM (n = 6); #$p < 0.05$, IL-5 versus medium; *$p < 0.05$, medium versus IL-33 or IL-5 + IL-33

.

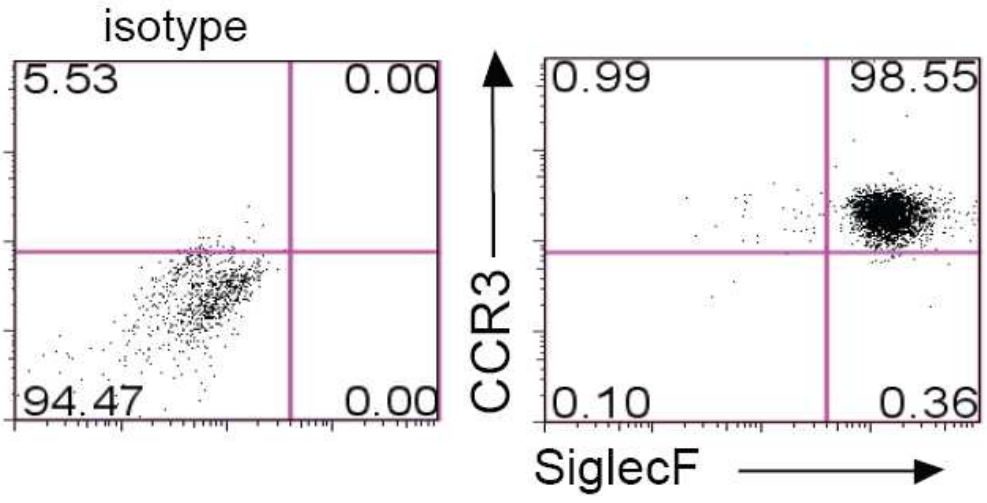


Fig. 5.5. The purity of murine bone marrow-derived eosinophils differentiated *in vitro*. After 48 hours of incubation in the presence of IL-5 bone marrow cells were sorted with FACS for $CCR3^{high}$Siglec-F^{+} cells (typically, the purity was > 98%).

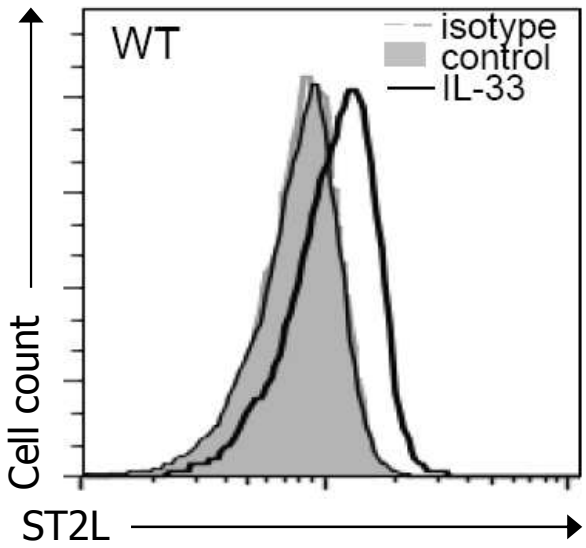


Fig. 5.6. IL-33 but not IL-5 induces ST2L expression on murine eosinophils. Mature murine FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) were cultured in medium with IL-5 (2ng/ml, control) or IL-5 (2ng/ml) + IL-33 (10ng/ml) over 2 days. Data are representative of two independent experiments. Both control medium and IL-33 variant also contain the same concentration of IL-5 (2ng/ml).

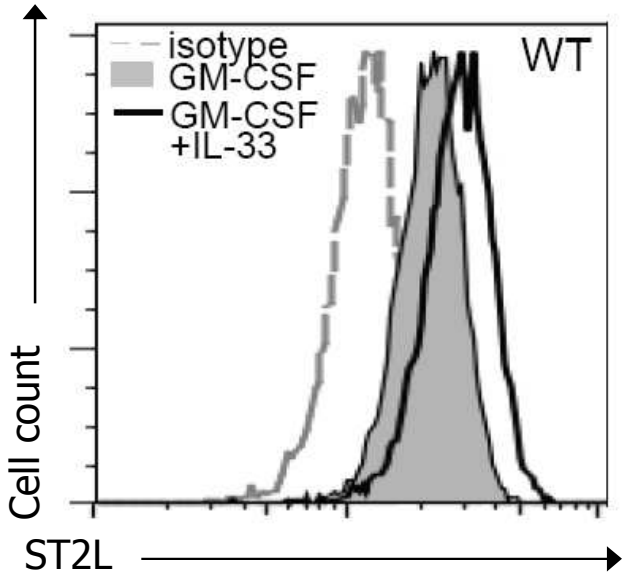


Fig. 5.7. IL-33 further increases the intensity of ST2L expression induced by GM-CSF. Mature murine FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) were cultured in medium with IL-5 (2ng/ml, control) or IL-5 + GM-CSF (10ng/ml) or IL-5 + GM-CSF (10ng/ml) + IL-33 (10ng/ml) over 2 days. Data are representative of two independent experiments.

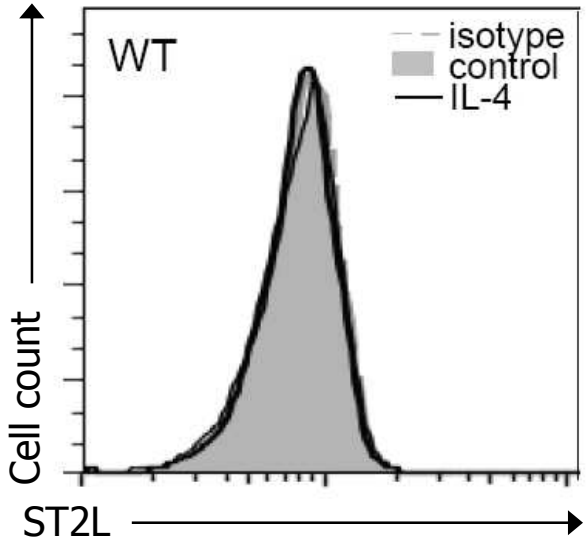


Fig. 5.8. IL-4 does not induce ST2L expression on murine eosinophils. Mature FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) were cultured in medium + IL-5 (2ng/ml, control) or IL-5 (2ng/ml) + IL-4 (10ng/ml) for 2 days. Data are representative of two independent experiments.

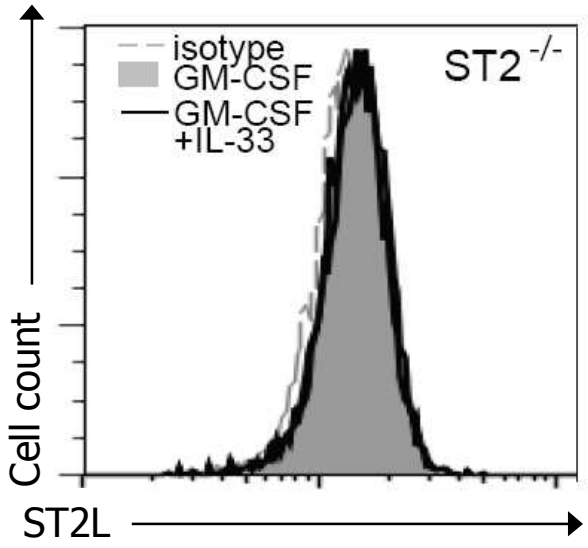


Fig. 5.9. ST2$^{-/-}$ eosinophils do not express ST2L. Mature murine FACS-sorted ST2$^{-/-}$ eosinophils (Siglec-F^{+}CCR3^{high}) were cultured in medium with IL-5 (2ng/ml, control) or IL-5 + GM-CSF (10ng/ml) or IL-5 + GM-CSF (10ng/ml) + IL-33 (10ng/ml) for 2 days. Data are representative of two independent experiments.

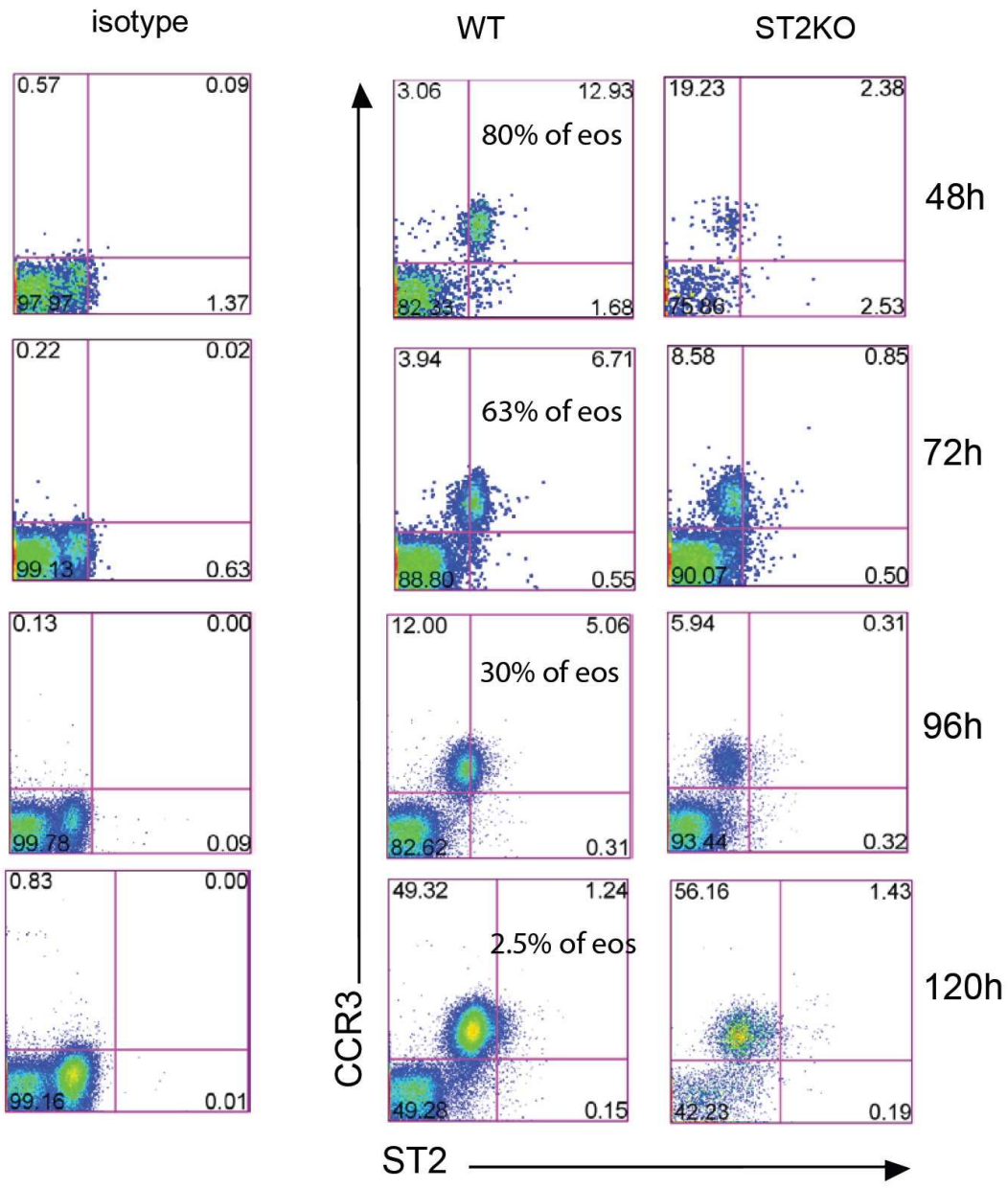


Fig. 5.10. Airway eosinophils express ST2L during OVA-induced airway inflammation model. WT and $ST2^{-/-}$ mice were sensitized with OVA + alum and subsequently challenged with OVA. BAL cells were collected 48, 72, 96 and 120h after the last OVA challenge and stained with anti F4/80 Ab, anti-CCR3 and anti-ST2L Abs. At 48h time point, up to 80% of BAL eosinophils ($F4/80^{-}CCR3^{+}$ cells) were $ST2L^{+}$. Pooled data from 10 mice at each time point shown.

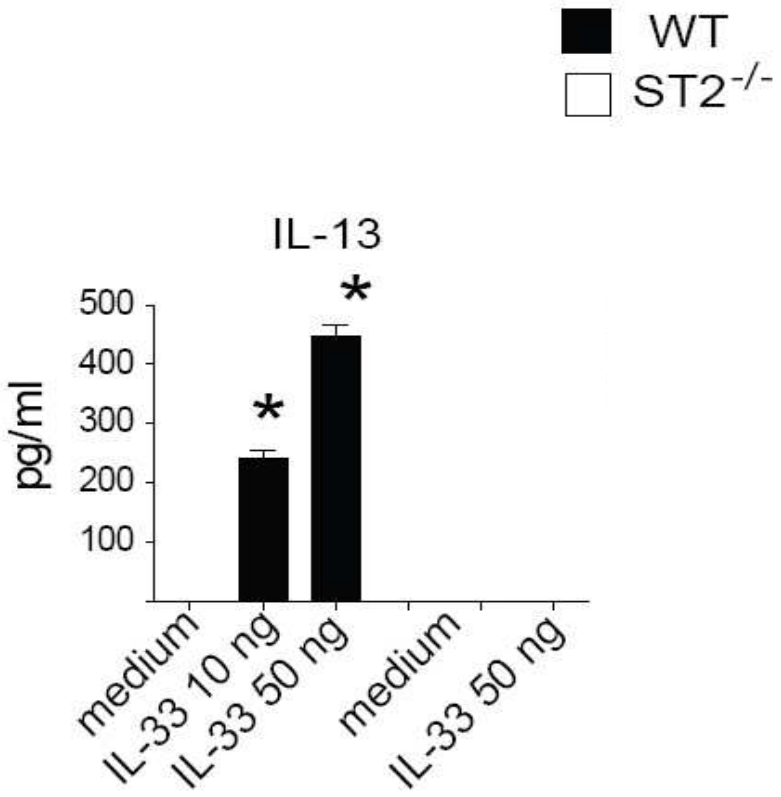


Fig. 5.11. IL-33 triggers IL-13 production by murine eosinophils *in vitro*. FACS-sorted WT and $ST2^{-/-}$ eosinophils (Siglec-$F^{+}CCR3^{high}$) were incubated for 48h in medium containing GM-CSF (1ng/ml, control medium) with or without IL-33. IL-13 concentrations in culture supernatants were analyzed by Luminex. Data are means ± SEM and are representative of three independent experiments. *$p < 0.05$, IL-33 versus control medium.

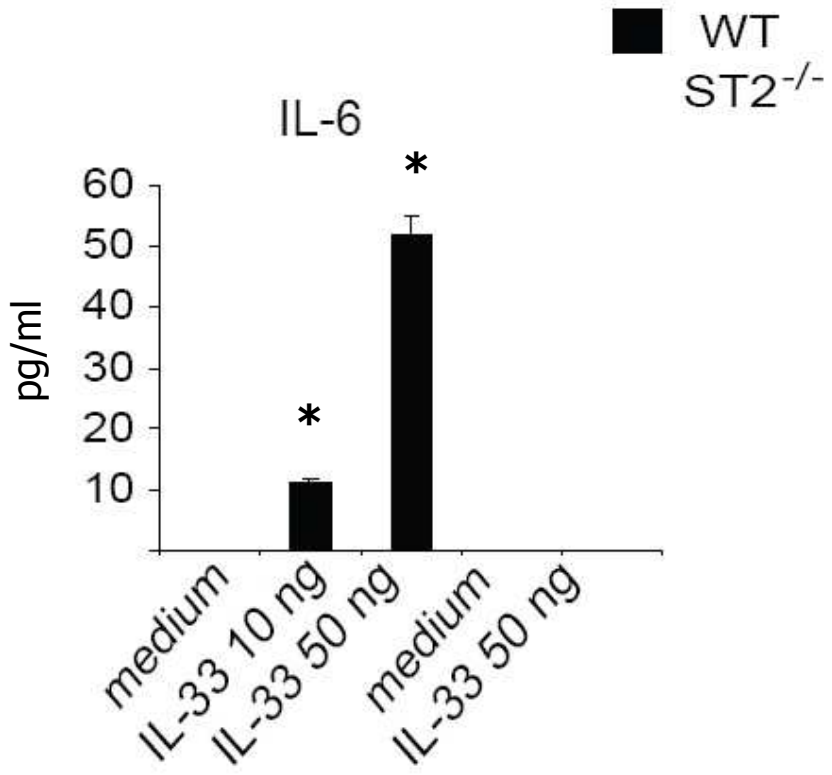


Fig. 5.12. IL-33 triggers IL-6 production by murine eosinophils *in vitro*. FACS-sorted WT and $ST2^{-/-}$ eosinophils (Siglec-$F^{+}CCR3^{high}$) were incubated for 48h in medium containing GM-CSF (1ng/ml, control medium) with or without IL-33. IL-6 concentrations in culture supernatants were analyzed by Luminex. Data are means ± SEM and are representative of three independent experiments. *$p < 0.05$, IL-33 versus control medium.

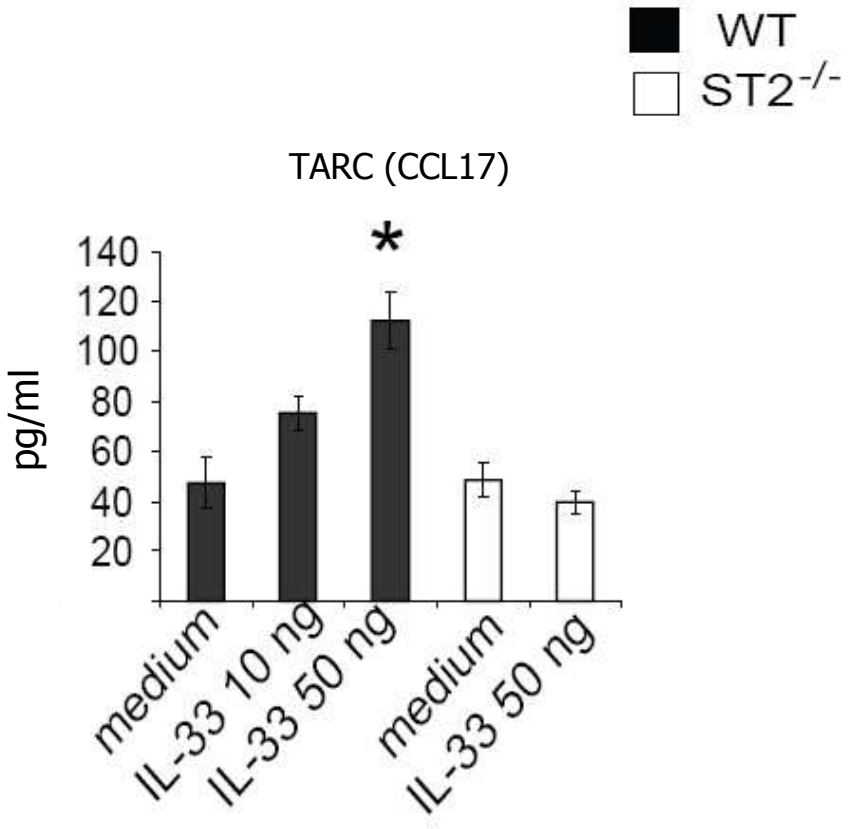


Fig. 5.13. IL-33 enhances TARC (CCL17) production by murine eosinophil *in vitro*. FACS-sorted WT and $ST2^{-/-}$ eosinophils (Siglec-$F^{+}CCR3^{high}$) were incubated for 48h in medium containing GM-CSF (1ng/ml, control medium) with or without IL-33. CCL17 (TARC) concentrations in culture supernatants were analyzed by ELISA. Data are means ± SEM and are representative of three independent experiments. *$p < 0.05$, IL-33 versus control medium.

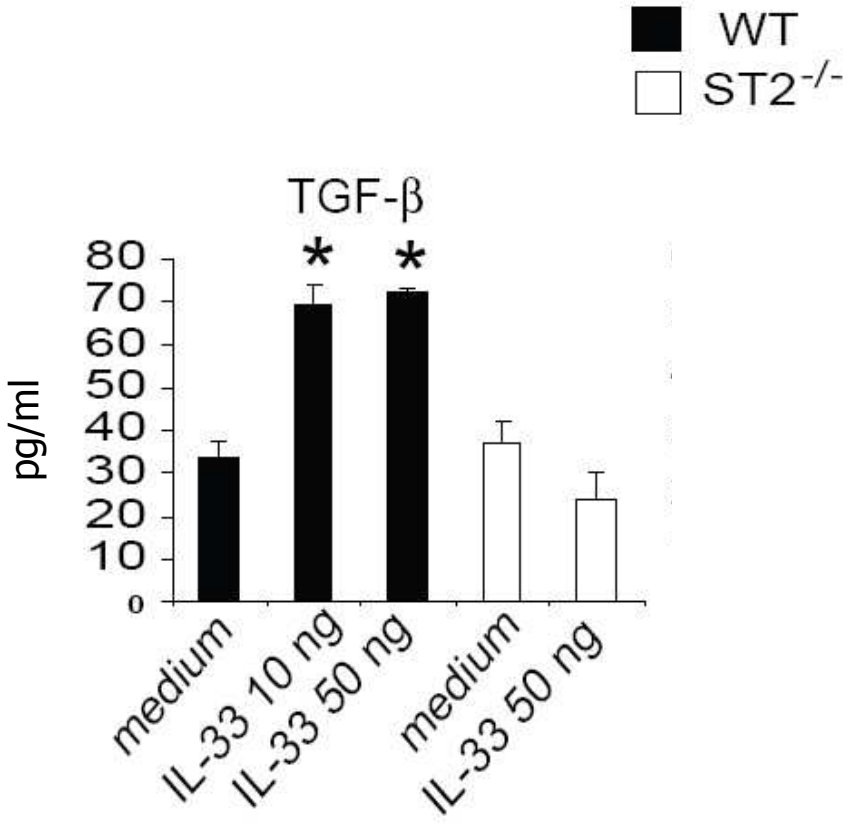


Fig. 5.14. IL-33 enhances TGF-β production by murine eosinophil *in vitro*. FACS-sorted WT or $ST2^{-/-}$ eosinophils (Siglec-$F^{+}CCR3^{high}$) were incubated for 48h in medium containing GM-CSF (1ng/ml, control medium) with or without IL-33. TGF-β concentrations in culture supernatants were analyzed by ELISA. Data are means ± SEM and are representative of three independent experiments. *$p < 0.05$, IL-33 versus control medium.

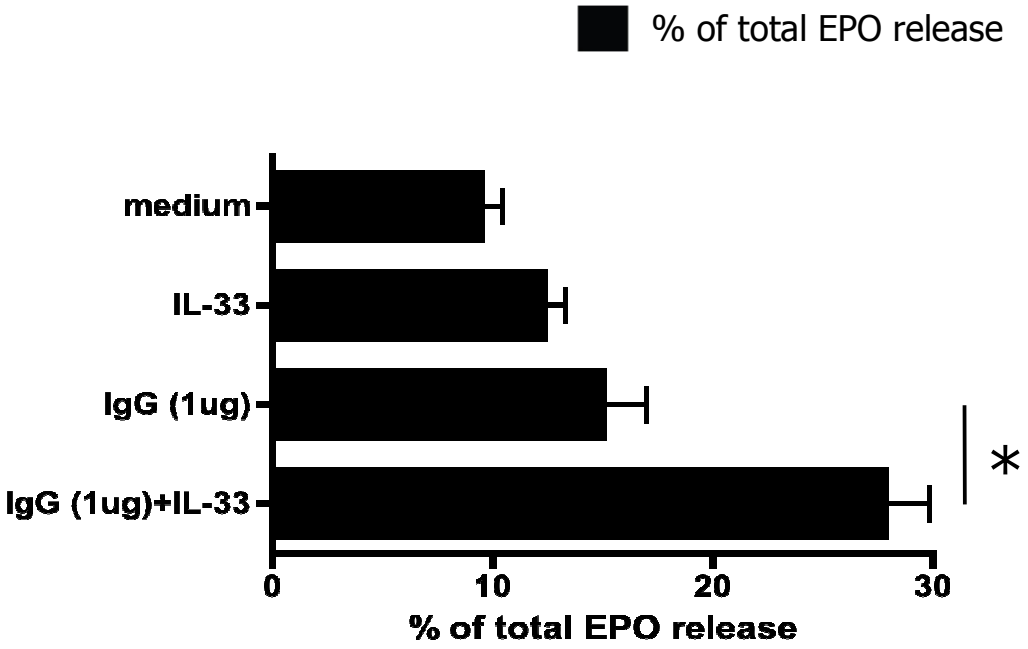


Fig. 5.15. IL-33 enhances IgG-induced degranulation of mouse eosinophils *in vitro*. FACS-sorted mature eosinophils (Siglec-$F^{+}CCR3^{high}$) were pre-incubated with GM-CSF or GM-CSF + IL-33 for 24h before washed with fresh RPMI medium and seeded into 96-well plates for degranulation assay. After 90 minutes EPO contents in supernatants was evaluated. Cell viability was assessed by counting in trypan blue. Total EPO release was calculated as the percentage of total EPO released from eosinophils that underwent 3 freeze-thaw cycles. The amount of EPO released from dead cells was subtracted from the values shown. Data are means (3 technical replicates) ± SEM and are representative of three independent experiments. *$p < 0.05$, IgG_1 (1μg/ml) + IL-33 (50ng/ml) versus IgG_1 alone (1μg/ml).

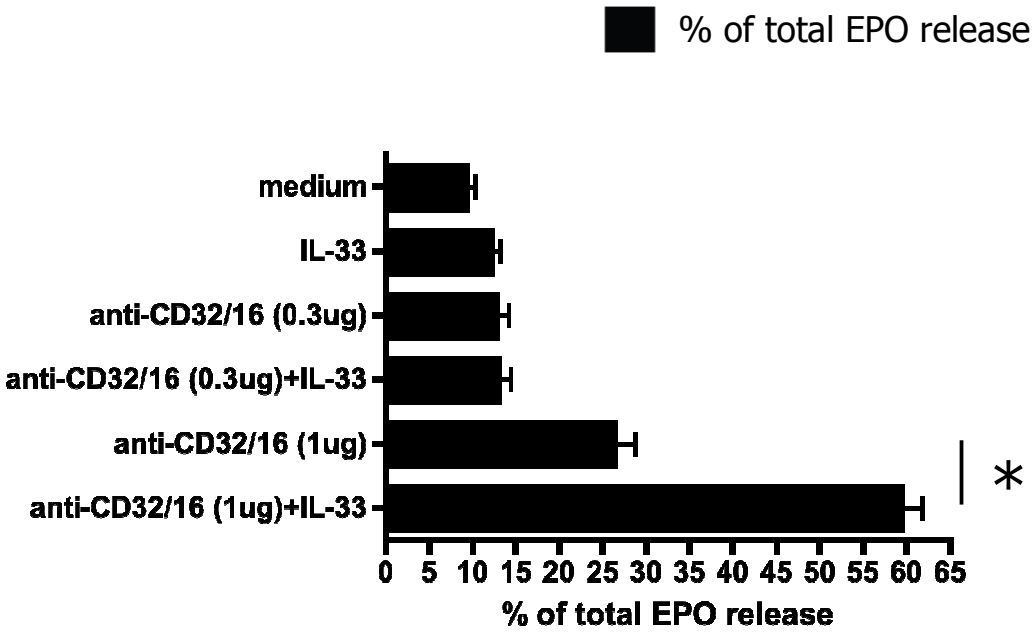


Fig. 5.16. IL-33 enhances αCD32/16-antibody-induced degranulation of mouse eosinophils *in vitro*. FACS-sorted mature eosinophils (Siglec-$F^{+}CCR3^{high}$) were pre-incubated with GM-CSF or GM-CSF + IL-33 for 24h before washed with fresh RPMI medium and seeded into 96-well plates for degranulation assay. After 90 minutes EPO contents in supernatants was evaluated. Cell viability was assessed by counting in trypan blue. Total EPO release was calculated as the percentage of total EPO released from eosinophils that underwent 3 freeze-thaw cycles. The amount of EPO released from dead cells was subtracted from the values shown. Data are means (3 technical replicates) ± SEM and are representative of three independent experiments. *$p < 0.05$; anti-CD32/16 antibody (1μg/ml) + IL-33 (50ng/ml) versus anti-CD32/16 antibody alone (1μg/ml).

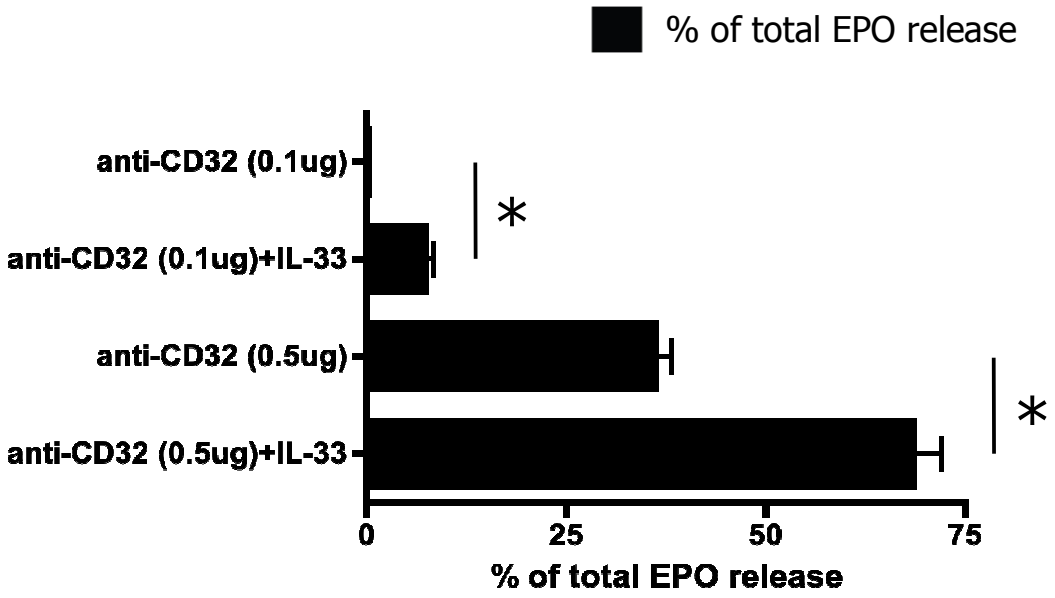


Fig. 5.17. IL-33 enhances αCD32-antibody-induced degranulation of human eosinophils *in vitro*. Mature eosinophils purified from peripheral blood were pre-incubated with GM-CSF or GM-CSF + IL-33 for 24h before washed with fresh RPMI medium and seeded into 96-well plates for degranulation assay. After 90 minutes EPO contents in supernatants was evaluated. Cell viability was assessed by counting in trypan blue. Total EPO release was calculated as the percentage of total EPO released from eosinophils that underwent 3 freeze-thaw cycles. The amount of EPO released from dead cells was subtracted from the values shown. Data are means ± SEM and are representative of three independent experiments. *$p < 0.05$; anti-CD32 antibody (0.5μg/ml) + IL-33 (50ng/ml) versus anti-CD32 antibody alone.

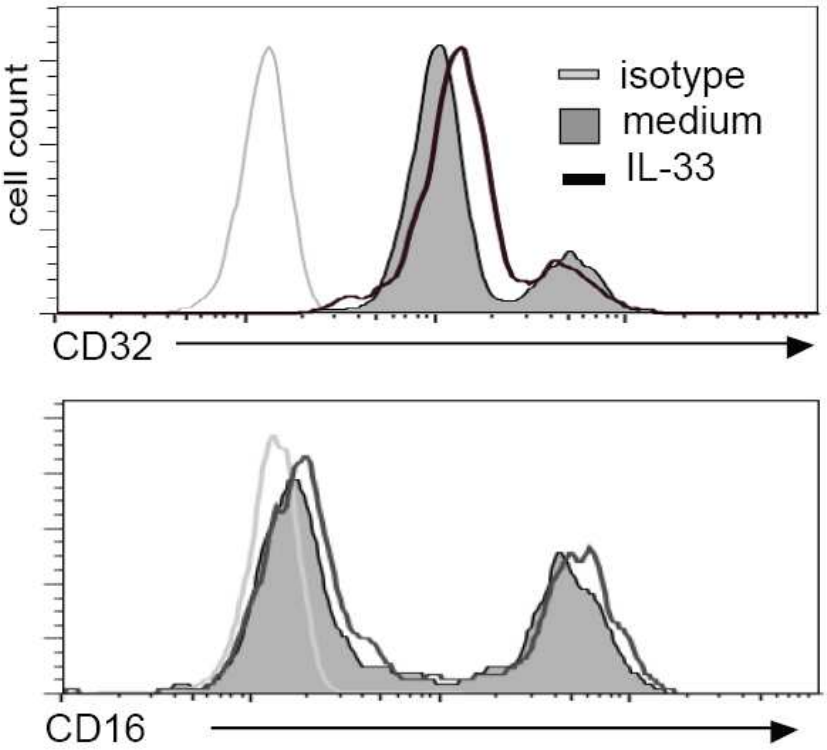


Fig. 5.18. IL-33 enchances CD32 (FcγII receptor) but not CD16 (FcγIIIR) expression by human eosinophils *in vitro*. Peripheral blood eosinophils were enriched according to MACS Miltenyi protocol, pre-incubated with GM-CSF (1ng/ml) + IL-33 (10ng/ml) or with GM-CSF (1ng/ml) alone (control medium) over 24 h and CD32 and CD16 expression was evaluated by FACS. Data are representative of three independent experiments.

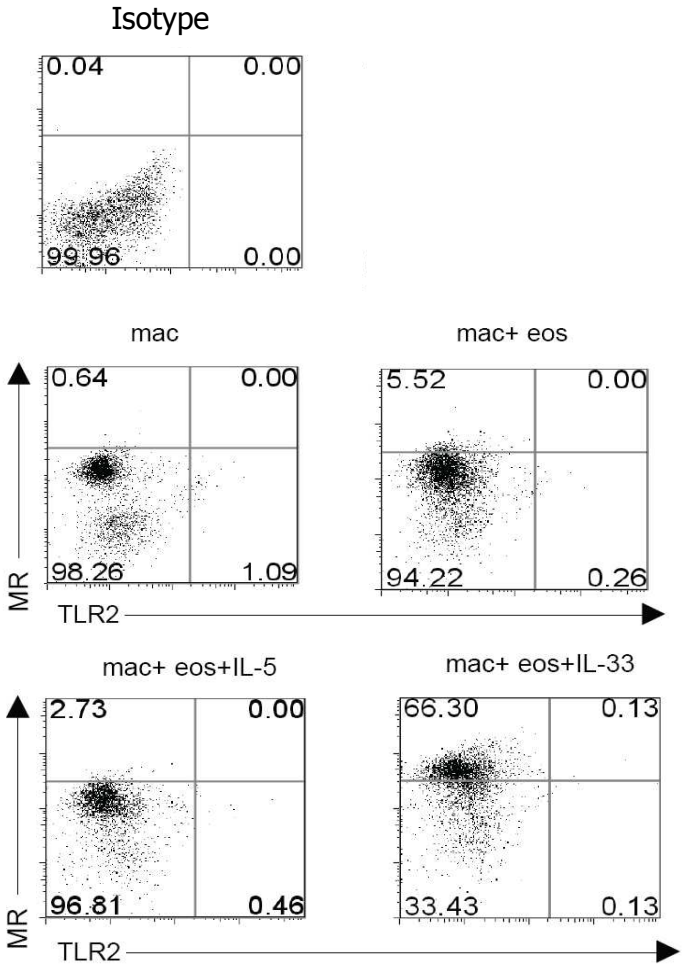


Fig. 5.19. IL-33-activated eosinophils increase the differentiation of alternatively activated MR^+ but not $TLR2^+$ macrophages *in vitro*. Murine macrophages from $ST2^{-/-}$ mice were cultured alone or co-cultured with FACS-sorted WT eosinophils (Siglec-F^+CCR3^{high}) in the presence of IL-5 (5ng/ml) or IL-33 (10ng/ml). After 48h macrophages were stained with anti-TLR2 and anti-mannose receptor (MR) antibodies. Data are representative of three independent experiments. eos = eosinophil; mac = macrophage.

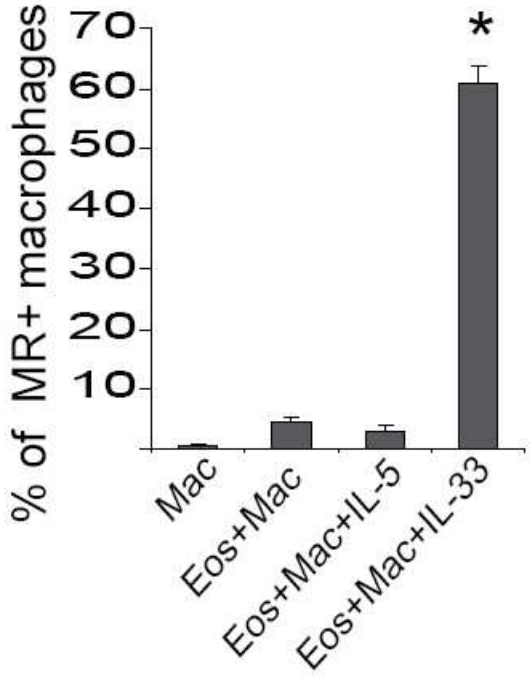


Fig. 5.20. IL-33-activated eosinophils increase the percentage of MR^+ alternatively activated macrophages *in vitro*. Murine macrophages from $ST2^{-/-}$ mice were cultured alone or co-cultured with FACS-sorted WT eosinophils (Siglec-F^+CCR3^{high}) in the presence of IL-5 (5ng/ml) or IL-33 (10ng/ml). After 48h macrophages were stained with anti-TLR2 and anti-mannose receptor (MR) antibodies. Data are means ± SEM of three independent experiments ($n = 3$), $*p < 0.05$, eosinophils + macrophages + IL-33 versus other cultures; eos = eosinophil; mac = macrophage.

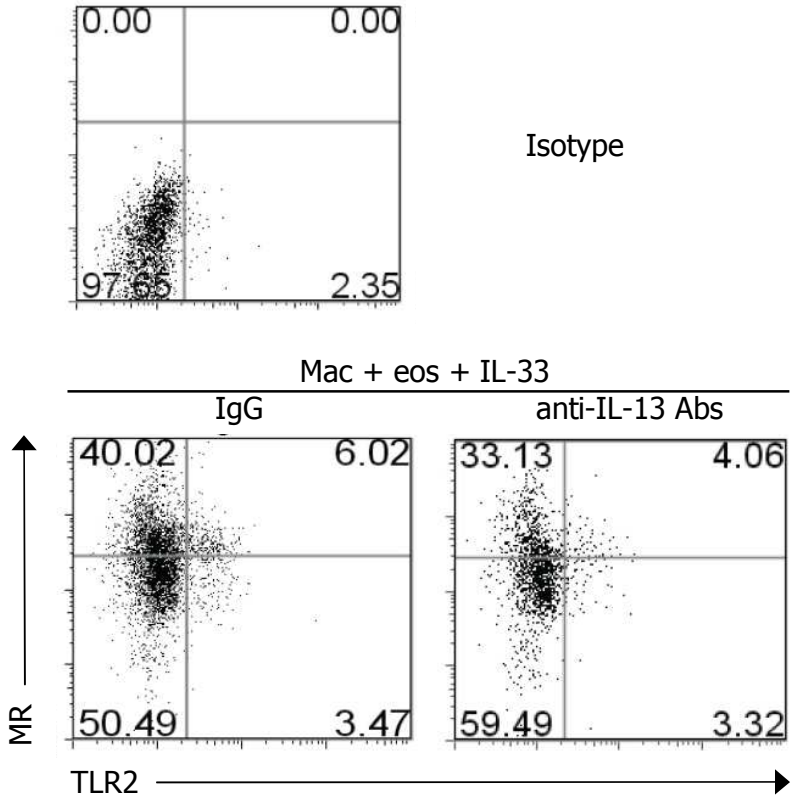


Fig. 5.21. The IL-33-triggered macrophage switching to AAM phenotype is dependent on IL-13 (representative FACS results). Murine macrophages from $ST2^{-/-}$ mice were co-cultured with FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) in the presence of IL-33 (10ng/ml) or IL-33 (10ng/ml) + anti-IL-13 Ab or isotype control (10μg/ml). After 48h macrophages were stained with anti-TLR2 and anti-mannose receptor (MR) antibodies. The IL-33-induced increase in MR^{+} macrophages markedly blocked by anti-IL-13 Ab. Data are representative of three independent experiments. Eos = eosinophil; Mac = macrophage.

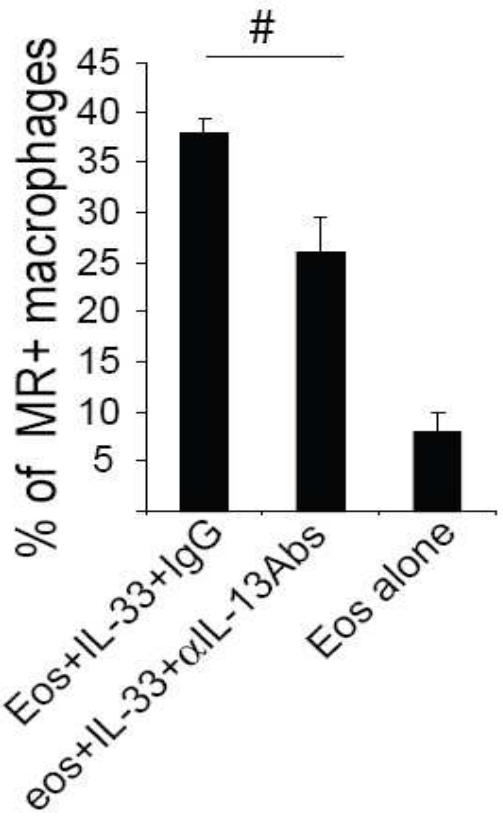


Fig. 5.22. The IL-33-triggered macrophage switching to AAM phenotype is dependent on IL-13 (quantitative data). Murine macrophages from $ST2^{-/-}$ mice were co-cultured with FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) in the presence of IL-33 (10ng/ml) or IL-33 (10ng/ml) + anti-IL-13 Ab or isotype control (10μg/ml). After 48h macrophages were stained with anti-TLR2 and anti-mannose receptor (MR) antibodies. Data are means ± SEM of three independent experiments ($n = 3$), $^{\#}p < 0.05$, eosinophils + anti-IL-13 versus eosinophils + IgG. Eos = eosinophil.

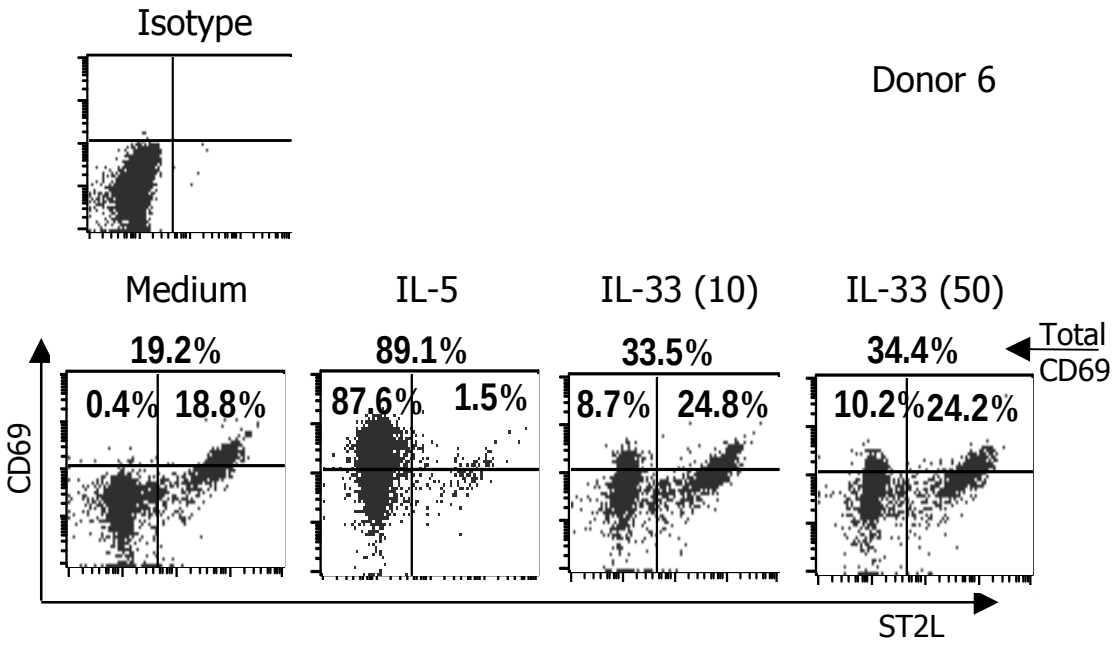


Fig. 5.23. IL-33 increases CD69 expression on human eosinophils (representative FACS results). PB eosinophils were cultured in complete medium or in medium complemented with IL-5 (10ng/ml) or IL-33 (10 or 50ng/ml) for 48hours, followed by surface FACS staining for CD69 and ST2L. Results representative of 6 donors of 3 independent experiments are shown.

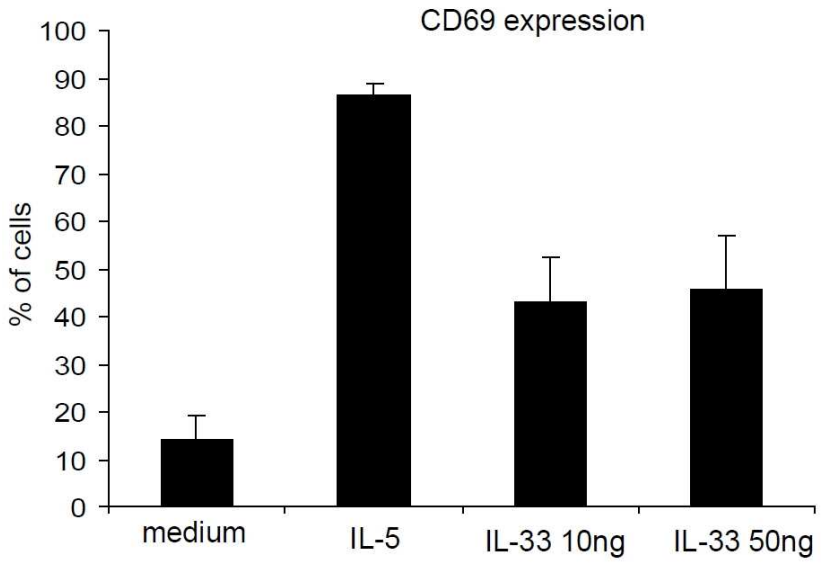


Fig. 5.24. IL-33 increases CD69 expression on human eosinophils (quantitative data). Human eosinophils were cultured in complete medium or in medium complemented with IL-5 (10ng/ml) or IL-33 (10 or 50ng/ml) for 48h, followed by surface FACS staining for CD69. Data are means ± SEM of 6 donors.

Chapter 6:

The role of IL-33-activated eosinophils in airway inflammation

6.1. The contribution of IL-33-activated eosinophils to airway inflammation

Given the role of the IL-33/ST2L system in eosinophil activation *in vitro*, I next investigated the contribution of IL-33-activated eosinophils to airway inflammation. FACS-sorted WT eosinophils ($CCR3^{high}$ Siglec-F^{+}) were stained with CFSE and adoptively transferred i.n. into $ST2^{-/-}$ mice. Subsequently, recipients received IL-33 or PBS i.n. for 3 consecutive days. Mice were culled 24h after the last IL-33 challenge and BAL fluid and cells were analyzed. Differential cell counts confirmed the presence of eosinophils in mice that received cells and a complete lack of eosinophils in the control groups given IL-33 or PBS only (**Fig. 6.1.**). Mice that received eosinophils plus IL-33 exhibited markedly higher total cell, eosinophil, macrophage and lymphocyte counts than those that received eosinophils plus PBS. BAL from the recipients of eosinophils plus IL-33 also contained significantly higher numbers of host eosinophils ($CFSE^{-}$) than the group given eosinophils plus PBS (**Figure 6.1.**). Moreover, IL-33-activated eosinophils increased CD4 T cell infiltration of the recipients' lungs as measured by FACS (**Figure 6.2.**). These data indicate that adoptively transferred eosinophils stimulated with IL-33 have a potential to exacerbate airway inflammation.

6.2. The contribution of IL-33-activated eosinophils to airway cytokine expression levels

Data presented in the section above suggest that IL-33, given to IL-33-unresponsive ($ST2^{-/-}$) recipients, that had received WT eosinophils, induced the production of mediators that were likely responsible for attracting recipients' inflammatory cells into the lungs. To further test this hypothesis I checked BAL fluids for the presence of cytokines and chemokines. BAL fluid analysis showed that, in contrast to animals that received PBS and IL-33 alone, both recipient groups that received eosinophils showed the presence of TARC (CCL17), MIP-1α (CCL3), eotaxin (CCL11), eotaxin-2 (CCL24), IL-13 and TGF-β (**Figure 6.3.**). However, mice that received eosinophils plus IL-33 produced significantly more CCL17, IL-13, TGF-β, CCL3 and CCL24 than those given eosinophils with PBS (**Figure 6.3.**). These data suggest that IL-33-activated eosinophils can indeed be a potent source of inflammatory mediators in the airways.

6.3. The effect of IL-33-activated eosinophils on the phenotype of alveolar macrophages

In section **5.3.3.** I showed that IL-33-activated eosinophils were able to induce differentiation of BM derived macrophages into M2, AAM in co-cultures *in vitro*. Having found an increased number of macrophages in BAL from mice that received eosinophils plus IL-33 I was interested in the actual phenotype of

these alveolar macrophages. To investigate this I stained BAL cells for F4/80, CCR3, MR, and TLR2. Alveolar macrophages from control mice had low expression of MR (a marker of M2 macrophage) that was consistent with their quiescent phenotype (26). The presence of adoptively transferred eosinophils in the airways increased the percentage of $F4/80^{+}CCR3^{-}$ MR^{+} macrophages. However, mice that received eosinophils + IL-33 had a significantly higher proportion of MR^{+} macrophages than mice that received eosinophils + PBS (**Figure 6.4.**). The percent of $TLR2^{+}$ (a marker of M1) macrophages was low and there was no difference between the groups (**Figure 6.5.**). These data therefore indicate that IL-33-activated eosinophils can increase the differentiation of alveolar macrophages toward the M2 phenotype *in vivo*.

Summary of Chapter 6

In summary, data presented in this chapter demonstrate that IL-33-activated eosinophils can significantly contribute to airway inflammation by releasing cytokines/chemokines, attracting other inflammatory cells and supporting the differentiation of alveolar macrophages towards M2, AAM.

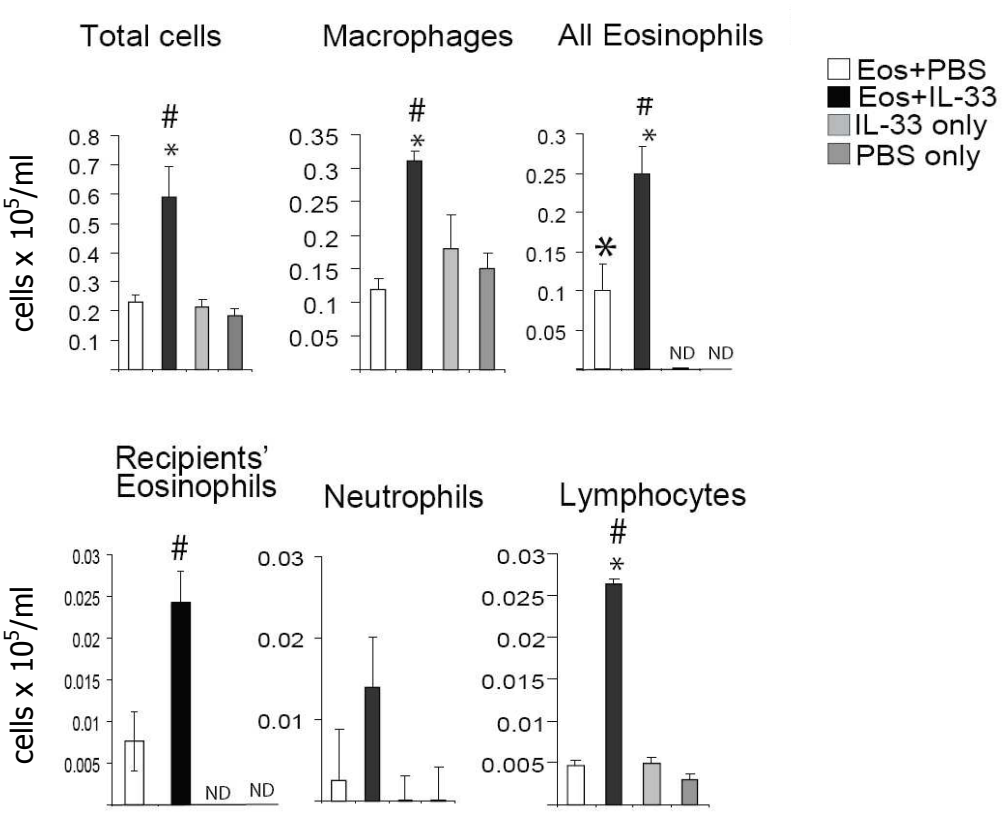


Fig. 6.1. Adoptive transfer of IL-33-activated eosinophils results in the infiltration of the airways by $ST2^{-/-}$ recipients' inflammatory cells. FACS-sorted WT eosinophils (Siglec-F^{+} $CCR3^{high}$, $10x10^{6}$/mouse) were stained with CFSE and adoptively transferred i.n. into $ST2^{-/-}$ recipients that were subsequently administered IL-33 for 3 days i.n. Control groups received eosinophils +PBS, PBS alone or IL-33 alone. Mice were culled 24 h after the last IL-33 administration. BAL fluid differential cell counts are shown. Data are means ± SEM ($n = 5$ mice/group) and are representative of two independent experiments; $*p < 0.05$, eosinophils versus PBS or IL-33 alone $^{\#}p < 0.05$, eosinophils + IL-33 versus eosinophils + PBS. Eos = eosinophils; Recipients' Eos = CFSE-negative eosinophils.

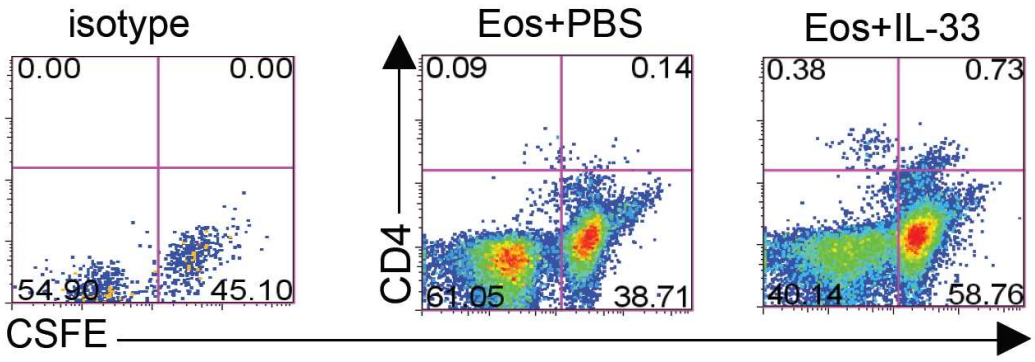


Fig. 6.2. IL-33 activated eosinophils attract CD4 T cells. FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) were stained with CFSE and adoptively transferred i.n. into $ST2^{-/-}$ recipients that were subsequently administered IL-33 or PBS daily for 3 days i.n. Mice were culled 24h following the last IL-33 aministration. FACS staining for CD4 is shown. Data are representative of 5 mice with similar results. Eos = eosinophil.

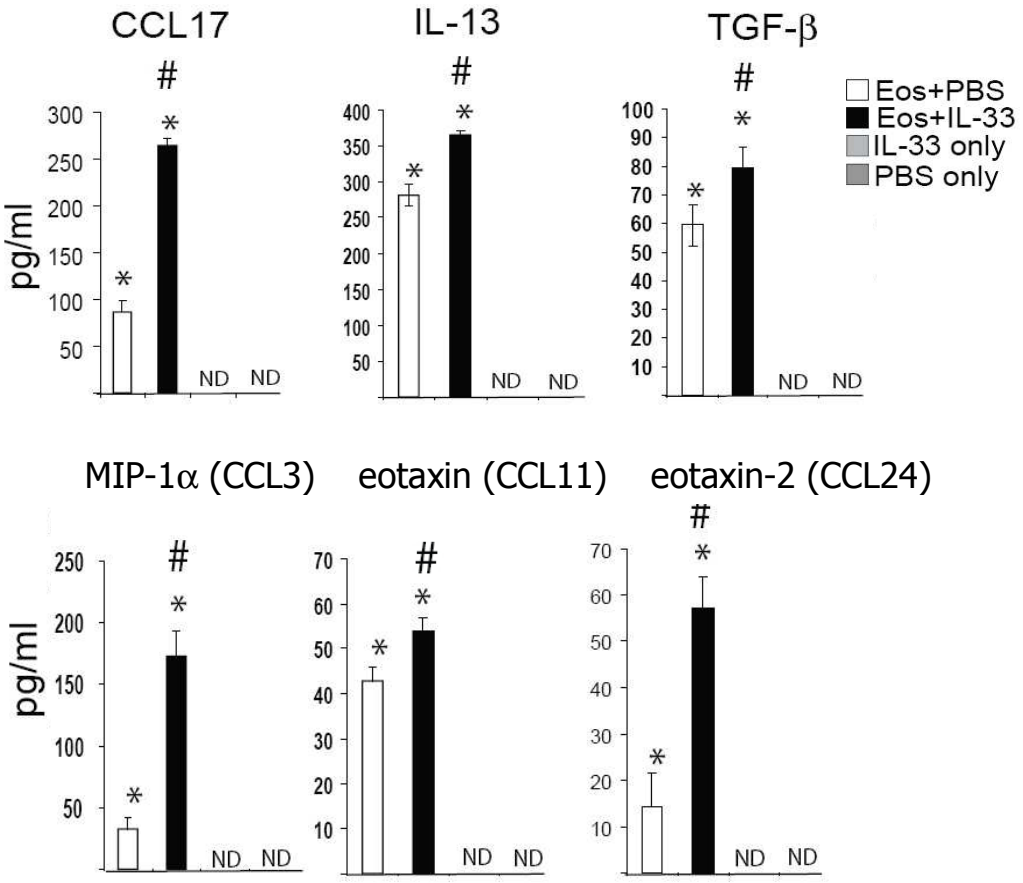


Fig. 6.3. Adoptively transferred IL-33-activated eosinophils induce inflammatory cytokine and chemokine production in the lungs of $ST2^{-/-}$ recipient mice. FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) were adoptively transferred i.n. into $ST2^{-/-}$ recipients that were subsequently administered IL-33 for 3 days i.n. Control groups received eosinophils +PBS, PBS alone or IL-33 alone. Mice were culled 24h after the last IL-33 administration. Cytokine and chemokine concentrations measured in BAL fluid by ELISA are shown. Data are means ± SEM (n = 5 mice/group) and are representative of two independent experiments; *$p < 0.05$, eosinophils versus PBS or IL-33 alone; #$p < 0.05$, eosinophils + IL-33 versus eosinophils + PBS; Eos = eosinophils

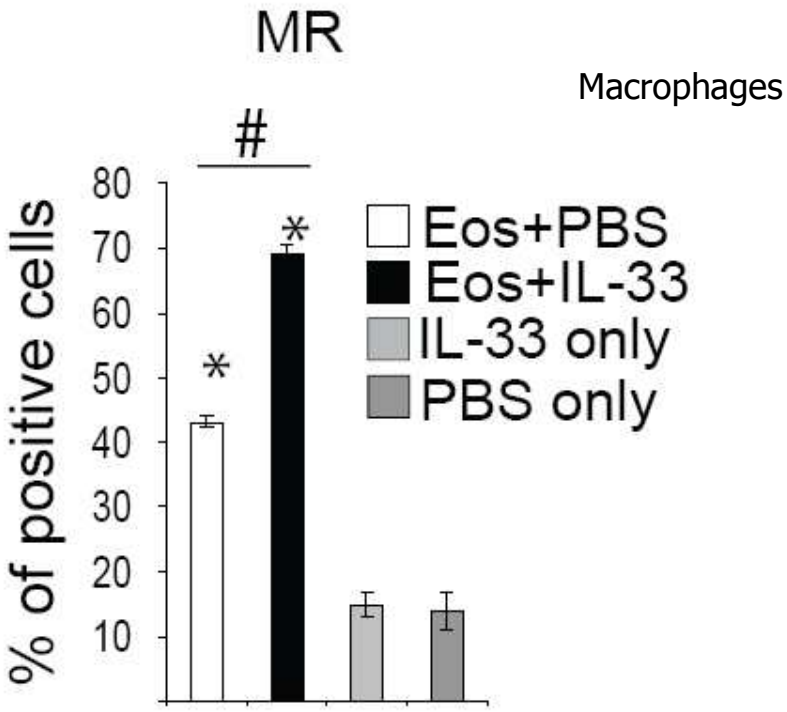


Fig. 6.4. Adoptively transferred IL-33-activated eosinophils markedly increase the proportion of alternatively activated macrophages (M2, AAM) in the lungs of $ST2^{-/-}$ recipient mice. FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) were stained with CFSE and adoptively transferred i.n. into $ST2^{-/-}$ recipients that were subsequently administered IL-33 for 3 days i.n. Control groups received eosinophils +PBS, PBS alone or IL-33 alone. Mice were culled 24h after the last IL-33 administration. MR expression on BAL fluid macrophages ($F4/80^{+}CCR3^{-}$) is shown. Data are means ± SEM (n = 5 mice/group) and are representative of two independent experiments; *$p < 0.05$, eosinophils versus PBS or IL-33 alone; #$p < 0.05$, eosinophils + IL-33 versus eosinophils + PBS; Eos = eosinophils.

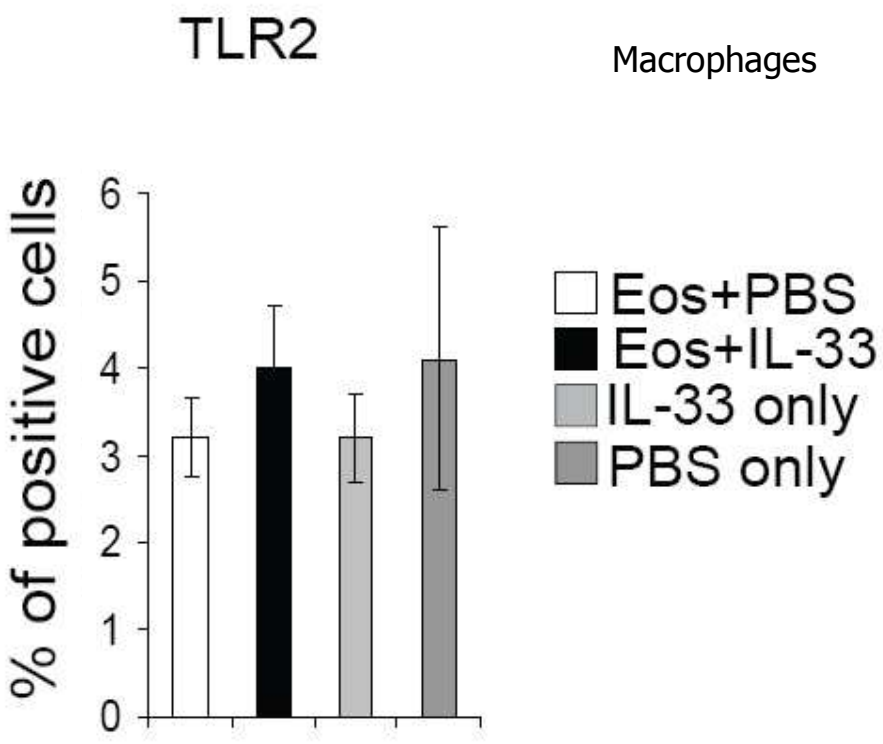


Fig. 6.5. IL-33-activated eosinophils do not alter the proportion of classically activated macrophages (M1) in the lungs of $ST2^{-/-}$ recipient mice. FACS-sorted WT eosinophils (Siglec-$F^{+}CCR3^{high}$) were adoptively transferred i.n. into $ST2^{-/-}$ recipients that were subsequently administered IL-33 for 3 days i.n. Control groups received eosinophils +PBS, PBS alone or IL-33 alone. Mice were culled 24h after the last IL-33 administration. TLR2 expression on BAL fluid macrophages ($F4/80^{+}CCR3^{-}$) is shown. Data are means ± SEM (n = 5 mice/group) and are representative of two independent experiments; Eos = eosinophils.

Chapter 7:

Discussion

Latterly, IL-33 has been implicated in type 2 lung inflammation mainly for its ability to affect T cell activation (80). However, the role of IL-33 in innate immune response in the lungs remains largely unknown. Studies *in vivo* showed that mast cells, basophils and NK cells are not essential for IL-33-induced airway inflammation ((185) and Pitman, N. et al., unpublished data).

It was recently demonstrated that IL-33 can be released from naïve lung explants upon stimulation with LPS and alum, likely via NLRP3-mediated inflammasome activation (15). A study from our laboratory reported that IL-33 protein is detected in the lungs of mice with OVA/alum-induced airway inflammation (43). Furthermore, immunohistochemistry studies carried out in our laboratory revealed that lung biopsies from asthma patients exhibit markedly elevated IL-33 expression in bronchial epithelial cells compared to healthy individuals, although a baseline IL-33 expression was observed in healthy controls as well. These data, in conjunction with recent reports suggesting that IL-33 can be rapidly released from epithelial cells in response to tissue injury or infection (6) seem to imply that IL-33 may play an important role in the pathogenesis of asthma, through the activation of lung resident cells that are readily expressing IL-33R, such as epithelial cells, fibroblasts and alveolar macrophages.

Indeed, the latest study into the role of IL-33 in airway inflammation carried out in our laboratory (26) confirmed early reports (4, 48) that IL-33 induces massive airway inflammation manifested by eosinophil and macrophage infiltration of the lung tissue and BAL fluid, as well as markedly elevated local

concentrations of IL-5 and IL-13 and induced goblet cell hyperplasia. Most intriguingly, our study revealed a significant alteration to the quiescent phenotype of alveolar macrophages from IL-33 treated mice which exhibited a profound increase in the expression of mannose receptor and IL-4Rα, as well as produced Eotaxin-2 and TARC which together constitute the characteristics of alternatively activated macrophages (M2, AAM). Moreover, we demonstrated previously that the differentiation of AAM was IL-33/ST2 dependent and also required IL-13/IL-4Rα but not IL-4 (26). AAM contributed to the IL-33-induced innate type 2 inflammation in the lungs by releasing chemokines, particularly Eotaxin-2 which neutralization reduced IL-33 triggered airway inflammation (26).

In order to confirm the role that alternatively activated alveolar macrophages play in IL-33-induced airway inflammation I employed a technique designed by Thepen et al. (157) which allows for the depletion of alveolar macrophages *in vivo* by means of intranasal administration of clodronate-containing liposomes that specifically target alveolar macrophages (**Figure 2.2.**). I was able to show that elimination of macrophages from the alveolar space leads to the amelioration of the IL-33-induced inflammation as reflected by decreased total BAL cell counts, as well as eosinophil counts in clodronate treated mice compared to PBS control (**Figure 3.2.**). Furthermore, I provided an insight into the mechanism by which IL-33 induces the differentiation of of alveolar macrophages toward AAM and thus inducing airway inflammation by demonstrating that IL-13 signaling pathway plays an essential role in this

process and its disruption significantly ameliorates IL-33-induced airway inflammation (**Figure 3.3.**).

By releasing chemokines, in particular eotaxin-2 (CCL24) (26), alveolar macrophages constitute a link between lung resident cells that act as the first line of response to IL-33 and several other cell types of the immune system, such as eosinophils, basophils mast cells and T cells, all of which can be recruited into the lungs and contribute to the IL-33-induced airway inflammation.

Given the prominent role of eosinophil leukocyte in asthma pathogenesis (186), in conjunction with more recent studies demonstrating that human eosinophils are able to express IL-33R (ST2L) *in vitro* (187), the contribution of IL-33/ST2 pathway to eosinophil differentiation and activation during the course of airway inflammation required further clarification.

Enhanced eosinophil differentiation is a hallmark feature of most clinical and experimental asthma phenotypes (143, 188) as well as IL-33-induced airway inflammation as demonstrated here and elsewhere (4, 48). Data obtained during this project indicate that IL-33 can act directly on bone marrow $CD117^{+}ST2L^{+}$ hematopoietic precursor cells and induce their differentiation toward eosinophils. This process depends on IL-5 and is IL-9 and IL-13 independent. Thus, IL-33-driven eosinophilia is likely a consequence of the cooperative autocrine action of IL-5 produced by $CD117^{+}$ hematopoietic precursors themselves that leads to their differentiation to eosinophils in bone

marrow, and the effect of IL-5 and/or chemokines produced by the cells on the periphery (4, 26, 29, 43) that mobilize mature eosinophils from the bone marrow compartment to the inflamed tissue. Further studies are needed to identify which progenitor cell population responds to IL-33 as both CMP and GMP express CD117 (90) (see **Figure 1.3.**).

Consistent with the above findings, recent study demonstrated that human hematopoietic $CD34^{+}$ cells from asthma patients express ST2L and respond to IL-33 by IL-5 production (189). This raises a possibility that IL-33 may drive eosinophil differentiation from hematopoietic precursors in clinical settings.

Furthermore, I show here that IL-33/ST2 system is essential for an optimal expression of CCR3 by differentiating eosinophils. It is well documented that CCR3 is of particular importance for eosinophil biology as it combines its action with IL-5 to facilitate mobilization of eosinophils from bone marrow to peripheral blood and it is ultimately responsible for eosinophil trafficking into the sites of inflammation (190). The lack of an optimal expression of CCR3 on $ST2^{-/-}$ eosinophils can likely be responsible for a decreased number of recruited eosinophils in BAL of $ST2^{-/-}$ compared to WT mice during OVA-induced airway inflammation model (**Figure 5.10.**), (26, 43). Of note, and in agreement with recent studies (191), IL-33 did not affect CCR3 expression on eosinophils that are already in the periphery (data not shown) or at sites of inflammation.

The accumulation of eosinophils in the airways is a prominent feature of most clinical asthma subtypes and experimental asthma models (143, 188). Many

human and animal studies point out the important function of eosinophils in the exacerbation of lung disease and airway remodeling (143). However, the precise factors that are responsible for the localized activation of eosinophils in the asthmatic lungs are not fully recognized. This become of particular importance in the light of recent finding that airway eosinophils are not susceptible to IL-5 as they shed their surface IL-5 receptor while they enter airway lumen (166). Given the abundant expression of IL-33 in the stromal tissue of asthmatic lungs (26-27), the possibility arises that IL-33 may trigger the activation of eosinophils that have been recruited to the airways.

Consistent with data published previously (191-192), I found that only limited proportion of mature mouse and PB eosinophils of most human donors expressed ST2L although they did express mRNA for both ST2L and sST2. By contrast, eosinophils that accumulated in airways during OVA-induced asthma model did express high levels of ST2L on the surface. My *in vitro* studies revealed that both IL-33 itself and GM-CSF are likely to upregulate ST2L on airway eosinophils. Intriguingly, IL-5 seems to play an inhibitory role in ST2L expression if acting alone on human eosinophils. This might prevent the activation of eosinophils driven by constitutive low levels of IL-5, GM-CSF and IL-3, which may be present under normal steady-state conditions.

The observation that in two out of six human donors PB eosinophils exhibited readily detectable ST2L expression could be attributed to the mild ongoing allergic reactions in those subjects. However, further studies in a bigger population of clinically diagnosed allergic individuals and healthy subjects are

required in order to establish a true variation of ST2L expression in human PB eosinophis.

Recent studies on eosinophil deficient mice show an important role of eosinophils in cytokine and chemokine production, and thereby their contribution to the perpetuation of inflammation (110, 145). Here, I show that IL-33 can be a trigger of these processes. IL-33/ST2-activated eosinophils contribute to the exacerbation of inflammation by increasing the number of macrophages and lymphocytes in the lungs which can be attributed to the increase in levels of cytokines and chemokines, including TARC, IL-13, TGF-β, MIP-1α and Eotaxin-2. My *in vitro* data show that murine eosinophils can be a potent source of TARC, IL-13, IL-6 and TGF-β upon IL-33 stimulation. Interestingly, pulmonary, eosinophil-dependent TARC release appears to be required for the localized recruitment of effector T cells during asthma model (145). I was able to demonstrate that IL-33 can be a factor that drives TARC production by eosinophils and this is followed by the accumulation of CD4 cells in the airways.

IL-33 *in vitro* seemed to have no effect on eotaxin-2 and MIP-1α production by eosinophils themselves. Thus, elevated levels of these two chemokines found in BAL of $ST2^{-/-}$ recipients that received WT eosinophil cells followed by IL-33 injection are probably due to an indirect effect of IL-33-stimulated eosinophils interacting with other cell population in the lungs, such as alveolar macrophages. In fact, the very presence of eosinophils in the airways triggers the differentiation of quiescent alveolar macrophages towards MR^{+} alternatively

activated macrophages, a phenotype that is pathogenic in type 2 diseases (26, 150). Studies focused on a mechanism behind this process revealed that IL-13, which is a well known AAM differentiation factor that is also released by eosinophils upon IL-33 stimulation can be, at least partially, responsible for the increased differentiation of AAM macrophages. Consistent with our data, Rankin et al. demonstrated that IL-33 induces the production of IL-13 from eosinophils which, in turn, can promote the development of cutaneous fibrosis (193). Moreover, the elegant studies by Pope et al. and Kurowska-Stolarska et al. suggest that BAL AAM macrophages may be the major producers of Eotaxin-2 in experimental asthma model (26, 127). Thus, it is likely that AAM are responsible for the elevated levels of Eotaxin-2 that I could observed in recipients given eosinophils plus IL-33.

There is growing evidence to support the notion that eosinophils also have the capacity to regulate mast cell function, which are producers of an array of chemokines, including MIP-1α (130, 194). Therefore; lung resident mast cells may likely release MIP-1α upon interaction with IL-33-activated eosinophils.

Furthermore, IL-33-activated WT eosinophils, consistent with the BAL chemokine profile they contribute to, could attract more host ($ST2^{-/-}$) eosinophils to the airways. Intriguingly, administration of IL-33 locally into the lungs also helped maintain significant numbers of adoptively transferred eosinophils in the airways. This may be explained by the effect of IL-33-induced chemokines that lock those cells in the airways. Furthermore, the survival of adoptively transferred eosinophils may be enhanced either as a result of an

interaction with other cells or as a direct effect of IL-33. The latter possibility is supported by observations in human eosinophils *in vitro* (187, 191-192).

Finally, my work provides evidence that IL-33 can significantly increase the eosinophil susceptibility to antibody-triggered degranulation by increasing the expression the expression of FcγIIR (CD32). Thus, the release of IL-33 from inflamed tissue may be a crucial factor responsible for cytotoxic granule-mediated tissue damage in clinical settings. In contrast to other studies (187), I did not detect any substantial eosinophil degranulation in the presence of IL-33 or IL-5 alone. In support of my findings, numerous studies suggest that cytokines, such as IL-5 for example, also have a limited contribution to the degranulation process, compared to potent degranulation factors such as immuno-complexes or complement molecules (195-196).

Taken together, IL-33/ST2 pathway regulates multiple features of eosinophil biology that can have a significant impact on allergic inflammation and asthma. In this project, I show that IL-33 can directly differentiate eosinophils from bone marrow precursors. Most importantly, I demonstrate that mature eosinophils can acquire high levels of IL-33 receptor (ST2L) at sites of airway inflammation. Finally my work provides new evidence that eosinophils activated through the IL-33/ST2 signaling pathway play an important role in airway inflammation *in vivo.* A possible mechanism by which IL-33 activates eosinophils and their contribution to airway inflammation is summarized in **Figure 7.1.**

Given the limited benefit of anti-IL-5 therapy in asthma patients, reflected by only moderate decrease in sputum eosinophil counts (only 55% reduction) and lack of clinical benefit (197-198), inhibition of the IL-33/ST2 pathway can be considered a promising therapeutic target. This notion is supported by an increasing number of studies showing that IL-33/ST2 pathway is equally important in the activation of other cell types involved in the pathogenesis of allergy and asthma, such as Th2 cells, mast cells, DC and macrophages (4, 26, 29, 43, 48, 68).

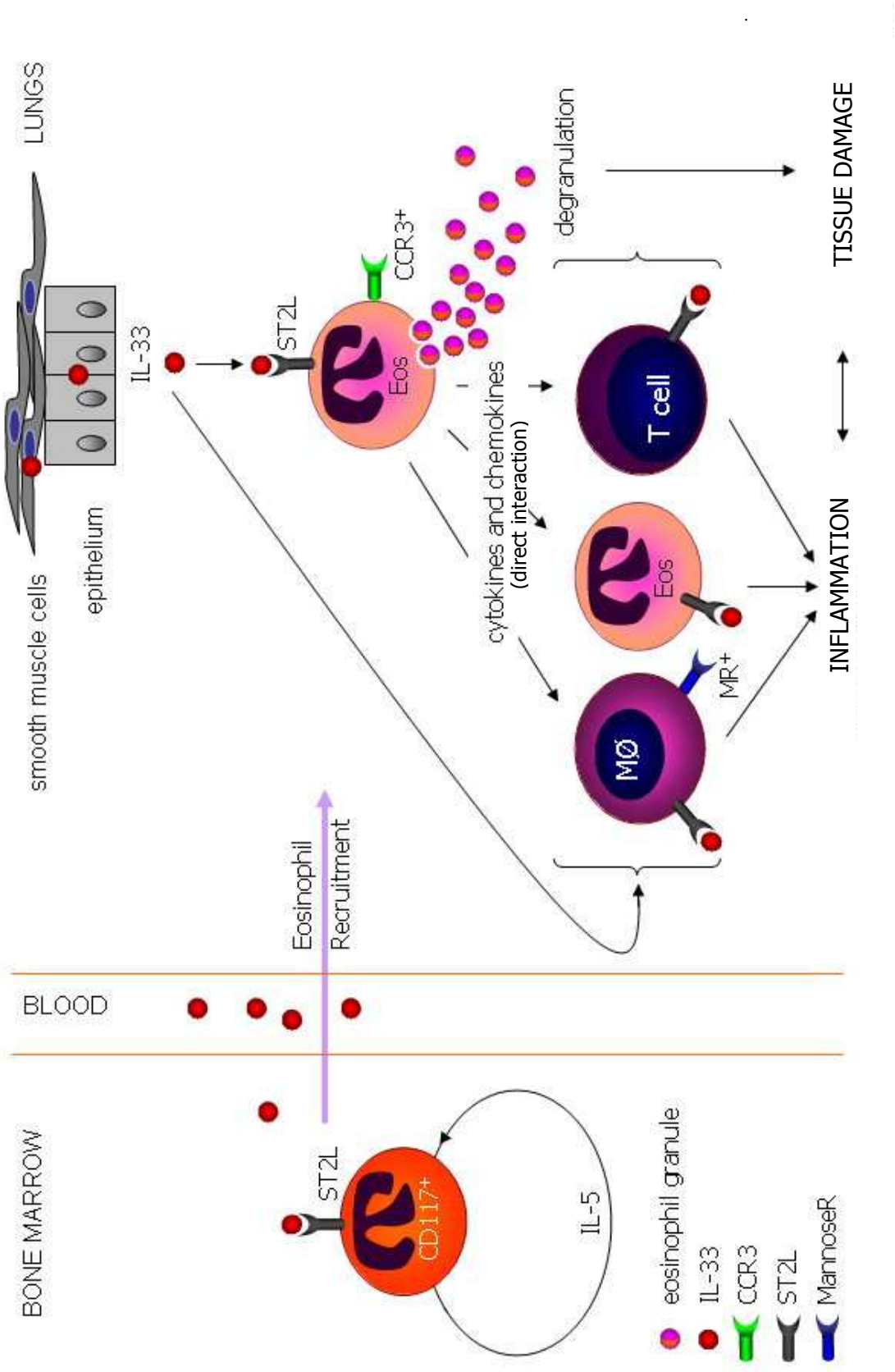


Figure 7.1. The contribution of IL33-induced eosinophils to airway inflammation

References:

1. Alves-Filho, J. C., F. Sonego, F. O. Souto, A. Freitas, W. A. Verri, Jr., M. Auxiliadora-Martins, A. Basile-Filho, A. N. McKenzie, D. Xu, F. Q. Cunha, and F. Y. Liew. 2010. Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nat Med* 16:708-712.

2. Onda, H., H. Kasuya, K. Takakura, T. Hori, T. Imaizumi, T. Takeuchi, I. Inoue, and J. Takeda. 1999. Identification of genes differentially expressed in canine vasospastic cerebral arteries after subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 19:1279-1288.

3. Baekkevold, E. S., M. Roussigne, T. Yamanaka, F. E. Johansen, F. L. Jahnsen, F. Amalric, P. Brandtzaeg, M. Erard, G. Haraldsen, and J. P. Girard. 2003. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol* 163:69-79.

4. Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D. M. Gorman, J. F. Bazan, and R. A. Kastelein. 2005. 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* 23:479-490.

5. Priestle, J. P., H. P. Schar, and M. G. Grutter. 1988. Crystal structure of the cytokine interleukin-1 beta. *EMBO J* 7:339-343.

6. Moussion, C., N. Ortega, and J. P. Girard. 2008. 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* 3:e3331.

7. Xu, D., H. R. Jiang, P. Kewin, Y. Li, R. Mu, A. R. Fraser, N. Pitman, M. Kurowska-Stolarska, A. N. McKenzie, I. B. McInnes, and F. Y. Liew. 2008. IL-33 exacerbates antigen-induced arthritis by activating mast cells. *Proc Natl Acad Sci U S A* 105:10913-10918.

8. Kuchler, A. M., J. Pollheimer, J. Balogh, J. Sponheim, L. Manley, D. R. Sorensen, P. M. De Angelis, H. Scott, and G. Haraldsen. 2008. Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation. *Am J Pathol* 173:1229-1242.

9. Ohno, T., K. Oboki, N. Kajiwara, E. Morii, K. Aozasa, R. A. Flavell, K. Okumura, H. Saito, and S. Nakae. 2009. Caspase-1, caspase-8, and calpain are dispensable for IL-33 release by macrophages. *J Immunol* 183:7890-7897.

10. Carriere, V., L. Roussel, N. Ortega, D. A. Lacorre, L. Americh, L. Aguilar, G. Bouche, and J. P. Girard. 2007. 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* 104:282-287.

11. Roussel, L., M. Erard, C. Cayrol, and J. P. Girard. 2008. Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. *EMBO Rep* 9:1006-1012.

12. Cayrol, C., and J. P. Girard. 2009. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc Natl Acad Sci U S A* 106:9021-9026.

13. Luthi, A. U., S. P. Cullen, E. A. McNeela, P. J. Duriez, I. S. Afonina, C. Sheridan, G. Brumatti, R. C. Taylor, K. Kersse, P. Vandenabeele, E. C. Lavelle, and S. J. Martin. 2009. Suppression of Interleukin-33 Bioactivity through Proteolysis by Apoptotic Caspases. *Immunity*.

14. Talabot-Ayer, D., C. Lamacchia, C. Gabay, and G. Palmer. 2009. Interleukin-33 is biologically active independently of caspase-1 cleavage. *J Biol Chem*.

15. Li, H., S. B. Willingham, J. P. Ting, and F. Re. 2008. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. *J Immunol* 181:17-21.

16. Hayakawa, M., H. Hayakawa, Y. Matsuyama, H. Tamemoto, H. Okazaki, and S. Tominaga. 2009. Mature interleukin-33 is produced by calpain-mediated cleavage in vivo. *Biochem Biophys Res Commun* 387:218-222.

17. Lamkanfi, M., and V. M. Dixit. 2009. IL-33 raises alarm. *Immunity* 31:5-7.

18. Sanada, S., D. Hakuno, L. J. Higgins, E. R. Schreiter, A. N. McKenzie, and R. T. Lee. 2007. IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. *J Clin Invest* 117:1538-1549.

19. Hudson, C. A., G. P. Christophi, R. C. Gruber, J. R. Wilmore, D. A. Lawrence, and P. T. Massa. 2008. Induction of IL-33 expression and activity in central nervous system glia. *J Leukoc Biol*.

20. Scaffidi, P., T. Misteli, and M. E. Bianchi. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418:191-195.

21. Eigenbrod, T., J. H. Park, J. Harder, Y. Iwakura, and G. Nunez. 2008. Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *J Immunol* 181:8194-8198.

22. Sakurai, T., G. He, A. Matsuzawa, G. Y. Yu, S. Maeda, G. Hardiman, and M. Karin. 2008. Hepatocyte necrosis induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis. *Cancer Cell* 14:156-165.

23. Kazama, H., J. E. Ricci, J. M. Herndon, G. Hoppe, D. R. Green, and T. A. Ferguson. 2008. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* 29:21-32.

24. Oppenheim, J. J., and D. Yang. 2005. Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol* 17:359-365.

25. Bianchi, M. E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 81:1-5.

26. Kurowska-Stolarska, M., B. Stolarski, P. Kewin, G. Murphy, C. J. Corrigan, S. Ying, N. Pitman, A. Mirchandani, B. Rana, N. van Rooijen, M. Shepherd, C. McSharry, I. B. McInnes, D. Xu, and F. Y. Liew. 2009. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol* 183:6469-6477.

27. Prefontaine, D., S. Lajoie-Kadoch, S. Foley, S. Audusseau, R. Olivenstein, A. J. Halayko, C. Lemiere, J. G. Martin, and Q. Hamid. 2009. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J Immunol* 183:5094-5103.

28. Prefontaine, D., J. Nadigel, F. Chouiali, S. Audusseau, A. Semlali, J. Chakir, J. G. Martin, and Q. Hamid. 2010. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* 125:752-754.

29. Pushparaj, P. N., H. K. Tay, C. H'Ng S, N. Pitman, D. Xu, A. McKenzie, F. Y. Liew, and A. J. Melendez. 2009. The cytokine interleukin-33 mediates anaphylactic shock. *Proc Natl Acad Sci U S A* 106:9773-9778.

30. Nishida, A., A. Andoh, H. Imaeda, O. Inatomi, H. Shiomi, and Y. Fujiyama. 2010. Expression of interleukin 1-like cytokine interleukin 33 and its receptor complex (ST2L and IL1RAcP) in human pancreatic myofibroblasts. *Gut* 59:531-541.

31. Seidelin, J. B., J. T. Bjerrum, M. Coskun, B. Widjaya, B. Vainer, and O. H. Nielsen. 2010. IL-33 is upregulated in colonocytes of ulcerative colitis. *Immunol Lett* 128:80-85.

32. Palmer, G., D. Talabot-Ayer, C. Lamacchia, D. Toy, C. A. Seemayer, S. Viatte, A. Finckh, D. E. Smith, and C. Gabay. 2009. Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis. *Arthritis Rheum* 60:738-749.

33. Choi, Y. S., H. J. Choi, J. K. Min, B. J. Pyun, Y. S. Maeng, H. Park, J. Kim, Y. M. Kim, and Y. G. Kwon. 2009. Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood* 114:3117-3126.

34. Ali, S., M. Huber, C. Kollewe, S. C. Bischoff, W. Falk, and M. U. Martin. 2007. 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*.

35. Chackerian, A. A., E. R. Oldham, E. E. Murphy, J. Schmitz, S. Pflanz, and R. A. Kastelein. 2007. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J Immunol* 179:2551-2555.

36. Iwahana, H., K. Yanagisawa, A. Ito-Kosaka, K. Kuroiwa, K. Tago, N. Komatsu, R. Katashima, M. Itakura, and S. Tominaga. 1999. 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* 264:397-406.

37. Tago, K., T. Noda, M. Hayakawa, H. Iwahana, K. Yanagisawa, T. Yashiro, and S. Tominaga. 2001. Tissue distribution and subcellular localization of a variant form of the human ST2 gene product, ST2V. *Biochem Biophys Res Commun* 285:1377-1383.

38. Iwahana, H., M. Hayakawa, K. Kuroiwa, K. Tago, K. Yanagisawa, S. Noji, and S. Tominaga. 2004. Molecular cloning of the chicken ST2 gene and a novel variant form of the ST2 gene product, ST2LV. *Biochim Biophys Acta* 1681:1-14.

39. Arend, W. P., G. Palmer, and C. Gabay. 2008. IL-1, IL-18, and IL-33 families of cytokines. *Immunol Rev* 223:20-38.

40. O'Neill, L. A. 2008. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol Rev* 226:10-18.

41. Hayakawa, H., M. Hayakawa, A. Kume, and S. Tominaga. 2007. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J Biol Chem* 282:26369-26380.

42. Palmer, G., B. P. Lipsky, M. D. Smithgall, D. Meininger, S. Siu, D. Talabot-Ayer, C. Gabay, and D. E. Smith. 2008. The IL-1 receptor accessory

protein (AcP) is required for IL-33 signaling and soluble AcP enhances the ability of soluble ST2 to inhibit IL-33. *Cytokine* 42:358-364.

43. Kurowska-Stolarska, M., P. Kewin, G. Murphy, R. C. Russo, B. Stolarski, C. C. Garcia, M. Komai-Koma, N. Pitman, Y. Li, W. Niedbala, A. N. McKenzie, M. M. Teixeira, F. Y. Liew, and D. Xu. 2008. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol* 181:4780-4790.

44. Bulek, K., S. Swaidani, J. Qin, Y. Lu, M. F. Gulen, T. Herjan, B. Min, R. A. Kastelein, M. Aronica, M. Kosz-Vnenchak, and X. Li. 2009. The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response. *J Immunol* 182:2601-2609.

45. Garlanda, C., H. J. Anders, and A. Mantovani. 2009. TIR8/SIGIRR: an IL-1R/TLR family member with regulatory functions in inflammation and T cell polarization. *Trends Immunol* 30:439-446.

46. Garlanda, C., F. Riva, N. Polentarutti, C. Buracchi, M. Sironi, M. De Bortoli, M. Muzio, R. Bergottini, E. Scanziani, A. Vecchi, E. Hirsch, and A. Mantovani. 2004. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. *Proc Natl Acad Sci U S A* 101:3522-3526.

47. Kroeger, K. M., B. M. Sullivan, and R. M. Locksley. 2009. IL-18 and IL-33 elicit Th2 cytokines from basophils via a MyD88- and p38{alpha}-dependent pathway. *J Leukoc Biol*.

48. Kondo, Y., T. Yoshimoto, K. Yasuda, S. Futatsugi-Yumikura, M. Morimoto, N. Hayashi, T. Hoshino, J. Fujimoto, and K. Nakanishi. 2008. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int Immunol* 20:791-800.

49. Funakoshi-Tago, M., K. Tago, M. Hayakawa, S. Tominaga, T. Ohshio, Y. Sonoda, and T. Kasahara. 2008. TRAF6 is a critical signal transducer in IL-33 signaling pathway. *Cell Signal* 20:1679-1686.

50. Theoharides, T. C., B. Zhang, D. Kempuraj, M. Tagen, M. Vasiadi, A. Angelidou, K. D. Alysandratos, D. Kalogeromitros, S. Asadi, N. Stavrianeas, E. Peterson, S. Leeman, and P. Conti. 2010. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. *Proc Natl Acad Sci U S A* 107:4448-4453.

51. Matsuyama, Y., H. Okazaki, H. Tamemoto, H. Kimura, Y. Kamata, K. Nagatani, T. Nagashima, M. Hayakawa, M. Iwamoto, T. Yoshio, S. Tominaga, and S. Minota. Increased levels of interleukin 33 in sera and synovial fluid from patients with active rheumatoid arthritis. *J Rheumatol* 37:18-25.

52. Theoharides, T. C., B. Zhang, D. Kempuraj, M. Tagen, M. Vasiadi, A. Angelidou, K. D. Alysandratos, D. Kalogeromitros, S. Asadi, N. Stavrianeas, E. Peterson, S. Leeman, and P. Conti. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. *Proc Natl Acad Sci U S A* 107:4448-4453.

53. Hotamisligil, G. S. 2006. Inflammation and metabolic disorders. *Nature* 444:860-867.

54. Wood, I. S., B. Wang, and P. Trayhurn. 2009. IL-33, a recently identified interleukin-1 gene family member, is expressed in human adipocytes. *Biochem Biophys Res Commun* 384:105-109.

55. Miller, A. M., D. L. Asquith, A. J. Hueber, L. A. Anderson, W. M. Holmes, A. N. McKenzie, D. Xu, N. Sattar, I. B. McInnes, and F. Y. Liew. Interleukin-33 Induces Protective Effects in Adipose Tissue Inflammation During Obesity in Mice. *Circ Res*.

56. Hansson, G. K., A. K. Robertson, and C. Soderberg-Naucler. 2006. Inflammation and atherosclerosis. *Annu Rev Pathol* 1:297-329.

57. King, T. E., Jr. 1999. A new look at the pathophysiology of asthma. *J Natl Med Assoc* 91:9S-15S.

58. Hang, L. W., T. C. Hsia, W. C. Chen, H. Y. Chen, and F. J. Tsai. 2003. TAP1 gene AccI polymorphism is associated with atopic bronchial asthma. *J Clin Lab Anal* 17:57-60.

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

60. Sakashita, M., T. Yoshimoto, T. Hirota, M. Harada, K. Okubo, Y. Osawa, S. Fujieda, Y. Nakamura, K. Yasuda, K. Nakanishi, and M. Tamari. 2008.

Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis. *Clin Exp Allergy* 38:1875-1881.

61. Reijmerink, N. E., D. S. Postma, M. Bruinenberg, I. M. Nolte, D. A. Meyers, E. R. Bleecker, and G. H. Koppelman. 2008. Association of IL1RL1, IL18R1, and IL18RAP gene cluster polymorphisms with asthma and atopy. *J Allergy Clin Immunol* 122:651-654 e658.

62. Ali, M., G. Zhang, W. R. Thomas, C. J. McLean, J. A. Bizzintino, I. A. Laing, A. C. Martin, J. Goldblatt, P. N. Le Souef, and C. M. Hayden. 2009. Investigations into the role of ST2 in acute asthma in children. *Tissue Antigens* 73:206-212.

63. Gudbjartsson, D. F., U. S. Bjornsdottir, E. Halapi, A. Helgadottir, P. Sulem, G. M. Jonsdottir, G. Thorleifsson, H. Helgadottir, V. Steinthorsdottir, H. Stefansson, C. Williams, J. Hui, J. Beilby, N. M. Warrington, A. James, L. J. Palmer, G. H. Koppelman, A. Heinzmann, M. Krueger, H. M. Boezen, A. Wheatley, J. Altmuller, H. D. Shin, S. T. Uh, H. S. Cheong, B. Jonsdottir, D. Gislason, C. S. Park, L. M. Rasmussen, C. Porsbjerg, J. W. Hansen, V. Backer, T. Werge, C. Janson, U. B. Jonsson, M. C. Ng, J. Chan, W. Y. So, R. Ma, S. H. Shah, C. B. Granger, A. A. Quyyumi, A. I. Levey, V. Vaccarino, M. P. Reilly, D. J. Rader, M. J. Williams, A. M. van Rij, G. T. Jones, E. Trabetti, G. Malerba, P. F. Pignatti, A. Boner, L. Pescollderungg, D. Girelli, O. Olivieri, N. Martinelli, B. R. Ludviksson, D. Ludviksdottir, G. I. Eyjolfsson, D. Arnar, G. Thorgeirsson, K. Deichmann, P. J. Thompson, M. Wjst, I. P. Hall, D. S. Postma, T. Gislason, J.

Gulcher, A. Kong, I. Jonsdottir, U. Thorsteinsdottir, and K. Stefansson. 2009. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. *Nat Genet* 41:342-347.

64. Oshikawa, K., K. Yanagisawa, S. Tominaga, and Y. Sugiyama. 2002. Expression and function of the ST2 gene in a murine model of allergic airway inflammation. *Clin Exp Allergy* 32:1520-1526.

65. Hoshino, K., S. Kashiwamura, K. Kuribayashi, T. Kodama, T. Tsujimura, K. Nakanishi, T. Matsuyama, K. Takeda, and S. Akira. 1999. 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* 190:1541-1548.

66. Mangan, N. E., A. Dasvarma, A. N. McKenzie, and P. G. Fallon. 2007. T1/ST2 expression on Th2 cells negatively regulates allergic pulmonary inflammation. *Eur J Immunol* 37:1302-1312.

67. Coyle, A. J., C. Lloyd, J. Tian, T. Nguyen, C. Erikkson, L. Wang, P. Ottoson, P. Persson, T. Delaney, S. Lehar, S. Lin, L. Poisson, C. Meisel, T. Kamradt, T. Bjerke, D. Levinson, and J. C. Gutierrez-Ramos. 1999. 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* 190:895-902.

68. Kearley, J., K. F. Buckland, S. A. Mathie, and C. M. Lloyd. 2009. Resolution of allergic inflammation and airway hyperreactivity is dependent

upon disruption of the T1/ST2-IL-33 pathway. *Am J Respir Crit Care Med* 179:772-781.

69. Liu, X., M. Li, Y. Wu, Y. Zhou, L. Zeng, and T. Huang. 2009. Anti-IL-33 antibody treatment inhibits airway inflammation in a murine model of allergic asthma. *Biochem Biophys Res Commun*.

70. Lohning, M., A. Stroehmann, A. J. Coyle, J. L. Grogan, S. Lin, J. C. Gutierrez-Ramos, D. Levinson, A. Radbruch, and T. Kamradt. 1998. 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* 95:6930-6935.

71. Rank, M. A., T. Kobayashi, H. Kozaki, K. R. Bartemes, D. L. Squillace, and H. Kita. 2009. IL-33-activated dendritic cells induce an atypical TH2-type response. *J Allergy Clin Immunol* 123:1047-1054.

72. Pearce, E. J., A. Cheever, S. Leonard, M. Covalesky, R. Fernandez-Botran, G. Kohler, and M. Kopf. 1996. Schistosoma mansoni in IL-4-deficient mice. *Int Immunol* 8:435-444.

73. Hogan, S. P., A. Mould, H. Kikutani, A. J. Ramsay, and P. S. Foster. 1997. Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyperreactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. *J Clin Invest* 99:1329-1339.

74. Hogarth, P. J., M. J. Taylor, and A. E. Bianco. 1998. IL-5-dependent immunity to microfilariae is independent of IL-4 in a mouse model of onchocerciasis. *J Immunol* 160:5436-5440.

75. Brewer, J. M., M. Conacher, C. A. Hunter, M. Mohrs, F. Brombacher, and J. Alexander. 1999. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J Immunol* 163:6448-6454.

76. Herrick, C. A., H. MacLeod, E. Glusac, R. E. Tigelaar, and K. Bottomly. 2000. Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J Clin Invest* 105:765-775.

77. Wenzel, S. E. 2006. Asthma: defining of the persistent adult phenotypes. *Lancet* 368:804-813.

78. Zhiguang, X., C. Wei, R. Steven, D. Wei, Z. Wei, M. Rong, L. Zhanguo, and Z. Lianfeng. Over-expression of IL-33 leads to spontaneous pulmonary inflammation in mIL-33 transgenic mice. *Immunol Lett* 131:159-165.

79. Schneider, E., A. F. Petit-Bertron, R. Bricard, M. Levasseur, A. Ramadan, J. P. Girard, A. Herbelin, and M. Dy. 2009. IL-33 activates unprimed murine basophils directly in vitro and induces their in vivo expansion indirectly by promoting hematopoietic growth factor production. *J Immunol* 183:3591-3597.

80. Kurowska-Stolarska, M., P. Kewin, G. Murphy, R. C. Russo, B. Stolarski, C. C. Garcia, M. Komai-Koma, N. Pitman, Y. Li, A. N. McKenzie, M. M. Teixeira, F. Y. Liew, and D. Xu. 2008. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol* 181:4780-4790.

81. Pecaric-Petkovic, T., S. A. Didichenko, S. Kaempfer, N. Spiegl, and C. A. Dahinden. 2009. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 113:1526-1534.

82. Suzukawa, M., M. Iikura, R. Koketsu, H. Nagase, C. Tamura, A. Komiya, S. Nakae, K. Matsushima, K. Ohta, K. Yamamoto, and M. Yamaguchi. 2008. An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J Immunol* 181:5981-5989.

83. Humphreys, N. E., D. Xu, M. R. Hepworth, F. Y. Liew, and R. K. Grencis. 2008. IL-33, a Potent Inducer of Adaptive Immunity to Intestinal Nematodes. *J Immunol* 180:2443-2449.

84. Neill, D. R., S. H. Wong, A. Bellosi, R. J. Flynn, M. Daly, T. K. Langford, C. Bucks, C. M. Kane, P. G. Fallon, R. Pannell, H. E. Jolin, and A. N. McKenzie. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.

85. Palacios, R., H. Karasuyama, and A. Rolink. 1987. Ly1+ PRO-B lymphocyte clones. Phenotype, growth requirements and differentiation in vitro and in vivo. *EMBO J* 6:3687-3693.

86. Yamaguchi, Y., T. Suda, J. Suda, M. Eguchi, Y. Miura, N. Harada, A. Tominaga, and K. Takatsu. 1988. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J Exp Med* 167:43-56.

87. Takatsu, K., N. Yamaguchi, Y. Hitoshi, E. Sonoda, S. Mita, and A. Tominaga. 1989. Signal transduction through interleukin-5 receptors. *Cold Spring Harb Symp Quant Biol* 54 Pt 2:745-751.

88. Sanderson, C. J. 1990. Eosinophil differentiation factor (interleukin-5). *Immunol Ser* 49:231-256.

89. Dyer, K. D., J. M. Moser, M. Czapiga, S. J. Siegel, C. M. Percopo, and H. F. Rosenberg. 2008. Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow. *J Immunol* 181:4004-4009.

90. Iwasaki, H., S. Mizuno, R. Mayfield, H. Shigematsu, Y. Arinobu, B. Seed, M. F. Gurish, K. Takatsu, and K. Akashi. 2005. Identification of eosinophil lineage-committed progenitors in the murine bone marrow. *J Exp Med* 201:1891-1897.

91. Mori, Y., H. Iwasaki, K. Kohno, G. Yoshimoto, Y. Kikushige, A. Okeda, N. Uike, H. Niiro, K. Takenaka, K. Nagafuji, T. Miyamoto, M. Harada, K. Takatsu, and K. Akashi. 2009. Identification of the human eosinophil lineage-committed progenitor: revision of phenotypic definition of the human common myeloid progenitor. *J Exp Med* 206:183-193.

92. Manz, M. G., T. Miyamoto, K. Akashi, and I. L. Weissman. 2002. Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci U S A* 99:11872-11877.

93. Nerlov, C., and T. Graf. 1998. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev* 12:2403-2412.

94. McKercher, S. R., G. W. Henkel, and R. A. Maki. 1999. The transcription factor PU.1 does not regulate lineage commitment but has lineage-specific effects. *J Leukoc Biol* 66:727-732.

95. Du, J., M. J. Stankiewicz, Y. Liu, Q. Xi, J. E. Schmitz, J. A. Lekstrom-Himes, and S. J. Ackerman. 2002. Novel combinatorial interactions of GATA-1, PU.1, and C/EBPepsilon isoforms regulate transcription of the gene encoding eosinophil granule major basic protein. *J Biol Chem* 277:43481-43494.

96. Ackerman, S. J., and B. S. Bochner. 2007. Mechanisms of eosinophilia in the pathogenesis of hypereosinophilic disorders. *Immunol Allergy Clin North Am* 27:357-375.

97. Hirai, H., P. Zhang, T. Dayaram, C. J. Hetherington, S. Mizuno, J. Imanishi, K. Akashi, and D. G. Tenen. 2006. C/EBPbeta is required for 'emergency' granulopoiesis. *Nat Immunol* 7:732-739.

98. Kincade, P. W. 2006. Supplying the demand for granulocytes. *Nat Immunol* 7:701-702.

99. Shivdasani, R. A., Y. Fujiwara, M. A. McDevitt, and S. H. Orkin. 1997. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J* 16:3965-3973.

100. Yamaguchi, Y., S. J. Ackerman, N. Minegishi, M. Takiguchi, M. Yamamoto, and T. Suda. 1998. Mechanisms of transcription in eosinophils: GATA-1, but not GATA-2, transactivates the promoter of the eosinophil granule major basic protein gene. *Blood* 91:3447-3458.

101. Yu, C., A. B. Cantor, H. Yang, C. Browne, R. A. Wells, Y. Fujiwara, and S. H. Orkin. 2002. 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* 195:1387-1395.

102. Yamaguchi, Y., H. Nishio, K. Kishi, S. J. Ackerman, and T. Suda. 1999. C/EBPbeta and GATA-1 synergistically regulate activity of the eosinophil granule major basic protein promoter: implication for C/EBPbeta activity in eosinophil gene expression. *Blood* 94:1429-1439.

103. Hirasawa, R., R. Shimizu, S. Takahashi, M. Osawa, S. Takayanagi, Y. Kato, M. Onodera, N. Minegishi, M. Yamamoto, K. Fukao, H. Taniguchi, H. Nakauchi, and A. Iwama. 2002. Essential and instructive roles of GATA factors in eosinophil development. *J Exp Med* 195:1379-1386.

104. Yamanaka, R., C. Barlow, J. Lekstrom-Himes, L. H. Castilla, P. P. Liu, M. Eckhaus, T. Decker, A. Wynshaw-Boris, and K. G. Xanthopoulos. 1997. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci U S A* 94:13187-13192.

105. McNagny, K., and T. Graf. 2002. Making eosinophils through subtle shifts in transcription factor expression. *J Exp Med* 195:F43-47.

106. Bedi, R., J. Du, A. K. Sharma, I. Gomes, and S. J. Ackerman. 2009. Human C/EBP-epsilon activator and repressor isoforms differentially reprogram myeloid lineage commitment and differentiation. *Blood* 113:317-327.

107. Yamanaka, R., J. Lekstrom-Himes, C. Barlow, A. Wynshaw-Boris, and K. G. Xanthopoulos. 1998. CCAAT/enhancer binding proteins are critical components of the transcriptional regulation of hematopoiesis (Review). *Int J Mol Med* 1:213-221.

108. Rosenberg, H. F., and J. I. Gallin. 1993. Neutrophil-specific granule deficiency includes eosinophils. *Blood* 82:268-273.

109. Milanovic, M., G. Terszowski, D. Struck, O. Liesenfeld, and D. Carstanjen. 2008. IFN consensus sequence binding protein (Icsbp) is critical for eosinophil development. *J Immunol* 181:5045-5053.

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

111. Zimmermann, N., G. K. Hershey, P. S. Foster, and M. E. Rothenberg. 2003. Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J Allergy Clin Immunol* 111:227-242; quiz 243.

112. Bochner, B. S., and R. P. Schleimer. 1994. The role of adhesion molecules in human eosinophil and basophil recruitment. *J Allergy Clin Immunol* 94:427-438; quiz 439.

113. Palframan, R. T., P. D. Collins, N. J. Severs, S. Rothery, T. J. Williams, and S. M. Rankin. 1998. Mechanisms of acute eosinophil mobilization from the bone marrow stimulated by interleukin 5: the role of specific adhesion molecules and phosphatidylinositol 3-kinase. *J Exp Med* 188:1621-1632.

114. Palframan, R. T., P. D. Collins, T. J. Williams, and S. M. Rankin. 1998. Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. *Blood* 91:2240-2248.

115. Voehringer, D., N. van Rooijen, and R. M. Locksley. 2007. Eosinophils develop in distinct stages and are recruited to peripheral sites by alternatively activated macrophages. *J Leukoc Biol* 81:1434-1444.

116. Kopf, M., F. Brombacher, P. D. Hodgkin, A. J. Ramsay, E. A. Milbourne, W. J. Dai, K. S. Ovington, C. A. Behm, G. Kohler, I. G. Young, and K. I. Matthaei. 1996. IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* 4:15-24.

117. Menzies-Gow, A., P. Flood-Page, R. Sehmi, J. Burman, Q. Hamid, D. S. Robinson, A. B. Kay, and J. Denburg. 2003. Anti-IL-5 (mepolizumab) therapy induces bone marrow eosinophil maturational arrest and decreases eosinophil progenitors in the bronchial mucosa of atopic asthmatics. *J Allergy Clin Immunol* 111:714-719.

118. Kay, A. B., and A. D. Klion. 2004. Anti-interleukin-5 therapy for asthma and hypereosinophilic syndrome. *Immunol Allergy Clin North Am* 24:645-666, vii.

119. Collins, P. D., S. Marleau, D. A. Griffiths-Johnson, P. J. Jose, and T. J. Williams. 1995. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med* 182:1169-1174.

120. Rankin, S. M., D. M. Conroy, and T. J. Williams. 2000. Eotaxin and eosinophil recruitment: implications for human disease. *Mol Med Today* 6:20-27.

121. Murphy, P. M. 1994. The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol* 12:593-633.

122. Daugherty, B. L., S. J. Siciliano, J. A. DeMartino, L. Malkowitz, A. Sirotina, and M. S. Springer. 1996. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. *J Exp Med* 183:2349-2354.

123. Ponath, P. D., S. Qin, T. W. Post, J. Wang, L. Wu, N. P. Gerard, W. Newman, C. Gerard, and C. R. Mackay. 1996. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J Exp Med* 183:2437-2448.

124. Fulkerson, P. C., H. Zhu, D. A. Williams, N. Zimmermann, and M. E. Rothenberg. 2005. CXCL9 inhibits eosinophil responses by a CCR3- and Rac2-dependent mechanism. *Blood* 106:436-443.

125. Kitaura, M., N. Suzuki, T. Imai, S. Takagi, R. Suzuki, T. Nakajima, K. Hirai, H. Nomiyama, and O. Yoshie. 1999. Molecular cloning of a novel human CC chemokine (Eotaxin-3) that is a functional ligand of CC chemokine receptor 3. *J Biol Chem* 274:27975-27980.

126. Voehringer, D., K. Shinkai, and R. M. Locksley. 2004. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* 20:267-277.

127. Pope, S. M., P. C. Fulkerson, C. Blanchard, H. S. Akei, N. M. Nikolaidis, N. Zimmermann, J. D. Molkentin, and M. E. Rothenberg. 2005. Identification of a cooperative mechanism involving interleukin-13 and eotaxin-2 in experimental allergic lung inflammation. *J Biol Chem* 280:13952-13961.

128. Zhang, M., T. Angata, J. Y. Cho, M. Miller, D. H. Broide, and A. Varki. 2007. Defining the in vivo function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils. *Blood* 109:4280-4287.

129. Zimmermann, N., M. L. McBride, Y. Yamada, S. A. Hudson, C. Jones, K. D. Cromie, P. R. Crocker, M. E. Rothenberg, and B. S. Bochner. 2008. Siglec-F antibody administration to mice selectively reduces blood and tissue eosinophils. *Allergy* 63:1156-1163.

130. Rothenberg, M. E., and S. P. Hogan. 2006. The eosinophil. *Annu Rev Immunol* 24:147-174.

131. Blanchard, C., and M. E. Rothenberg. 2009. Biology of the eosinophil. *Adv Immunol* 101:81-121.

132. Gouon-Evans, V., M. E. Rothenberg, and J. W. Pollard. 2000. Postnatal mammary gland development requires macrophages and eosinophils. *Development* 127:2269-2282.

133. Jordan, H. E., and C. C. Speidel. 1923. Blood Cell Formation and Distribution in Relation to the Mechanism of Thyroid-Accelerated Metamorphosis in the Larval Frog. *J Exp Med* 38:529-541.

134. Yang, J., A. Torio, R. B. Donoff, G. T. Gallagher, R. Egan, P. F. Weller, and D. T. Wong. 1997. Depletion of eosinophil infiltration by anti-IL-5 monoclonal antibody (TRFK-5) accelerates open skin wound epithelial closure. *Am J Pathol* 151:813-819.

135. Wehling, N., G. D. Palmer, C. Pilapil, F. Liu, J. W. Wells, P. E. Muller, C. H. Evans, and R. M. Porter. 2009. Interleukin-1beta and tumor necrosis factor alpha inhibit chondrogenesis by human mesenchymal stem cells through NF-kappaB-dependent pathways. *Arthritis Rheum* 60:801-812.

136. Blumenthal, R. D., M. Samoszuk, A. P. Taylor, G. Brown, R. Alisauskas, and D. M. Goldenberg. 2000. Degranulating eosinophils in human endometriosis. *Am J Pathol* 156:1581-1588.

137. Puxeddu, I., N. Berkman, A. H. Nissim Ben Efraim, D. E. Davies, D. Ribatti, G. J. Gleich, and F. Levi-Schaffer. 2009. The role of eosinophil major basic protein in angiogenesis. *Allergy* 64:368-374.

138. Trivedi, S. G., and C. M. Lloyd. 2007. Eosinophils in the pathogenesis of allergic airways disease. *Cell Mol Life Sci* 64:1269-1289.

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

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

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

142. Akuthota, P., H. B. Wang, L. A. Spencer, and P. F. Weller. 2008. Immunoregulatory roles of eosinophils: a new look at a familiar cell. *Clin Exp Allergy* 38:1254-1263.

143. Hogan, S. P., H. F. Rosenberg, R. Moqbel, S. Phipps, P. S. Foster, P. Lacy, A. B. Kay, and M. E. Rothenberg. 2008. Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy* 38:709-750.

144. Chu, V. T., A. Frohlich, G. Steinhauser, T. Scheel, T. Roch, S. Fillatreau, J. J. Lee, M. Lohning, and C. Berek. 2011. Eosinophils are required for the maintenance of plasma cells in the bone marrow. *Nat Immunol* 12:151-159.

145. Jacobsen, E. A., S. I. Ochkur, R. S. Pero, A. G. Taranova, C. A. Protheroe, D. C. Colbert, N. A. Lee, and J. J. Lee. 2008. Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *J Exp Med* 205:699-710.

146. Shi, H. Z. 2004. Eosinophils function as antigen-presenting cells. *J Leukoc Biol* 76:520-527.

147. Gordon, S., and P. R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 5:953-964.

148. Strauss-Ayali, D., S. M. Conrad, and D. M. Mosser. 2007. Monocyte subpopulations and their differentiation patterns during infection. *J Leukoc Biol* 82:244-252.

149. Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau, and F. Geissmann. 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 317:666-670.

150. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.

151. Benoit, M., B. Desnues, and J. L. Mege. 2008. Macrophage polarization in bacterial infections. *J Immunol* 181:3733-3739.

152. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 25:677-686.

153. Kreider, T., R. M. Anthony, J. F. Urban, Jr., and W. C. Gause. 2007. Alternatively activated macrophages in helminth infections. *Curr Opin Immunol* 19:448-453.

154. Nair, M. G., I. J. Gallagher, M. D. Taylor, P. Loke, P. S. Coulson, R. A. Wilson, R. M. Maizels, and J. E. Allen. 2005. Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infect Immun* 73:385-394.

155. Lambrecht, B. N. 2006. Alveolar macrophage in the driver's seat. *Immunity* 24:366-368.

156. Holt, P. G. 1978. Inhibitory activity of unstimulated alveolar macrophages on T-lymphocyte blastogenic response. *Am Rev Respir Dis* 118:791-793.

157. Thepen, T., N. Van Rooijen, and G. Kraal. 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J Exp Med* 170:499-509.

158. Morris, D. G., X. Huang, N. Kaminski, Y. Wang, S. D. Shapiro, G. Dolganov, A. Glick, and D. Sheppard. 2003. Loss of integrin alpha(v)beta6-mediated TGF-beta activation causes Mmp12-dependent emphysema. *Nature* 422:169-173.

159. Broug-Holub, E., G. B. Toews, J. F. van Iwaarden, R. M. Strieter, S. L. Kunkel, R. Paine, 3rd, and T. J. Standiford. 1997. Alveolar macrophages are required for protective pulmonary defenses in murine Klebsiella pneumonia: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. *Infect Immun* 65:1139-1146.

160. Zhao, M., L. G. Fernandez, A. Doctor, A. K. Sharma, A. Zarbock, C. G. Tribble, I. L. Kron, and V. E. Laubach. 2006. Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol* 291:L1018-1026.

161. Pribul, P. K., J. Harker, B. Wang, H. Wang, J. S. Tregoning, J. Schwarze, and P. J. Openshaw. 2008. Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development. *J Virol* 82:4441-4448.

162. Song, C., L. Luo, Z. Lei, B. Li, Z. Liang, G. Liu, D. Li, G. Zhang, B. Huang, and Z. H. Feng. 2008. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol* 181:6117-6124.

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

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

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

166. Liu, L. Y., J. B. Sedgwick, M. E. Bates, R. F. Vrtis, J. E. Gern, H. Kita, N. N. Jarjour, W. W. Busse, and E. A. Kelly. 2002. Decreased expression of membrane IL-5 receptor alpha on human eosinophils: I. Loss of membrane IL-5 receptor alpha on airway eosinophils and increased soluble IL-5 receptor alpha in the airway after allergen challenge. *J Immunol* 169:6452-6458.

167. Stein, M. L., J. M. Villanueva, B. K. Buckmeier, Y. Yamada, A. H. Filipovich, A. H. Assa'ad, and M. E. Rothenberg. 2008. Anti-IL-5 (mepolizumab) therapy reduces eosinophil activation ex vivo and increases IL-5 and IL-5 receptor levels. *J Allergy Clin Immunol* 121:1473-1483, 1483 e1471-1474.

168. Komai-Koma, M., D. Xu, Y. Li, A. N. McKenzie, I. B. McInnes, and F. Y. Liew. 2007. IL-33 is a chemoattractant for human Th2 cells. *Eur J Immunol* 37:2779-2786.

169. Townsend, M. J., P. G. Fallon, D. J. Matthews, H. E. Jolin, and A. N. McKenzie. 2000. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med* 191:1069-1076.

170. Fallon, P. G., H. E. Jolin, P. Smith, C. L. Emson, M. J. Townsend, R. Fallon, P. Smith, and A. N. McKenzie. 2002. IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity* 17:7-17.

171. McKenzie, G. J., A. Bancroft, R. K. Grencis, and A. N. McKenzie. 1998. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr Biol* 8:339-342.

172. Badewa, A. P., C. E. Hudson, and A. S. Heiman. 2002. Regulatory effects of eotaxin, eotaxin-2, and eotaxin-3 on eosinophil degranulation and superoxide anion generation. *Exp Biol Med (Maywood)* 227:645-651.

173. Stock, P., O. Akbari, G. Berry, G. J. Freeman, R. H. Dekruyff, and D. T. Umetsu. 2004. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol* 5:1149-1156.

174. Zucker-Franklin, D., and G. Grusky. 1976. The identification of eosinophil colonies in soft-agar cultures by differential staining for peroxidase. *J Histochem Cytochem* 24:1270-1272.

175. Ten, R. M., L. R. Pease, D. J. McKean, M. P. Bell, and G. J. Gleich. 1989. Molecular cloning of the human eosinophil peroxidase. Evidence for the existence of a peroxidase multigene family. *J Exp Med* 169:1757-1769.

176. Zardini, D. M., P. Heuschling, A. Gallois, J. L. Bueb, and E. J. Tschirhart. 1997. Human umbilical cord blood-derived eosinophils cultured in the presence of IL-3 and IL-5 respond to fMLP with [Ca2+]i variation and O2- production. *J Immunol Methods* 205:1-9.

177. Lamkhioued, B., S. G. Abdelilah, Q. Hamid, N. Mansour, G. Delespesse, and P. M. Renzi. 2003. The CCR3 receptor is involved in eosinophil differentiation and is up-regulated by Th2 cytokines in CD34+ progenitor cells. *J Immunol* 170:537-547.

178. Zhiguang, X., C. Wei, R. Steven, D. Wei, Z. Wei, M. Rong, L. Zhanguo, and Z. Lianfeng. 2010. Over-expression of IL-33 leads to spontaneous pulmonary inflammation in mIL-33 transgenic mice. *Immunol Lett* 131:159-165.

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

180. Cox, D., L. Earle, S. A. Jimenez, K. M. Leiferman, G. J. Gleich, and J. Varga. 1995. Elevated levels of eosinophil major basic protein in the sera of patients with systemic sclerosis. *Arthritis Rheum* 38:939-945.

181. Lee, J. J., E. A. Jacobsen, M. P. McGarry, R. P. Schleimer, and N. A. Lee. 2010. Eosinophils in health and disease: the LIAR hypothesis. *Clin Exp Allergy* 40:563-575.

182. Nopp, A., J. Lundahl, and G. Hallden. 2000. Quantitative, rather than qualitative, differences in CD69 upregulation in human blood eosinophils upon activation with selected stimuli. *Allergy* 55:148-156.

183. Luttmann, W., B. Knoechel, M. Foerster, H. Matthys, J. C. Virchow, Jr., and C. Kroegel. 1996. Activation of human eosinophils by IL-13. Induction of CD69 surface antigen, its relationship to messenger RNA expression, and promotion of cellular viability. *J Immunol* 157:1678-1683.

184. Hartnell, A., D. S. Robinson, A. B. Kay, and A. J. Wardlaw. 1993. CD69 is expressed by human eosinophils activated in vivo in asthma and in vitro by cytokines. *Immunology* 80:281-286.

185. Kondo, Y., T. Yoshimoto, K. Yasuda, S. Futatsugi-Yumikura, M. Morimoto, N. Hayashi, T. Hoshino, J. Fujimoto, and K. Nakanishi. 2008. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int Immunol*.

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

187. Cherry, W. B., J. Yoon, K. R. Bartemes, K. Iijima, and H. Kita. 2008. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *J Allergy Clin Immunol* 121:1484-1490.

188. Douwes, J., and N. Pearce. 2002. Asthma and the westernization 'package'. *Int J Epidemiol* 31:1098-1102.

189. Allakhverdi, Z., M. R. Comeau, D. E. Smith, D. Toy, L. M. Endam, M. Desrosiers, Y. J. Liu, K. J. Howie, J. A. Denburg, G. M. Gauvreau, and G. Delespesse. 2009. CD34+ hemopoietic progenitor cells are potent effectors of allergic inflammation. *J Allergy Clin Immunol* 123:472-478.

190. Humbles, A. A., B. Lu, D. S. Friend, S. Okinaga, J. Lora, A. Al-Garawi, T. R. Martin, N. P. Gerard, and C. Gerard. 2002. 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* 99:1479-1484.

191. Suzukawa, M., R. Koketsu, M. Iikura, S. Nakae, K. Matsumoto, H. Nagase, H. Saito, K. Matsushima, K. Ohta, K. Yamamoto, and M. Yamaguchi. 2008. Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Lab Invest* 88:1245-1253.

192. Chow, J. Y., C. K. Wong, P. F. Cheung, and C. W. Lam. 2009. Intracellular signaling mechanisms regulating the activation of human eosinophils by the novel Th2 cytokine IL-33: implications for allergic inflammation. *Cell Mol Immunol*.

193. Rankin, A. L., J. B. Mumm, E. Murphy, S. Turner, N. Yu, T. K. McClanahan, P. A. Bourne, R. H. Pierce, R. Kastelein, and S. Pflanz. 2010. IL-33 induces IL-13-dependent cutaneous fibrosis. *J Immunol* 184:1526-1535.

194. Piliponsky, A. M., G. J. Gleich, I. Bar, and F. Levi-Schaffer. 2002. Effects of eosinophils on mast cells: a new pathway for the perpetuation of allergic inflammation. *Mol Immunol* 38:1369.

195. Fujisawa, T., R. Abu-Ghazaleh, H. Kita, C. J. Sanderson, and G. J. Gleich. 1990. Regulatory effect of cytokines on eosinophil degranulation. *J Immunol* 144:642-646.

196. Clark, K., L. Simson, N. Newcombe, A. M. Koskinen, J. Mattes, N. A. Lee, J. J. Lee, L. A. Dent, K. I. Matthaei, and P. S. Foster. 2004. Eosinophil degranulation in the allergic lung of mice primarily occurs in the airway lumen. *J Leukoc Biol* 75:1001-1009.

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

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